

Tools and Techniques for Assessing Recovery Potential of Aerobic Sludge Biomass Stressed with Copper(II), Amoxicillin IV, Chlorpyrifos, and Piggery Wastewater

A Thesis

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Doctor of Philosophy

by

Rajneesh Kumar



**Centre for the Environment
Indian Institute of Technology Guwahati
Guwahati-781039, Assam, India**

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परिवार, प्रकृति की उत्कृष्ट कृतियों में से एक है।

—जॉर्ज संतयान (1863–1952)

दार्शनिक

परम् पूजनीय माता–पिता
एवं
आदरणीय गुरुओं
को समर्पित





Statement

I hereby declare that the work embodied in this thesis entitled **Tools and Techniques for Assessing Recovery Potential of Aerobic Sludge Biomass Stressed with Copper(II), Amoxicillin IV, Chlorpyrifos, and Piggery Wastewater** carried out by me in the Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India, under the supervision of **Prof. Mohammad Jawed** and **Prof. Gurvinder Kaur Saini**. Wherever I have consulted the research findings of others in the creation of this work, I have given due credit by citing them in the text of the thesis and giving their details in the references.

Rajneesh Kumar

Roll No.146152010

Centre for the Environment

Indian Institute of Technology Guwahati

Guwahati - 781039, Assam, India

Date : May 2022

Place: Guwahati





भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati
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Certificate

This is to certify that work described in this thesis entitled **Tools and Techniques for Assessing Recovery Potential of Aerobic Sludge Biomass Stressed with Copper(II), Amoxicillin IV, Chlorpyrifos, and Piggery Wastewater** by **Rajneesh Kumar** (Roll No. 146152010) is an authentic record of the results obtained from the research work carried out under my supervision in the Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India. I certify that he has fulfilled all the requirements according to the rules of this institute regarding the investigations embodied in his thesis and this work has not been submitted elsewhere for a degree.

Dr. Mohammad Jawed

Professor

Department of Civil Engineering

Indian Institute of Technology Guwahati

Guwahati - 781039, Assam, India

Date : May 2022

Place: Guwahati

Dr. Gurvinder Kaur Saini

Professor

Department of Biosciences and Bioengineering

Indian Institute of Technology Guwahati

Guwahati - 781039, Assam, India

Date : May 2022

Place: Guwahati



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Contents

List of Figures	v
List of Tables	xiii
Nomenclature	xix
Abstract	xxiii
1 Introduction and Literature Review	1–71
1.1 Prologue	1
1.2 Thesis organization	2
1.3 General aspects	2
1.4 Domestic wastewater composition	3
1.4.1 Organic matter	3
1.5 Biological treatment options for organic wastewater	4
1.5.1 Aerobic bio-degradation	5
1.5.1.1 Aerobic suspended growth system	5
1.5.1.2 Aerobic attached-growth system	9
1.5.2 Anaerobic bio-degradation	10
1.5.2.1 Anaerobic suspended growth system	10
1.5.2.2 Anaerobic attached-growth system	12
1.6 Microorganisms present in activated sludge	13
1.6.1 Algae	13
1.6.2 Archaea	13
1.6.3 Bacteria	13
1.6.4 Protozoa	13
1.6.5 Fungi	13
1.6.6 Metazoa	14
1.7 Significance of microorganism	14
1.7.1 Formation of flocs	14
1.7.2 Enzymes action	15
1.7.3 Microbial community	16
1.8 Source of heavy metals, pharmaceuticals, and pesticides in sewage	17
1.8.1 Heavy metals	18
1.8.2 Pharmaceuticals	20
1.8.3 Pesticides	22
1.9 Impact of heavy metals, pharmaceuticals, and pesticides	23
1.9.1 Heavy metals	23
1.9.2 Antibiotics	30
1.9.3 Pesticides	34
1.9.4 Real wastewater	36
1.9.5 Other factors and pollutants	39
1.10 Interaction of heavy metals, antibiotics, and pesticides with aerobic sludge biomass	42
1.11 Summary	47
1.12 Lacunae	49

1.13	Objectives of the study	51
1.14	Research methodology	52
	References	53
2	Impacts of Heavy Metal (Copper) Stress on Aerobic Sludge Biomass	71–137
2.1	Background	71
2.2	Materials and Methods	73
2.2.1	Chemicals and reagents	73
2.2.2	Aerobic sludge biomass	73
2.2.3	Reactor selection	73
2.2.4	Reactor setup and operation	74
2.2.5	Analytical methods	76
2.2.5.1	Temperature and pH	77
2.2.5.2	Determination of Cu(II)	77
2.2.5.3	Chemical oxygen demand	78
2.2.5.4	Alkalinity	78
2.2.5.5	Solids analysis	78
2.2.5.6	Interface settling velocity and sludge volume index	79
2.2.5.7	Aerobic sludge biomass activity test	79
2.2.5.8	Filament index	80
2.2.5.9	Photographic observation	80
2.2.5.10	Gram's staining and microscopic examination	80
2.2.5.11	Field emission scanning electron microscope and energy-dispersive X-ray	81
2.2.5.12	Enzyme activity	81
2.2.5.13	Amplified ribosomal DNA restriction analysis (ARDRA)	83
2.3	Results and Discussion	84
2.3.1	Stressed phase operation	84
2.3.1.1	Environmental and performance parameters	84
2.3.1.2	Biomass related general parameters	92
2.3.1.3	Biomass related microbiological parameters	100
2.3.2	Recovery phase operation	110
2.3.2.1	Environmental and performance parameters	110
2.3.2.2	Biomass related general parameters	117
2.3.2.3	Biomass related microbiological parameters	124
2.4	Summary	132
	References	134
3	Effects of Antibiotic (Amoxicillin IV) Stress on Aerobic Sludge Biomass	137–201
3.1	Background	137
3.2	Materials and Methods	139
3.2.1	Chemicals and reagents	139
3.2.2	Aerobic sludge biomass	139
3.2.3	Reactor setup and operation	139
3.2.4	Analytical methods	142
3.2.4.1	Cell viability test	143
3.2.4.2	Extracellular polymeric substance	143
3.2.4.3	Determination of AMX	144
3.3	Results and Discussion	144
3.3.1	Stressed phase operation	144

3.3.1.1	<i>Environmental and performance parameters</i>	144
3.3.1.2	<i>Biomass related general parameters</i>	153
3.3.1.3	<i>Biomass related microbiological parameters</i>	159
3.3.2	Recovery phase operation	171
3.3.2.1	<i>Environmental and performance parameters</i>	171
3.3.2.2	<i>Biomass related general parameters</i>	178
3.3.2.3	<i>Biomass related microbiological parameters</i>	185
3.4	Summary	197
	References	199
4	Effects of Pesticide (Chlorpyrifos) Stress on Aerobic Sludge Biomass	201–262
4.1	Background	201
4.2	Materials and Methods	202
4.2.1	Chemicals and reagents	202
4.2.2	Source of aerobic sludge biomass	202
4.2.3	Reactor setup and operation	202
4.2.4	Analytical methods	205
4.2.4.1	<i>Determination of CPS</i>	205
4.3	Results and Discussion	206
4.3.1	Stressed phase operation	206
4.3.1.1	<i>Environmental and performance parameters</i>	206
4.3.1.2	<i>Biomass related general parameters</i>	214
4.3.1.3	<i>Biomass related microbiological parameters</i>	221
4.3.2	Recovery phase operation	233
4.3.2.1	<i>Environmental and performance parameters</i>	233
4.3.2.2	<i>Biomass related general parameters</i>	241
4.3.2.3	<i>Biomass related microbiological parameters</i>	246
4.4	Summary	258
	References	259
5	Impacts of Piggery Wastewater Stress on Aerobic Sludge Biomass	263–350
5.1	Background	263
5.2	Materials and methods	264
5.2.1	Chemicals and reagents	264
5.2.2	Aerobic sludge biomass	265
5.2.3	Source of piggery wastewater	265
5.2.4	Reactor setup and operation	266
5.2.4.1	<i>Stressed phase I operation with PWW</i>	266
5.2.4.2	<i>Stressed phase II operation with PWW+Cu(II)</i>	267
5.2.4.3	<i>Recovery phase operation</i>	269
5.2.5	Analytical methods	270
5.3	Results and Discussion	270
5.3.1	Stressed phase I operation with PWW	271
5.3.1.1	<i>Environmental and performance parameters</i>	271
5.3.1.2	<i>Biomass related general parameters</i>	276
5.3.1.3	<i>Biomass related microbiological parameters</i>	280
5.3.2	Stressed phase II operation with PWW+Cu(II)	289
5.3.2.1	<i>Environmental and performance parameters</i>	289
5.3.2.2	<i>Biomass related general parameters</i>	297
5.3.2.3	<i>Biomass related microbiological parameters</i>	304

5.3.3	Recovery phase operation	315
5.3.3.1	<i>Environmental and performance parameters</i>	315
5.3.3.2	<i>Biomass related general parameters</i>	323
5.3.3.3	<i>Biomass related microbiological parameters</i>	330
5.4	Summary	344
	References	347
6	Tools and Techniques to Assess Health of Aerobic Sludge Biomass at Treatment Plant Site	351–373
6.1	Critical review of tests/observations/data obtained for stressed aerobic sludge biomass	353
6.1.1	MLSS and MLVSS tests	353
6.1.2	SVI and ISV tests	360
6.1.3	Microscopic and photographic observations	361
6.1.4	Aerobic sludge biomass activity (ASBA) tests	365
6.1.5	Enzymatic tests	367
6.1.6	Amplified ribosomal DNA restriction analysis (ARDRA)	370
6.2	Summary	372
7	Summary and Limitations	373–387
7.1	Impacts of copper(II) stress on aerobic sludge biomass	373
7.1.1	Stressed phase operation	373
7.1.2	Recovery phase operation	374
7.2	Effects of amoxicillin IV stress on aerobic sludge biomass	375
7.2.1	Stressed phase operation	375
7.2.2	Recovery phase operation	377
7.3	Effects of chlorpyrifos stress on aerobic sludge biomass	378
7.3.1	Stressed phase operation	378
7.3.2	Recovery phase operation	379
7.4	Impacts of piggery wastewater stress on aerobic sludge biomass	380
7.4.1	Stressed phase I operation	381
7.4.2	Stressed phase II operation	382
7.4.3	Recovery phase operation	383
7.5	Tools and techniques to assess health of aerobic sludge biomass at treatment plant site	384
7.6	Limitations	385
	Appendix-I	387
	Appendix-II	393
	Appendix-III	403
	Appendix-IV	417
	Appendix-V	423

List of Figures

Fig. 1.1	Biological wastewater treatment processes for organic matter removal from wastewater.	4
Fig. 1.2	Schematic representation of organic removal by aerobic process.	5
Fig. 1.3	Schematic diagram of activated sludge process.	6
Fig. 1.4	Pathway of BOD removal by microorganism.	6
Fig. 1.5	Aerobic suspended growth biological reactors.	7
Fig. 1.6	Schematic representation of organic removal by anaerobic process.	10
Fig. 1.7	Anaerobic suspended growth biological reactors.	11
Fig. 1.8	Schematic presentation of an activated sludge floc.	14
Fig. 1.9	Source of toxic pollutants to sewage treatment plant.	18
Fig. 1.10	Various types of metal-microbe interactions.	43
Fig. 1.11	A summary of toxic influences of metals on the microbial cell.	45
Fig. 1.12	Main classes of antimicrobial drugs, its targets, and mode of action on a bacterial cell.	47
Fig. 1.13	Flow chart of research methodology.	53
Fig. 2.1	Schematic sequence of operation of reactor.	73
Fig. 2.2	Schematic diagram of reactor setup used for experiment.	75
Fig. 2.3	Schematic representation of cycle time for reactor operation.	76
Fig. 2.4	Set-up used for photographic observation.	80
Fig. 2.5	Variation in environmental and performance related parameters for reactor R_{Cu0} during 0-25 d of operation.	85
Fig. 2.6	Variation in environmental and performance related parameters for reactor R_{Cu10} during 0-25 d of stressed phase operation.	86
Fig. 2.7	Variation in environmental and performance related parameters for reactor R_{Cu20} during 0-25 d of stressed phase operation.	87
Fig. 2.8	Variation in environmental and performance related parameters for reactor R_{Cu40} during 0-25 d of stressed phase operation.	88
Fig. 2.9	Variation in environmental and performance related parameters for reactor R_{Cu80} during 0-25 d of stressed phase operation.	89
Fig. 2.10	Variation in MLSS, MLVSS, and SVI values for reactor biomass from (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} during 0-25 d of stressed phase operation.	94
Fig. 2.11	Typical settling test results for reactor (a) R_{Cu0} and (b) R_{Cu10} biomasses during 0-25 d of stressed phase operation.	97
Fig. 2.12	Variation in interface settling velocities of reactor biomasses during 0-25 d of stressed phase operation.	97
Fig. 2.13	Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactor (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} on 20 th d during steady-state condition of stressed phase operation.	98
Fig. 2.14	FESEM micro-images of aerobic sludge biomass withdrawn on 25 th d of stressed phase operation from reactors (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , and (d) R_{Cu40}	103

Fig. 2.15	EDX analysis of aerobic sludge biomass withdrawn on 25 th d of stressed phase operation from reactor R _{Cu40} (a) EDX mapping and (b) EDX spectrum.	103
Fig. 2.16	Enzyme activities of aerobic sludge biomass (a) E _{Catalase} , (b) E _{Dehydrogenase} , (c) E _{Protease} , and (d) E _{Urease} estimated during stressed phase operation.	104
Fig. 2.17	Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase treated with (a) <i>AluI</i> and (b) <i>MspI</i> restriction enzymes.	107
Fig. 2.18	Variation in environmental and performance related parameters for reactor R _{Cu0} during 26-54 d of operation along with 0-25 d of operation [without Cu(II) in the feed].	111
Fig. 2.19	Variation in environmental and performance related parameters for reactor R _{Cu10} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.	112
Fig. 2.20	Variation in environmental and performance related parameters for reactor R _{Cu20} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.	113
Fig. 2.21	Variation in environmental and performance related parameters for reactor R _{Cu40} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.	114
Fig. 2.22	Variation in environmental and performance related parameters for reactor R _{Cu80} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.	115
Fig. 2.23	Variation in MLSS, MLVSS, and SVI values for reactor biomass from (a) R _{Cu0} , (b) R _{Cu10} , (c) R _{Cu20} , (d) R _{Cu40} , and (e) R _{Cu80} during 26-54 d under recovery phase operation along with data for stressed phase operation.	119
Fig. 2.24	Typical settling test results for reactor (a) R _{Cu0} and (b) R _{Cu10} biomasses during 26-54 d of recovery phase operation.	121
Fig. 2.25	Variation in interface settling velocities of reactor biomasses during 26-54 d under recovery phase operation along with data for stressed phase operation.	122
Fig. 2.26	Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactors (a) R _{Cu0} , (b) R _{Cu10} , (c) R _{Cu20} , (d) R _{Cu40} , and (e) R _{Cu80} on 51 st d towards the end of recovery phase operation.	123
Fig. 2.27	Enzyme activities of aerobic sludge biomass assessed during recovery phase operation (a) E _{Catalase} , (b) E _{Dehydrogenase} , (c) E _{Protease} , and (d) E _{Urease} along with data for stressed phase operation.	128
Fig. 2.28	Digestion pattern and dendrogram of aerobic sludge biomass treated with restriction enzyme <i>AluI</i> : (a) under stressed phase and (b) under recovery phase; and <i>MspI</i> : (c) under stressed phase (d) under recovery phase.	130
Fig. 3.1	Schematic diagram of experimental set-up.	140
Fig. 3.2	Schematic representation of cycle time for reactors.	141
Fig. 3.3	Variation in environmental and performance related parameters for reactor R _{amx0} during 0-43 d of operation.	145
Fig. 3.4	Variation in environmental and performance related parameters for reactor R _{amx500} during 0-43 d of operation.	146
Fig. 3.5	Variation in environmental and performance related parameters for reactor R _{amx1000} during 0-43 d of operation.	147

Fig. 3.6	Variation in environmental and performance related parameters for reactor $R_{amx2000}$ during 0-43 d of operation.	148
Fig. 3.7	Variation in MLSS, MLVSS, and SVI values for reactors (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ during 0-43 d under stressed phase operation.	154
Fig. 3.8	Typical settling test results for reactor (a) R_{amx0} and (b) $R_{amx2000}$ biomasses during 0-43 d under stressed phase operation.	156
Fig. 3.9	Variation in interface settling velocities of reactor biomasses during 0-43 d during stressed phase operation.	157
Fig. 3.10	Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactors (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ on 43 rd d of stressed phase operation.	158
Fig. 3.11	(a) PS, (b) PN, and (c) EPS contents in aerobic sludge biomass estimated during stressed phase operation.	165
Fig. 3.12	Enzyme activities of aerobic sludge biomass assessed during stressed phase operation (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease}	167
Fig. 3.13	Digestion pattern and dendrogram of aerobic sludge biomass treated with (a) <i>AluI</i> , (b) <i>HhaI</i> , and (c) <i>MspI</i> restriction enzymes under stressed phase operation.	169
Fig. 3.14	Variation in environmental and performance related parameters for reactor R_{amx0} during 44-78 d of operation along with 0-43 d of operation [without AMX in the feed].	172
Fig. 3.15	Variation in environmental and performance related parameters for reactor R_{amx500} operated during 44-78 d of recovery phase operation along with data for stressed phase operation.	173
Fig. 3.16	Variation in environmental and performance related parameters for reactor $R_{amx1000}$ operated during 44-78 d of recovery phase operation along with data for stressed phase operation.	174
Fig. 3.17	Variation in environmental and performance related parameters for reactor $R_{amx2000}$ operated during 44-78 d of recovery phase operation along with data for stressed phase operation.	175
Fig. 3.18	Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ biomasses during 44-78 d of recovery phase operation along with data for stressed phase operation.	180
Fig. 3.19	Typical settling test results for reactor (a) R_{amx0} and (b) $R_{amx2000}$ biomasses during 44-78 d of recovery phase operation.	182
Fig. 3.20	Variation in interface settling velocities of reactor biomasses during 44-78 d of recovery phase operation along with data for stressed phase operation.	183
Fig. 3.21	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ on 78 th d of recovery phase operation.	184
Fig. 3.22	(a) PS, (b) PN, and (c) EPS contents in aerobic sludge biomass during stressed and recovery phase operations.	191
Fig. 3.23	Enzyme activities of aerobic sludge biomass in recovery phase operation (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} along with data for stressed phase operation.	193

Fig. 3.24	Digestion pattern and dendrogram of aerobic sludge biomass under stressed and recovery phase operation treated with <i>AluI</i> : (a) stressed phase (b) recovery phase; <i>HhaI</i> : (c) stressed phase (d) recovery phase; and <i>MspI</i> : (e) stressed phase (f) recovery phase restriction enzymes. . .	195
Fig. 4.1	Schematic diagram of reactor setup used for experiment.	203
Fig. 4.2	Schematic representation of cycle time for reactor operation.	204
Fig. 4.3	Variation in environmental and performance related parameters for reactor R_{cps0} during 0-34 d of operation.	207
Fig. 4.4	Variation in environmental and performance related parameters for reactor R_{cps50} during 0-34 d of operation.	208
Fig. 4.5	Variation in environmental and performance related parameters for reactor R_{cps100} during 0-34 d of operation.	209
Fig. 4.6	Variation in environmental and performance related parameters for reactor R_{cps200} during 0-34 d of operation.	210
Fig. 4.7	Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} biomasses during 0-34 d under stressed phase operation.	216
Fig. 4.8	Typical settling test results for reactor (a) R_{cps0} and (b) R_{cps200} biomasses during 0-34 d under stressed phase operation.	218
Fig. 4.9	Variation in interface settling velocities of reactor biomasses during 0-34 d under stressed phase operation.	219
Fig. 4.10	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} on 34 th d of stressed phase operation.	220
Fig. 4.11	Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase operation.	226
Fig. 4.12	Enzyme activities of aerobic sludge biomass (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} assessed during stressed phase operation. . .	227
Fig. 4.13	Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase operation treated with (a) <i>AluI</i> , (b) <i>HhaI</i> , and (c) <i>MspI</i> restriction enzymes.	230
Fig. 4.14	Variation in environmental and performance related parameters for reactor R_{cps0} during 35-76 d of operation along with 0-34 d of operation (without CPS in the feed).	234
Fig. 4.15	Variation in environmental and performance related parameters for reactor R_{cps50} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.	235
Fig. 4.16	Variation in environmental and performance related parameters for reactor R_{cps100} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.	236
Fig. 4.17	Variation in environmental and performance related parameters for reactor R_{cps200} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.	237
Fig. 4.18	Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} biomasses during 35-76 d of recovery phase operation along with stressed phase operation.	242
Fig. 4.19	Typical interface settling test results for reactor (a) R_{cps0} and (b) R_{cps200} biomasses during 35-76 d of recovery phase operation.	243

Fig. 4.20	Variation in interface settling velocities of reactor biomasses during 35-76 d of recovery phase operation along with data for stressed phase operation.	244
Fig. 4.21	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} on 76 th d of recovery phase operation.	245
Fig. 4.22	Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during recovery phase operation along with data for stressed phase operation.	251
Fig. 4.23	Enzyme activities of aerobic sludge biomass (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} assessed during recovery phase operation along with data for stressed phase operation.	253
Fig. 4.24	Digestion pattern and dendrogram of aerobic sludge biomass treated with restriction enzyme <i>AluI</i> : (a) under stressed phase and (b) under recovery phase; <i>HhaI</i> : (c) under stressed phase (d) under recovery phase; and <i>MspI</i> : (e) under stressed phase (f) under recovery phase.	255
Fig. 5.1	Schematic diagram of reactor setup used for experiment during stressed phase I operation with PWW.	266
Fig. 5.2	Schematic representation of cycle time for reactors during stressed phase I operation with PWW.	267
Fig. 5.3	Schematic diagram of reactor setup used for experiment during stressed phase II operation with PWW+Cu(II).	268
Fig. 5.4	Schematic representation of cycle time for reactors during stressed phase II operation with PWW+Cu(II).	269
Fig. 5.5	Variation in environmental and performance related parameters for reactor R_c during 0-41 d of stressed phase I operation.	272
Fig. 5.6	Variation in environmental and performance related parameters for reactor R_s during 0-41 d of stressed phase I operation with PWW feed.	273
Fig. 5.7	Variation in environmental and performance related parameters for reactor R_r during 0-41 d of stressed phase I operation with PWW feed.	274
Fig. 5.8	Variation in MLSS, MLVSS, and SVI values for reactors (a) R_c , (b) R_s , and (c) R_r during stressed phase I operation with PWW feed.	277
Fig. 5.9	Variation in interface settling velocities for reactor biomasses during stressed phase I operation with PWW feed.	278
Fig. 5.10	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactors (a) R_c , (b) R_s , and (c) R_r on 41 st d of stressed phase I operation with PWW feed.	279
Fig. 5.11	Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase I operation with PWW feed.	284
Fig. 5.12	Enzyme activities of aerobic sludge biomass (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} assessed during stressed phase I operation with PWW feed.	286
Fig. 5.13	Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase I operation with PWW feed treated with (a) <i>AluI</i> , (b) <i>HhaI</i> , and (c) <i>MspI</i> restriction enzymes.	287
Fig. 5.14	Variation in environmental and performance related parameters for reactor R_c during 42-72 d of stressed phase II operation.	290

Fig. 5.15	Variation in environmental and performance related parameters for reactor R_{sCu40} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.	291
Fig. 5.16	Variation in environmental and performance related parameters for reactor R_{sCu80} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.	292
Fig. 5.17	Variation in environmental and performance related parameters for reactor R_{rCu40} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.	293
Fig. 5.18	Variation in environmental and performance related parameters for reactor R_{rCu80} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.	294
Fig. 5.19	Variation in MLSS, MLVSS, and SVI for reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.	299
Fig. 5.20	Variation in interface settling velocities for reactor biomasses during stressed phase II operation with PWW+Cu(II) feed.	301
Fig. 5.21	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} on 72 nd d of stressed phase II operation with PWW+Cu(II) feed.	302
Fig. 5.22	Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase II operation with PWW+Cu(II) feed.	308
Fig. 5.23	Enzyme activities of aerobic sludge biomass (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} assessed during stressed phase II operation with PWW+Cu(II) feed.	310
Fig. 5.24	Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase II operation with PWW+Cu(II) feed treated with (a) <i>AluI</i> , (b) <i>HhaI</i> , and (c) <i>MspI</i> restriction enzymes.	312
Fig. 5.25	Variation in environmental and performance related parameters for reactor R_c during 73-120 d of under recovery phase operation along with data for stressed phase I and II operations.	316
Fig. 5.26	Variation in environmental and performance related parameters for reactor R_{sCu40} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.	317
Fig. 5.27	Variation in environmental and performance related parameters for reactor R_{sCu80} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.	318
Fig. 5.28	Variation in environmental and performance related parameters for reactor R_{rCu40} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.	319
Fig. 5.29	Variation in environmental and performance related parameters for reactor R_{rCu80} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.	320
Fig. 5.30	Variation in MLSS, MLVSS, and SVI values for reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , (e) R_{rCu80} during 73-120 d of recovery phase operation along with stressed phase I and II operations.	325
Fig. 5.31	Variation in interface settling velocities of reactor biomass during 73-120 d of recovery phase operation along with data for stressed phase I and II operations.	327

Fig. 5.32	Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} on 120 th d of recovery phase operation.	328
Fig. 5.33	Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during recovery phase operation along with data for stressed phase I and II operations.	336
Fig. 5.34	Enzyme activities of aerobic sludge biomass in recovery phase operation (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} along with data for stressed phase I and II operations.	338
Fig. 5.35	Digestion pattern of aerobic sludge biomass treated with restriction enzymes <i>AluI</i> , <i>HhaI</i> , and <i>MspI</i> during stressed phase I, II and recovery phase operations.	341
Fig. 5.36	Dendrogram of aerobic sludge biomass treated with restriction enzymes during stressed phase I operation (a) <i>AluI</i> , (b) <i>HhaI</i> , and (c) <i>MspI</i> ; stressed phase II operation (d) <i>AluI</i> , (e) <i>HhaI</i> , and (f) <i>MspI</i> ; and recovery phase operation (g) <i>AluI</i> , (h) <i>HhaI</i> , and (i) <i>MspI</i>	342





List of Tables

Table 1.1	Typical composition of untreated domestic wastewater.	3
Table 1.2	Class of enzymes and their roles.	15
Table 1.3	Summary of studied parameters to evaluate impact of different heavy metals on biological reactors.	25
Table 1.4	Parameters affected in presence of pharmaceutical compounds during wastewater treatment.	31
Table 1.5	Parameters studied with various concentration of agrochemicals in different reactors.	35
Table 1.6	Parameters studied during real wastewater treatment.	37
Table 1.7	Summary of parameters studied for treatment of various toxic compounds.	41
Table 2.1	Analysis frequency of initial and effluent sample parameters.	77
Table 2.2	Summary of environmental and performance related parameters for reactors operated during 0-25 d of stressed phase operation.	90
Table 2.3	Summary of environmental and performance related parameters for reactors operated during 19-25 d of steady-state of stressed phase operation.	93
Table 2.4	Summary of solids characteristics estimated during stressed phase operation.	95
Table 2.5	Aerobic sludge biomass activity test results carried out towards the end of stressed phase operation.	99
Table 2.6	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} during stressed phase operation.	101
Table 2.7	Photographic images of aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} during stressed phase operation.	102
Table 2.8	Filament index of aerobic sludge biomasses estimated during stressed phase operation.	102
Table 2.9	Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase operation.	105
Table 2.10	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase operation.	108
Table 2.11	Summary of environmental and performance related parameters for reactors operated during 26-54 d under recovery phase operation along with data for stressed phase operation (0-25 d).	116
Table 2.12	Summary of environmental and performance related parameters for reactors operated during steady-state of recovery phase operation (48-54 d) along with data for stressed phase operation (19-25 d).	118
Table 2.13	Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.	120

Table 2.14	Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.	123
Table 2.15	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} in recovery phase operation along with microscopic images for stressed phase operation.	125
Table 2.16	Photographic images of aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} in recovery phase operation along with images for stressed phase operation.	126
Table 2.17	Filament index of aerobic sludge biomass estimated during recovery phase operation along with data for stressed phase operation.	127
Table 2.18	Summary of enzyme activities of aerobic sludge biomass assessed during recovery phase operation along with data for stressed phase operation.	129
Table 2.19	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase operation.	131
Table 3.1	Analysis frequency of initial and effluent sample parameters.	142
Table 3.2	Summary of environmental and performance related parameters for reactors operated during 0-43 d of stressed phase operation.	149
Table 3.3	Summary of environmental and performance related parameters for reactors operated during 37-43 d of steady-state condition of stressed phase operation.	152
Table 3.4	Summary of solids characteristics estimated during stressed phase operation.	155
Table 3.5	Aerobic sludge biomass activity test results carried out at the end of stressed phase operation.	158
Table 3.6	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ during stressed phase operation.	160
Table 3.7	Photographic images of aerobic sludge biomasses withdrawn from reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ during stressed phase operation.	162
Table 3.8	Filament index of aerobic sludge biomass estimated during stressed phase operation.	163
Table 3.9	Fluorescence microscopic images of stained aerobic sludge biomasses withdrawn from reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ during stressed phase operation.	163
Table 3.10	PS, PN, and EPS contents in aerobic sludge biomass estimated during stressed phase operation.	166
Table 3.11	Summary of enzyme activities of aerobic sludge biomass during stressed phase operation.	167
Table 3.12	Similarity matrix for stressed phase aerobic sludge biomass samples resulting from digestion by restriction enzymes.	170
Table 3.13	Summary of environmental and performance related parameters for reactors operated during 44-78 d of recovery phase operation along with data for stressed phase operation (0-43 d).	176

Table 3.14	Summary of environmental and performance related parameters for reactors operated during steady-state of recovery phase operation (72-78 d) along with data for stressed phase operation (37-44 d).	179
Table 3.15	Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.	181
Table 3.16	Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.	184
Table 3.17	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with microscopic images of biomasses withdrawn during stressed phase operation.	186
Table 3.18	Photographic images of aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with images of biomasses withdrawn during stressed phase operation.	187
Table 3.19	Filament index of aerobic sludge biomass estimated during stressed and recovery phase operations.	188
Table 3.20	Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with images for stressed phase operation.	189
Table 3.21	PS, PN, and EPS contents in aerobic sludge biomass estimated during stressed and recovery phase operations.	192
Table 3.22	Summary of enzyme activities of aerobic sludge biomass during recovery phase operation along with data for stressed phase operation.	194
Table 3.23	Similarity matrix resulting from digestion of aerobic sludge biomass samples collected during recovery phase operation by restriction enzymes along with data for stressed phase operation.	196
Table 4.1	Analysis frequency of initial and effluent sample parameters.	206
Table 4.2	Summary of environmental and performance related parameters for reactors operated during 0-34 d of stressed phase operation.	211
Table 4.3	Summary of environmental and performance related parameters for reactors operated during 25-34 d of steady-state condition of stressed phase operation.	215
Table 4.4	Summary of solids characteristics estimated during stressed phase operation.	217
Table 4.5	Aerobic sludge biomass activity test results carried out towards the end of stressed phase operation.	220
Table 4.6	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operation.	222
Table 4.7	Photographic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operations.	223
Table 4.8	Fluorescence microscopic images of stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operations.	224
Table 4.9	Filament index of aerobic sludge biomass estimated during stressed phase operation.	225

Table 4.10	PS, PN, and EPS contents in aerobic sludge biomass estimated under stressed phase operation.	227
Table 4.11	Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase operation.	228
Table 4.12	Similarity matrix resulting from digestion of aerobic sludge biomass samples withdrawn during stressed phase operation by restriction enzymes.	231
Table 4.13	Summary of environmental and performance related parameters for reactors operated during 35-76 d of recovery phase operation along with the data for stressed phase operation (0-34 d).	238
Table 4.14	Summary of environmental and performance related parameters for reactors operated during steady-state condition of recovery phase operation (68-76 d) along with the data for stressed phase operation (25-34 d).	240
Table 4.15	Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.	243
Table 4.16	Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.	245
Table 4.17	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with microscopic images of biomass withdrawn during stressed phase operation.	247
Table 4.18	Photographic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with images of biomasses withdrawn during stressed phase operation.	248
Table 4.19	Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with images of biomasses withdrawn during stressed phase operation.	249
Table 4.20	Filament index of aerobic sludge biomasses estimated during stressed and recovery phase operations.	250
Table 4.21	PS, PN, and EPS contents in aerobic sludge biomass estimated under stressed and recovery phase operation.	252
Table 4.22	Summary of enzyme activities of aerobic sludge biomass assessed during stressed and recovery phase operations.	254
Table 4.23	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase operation.	256
Table 5.1	Characteristics of raw piggery wastewater collected from pig farm.	265
Table 5.2	Analysis frequency of initial and effluent sample parameters.	270
Table 5.3	Summary of environmental and performance related parameters for reactors operated during 0-41 d of stressed phase I operation with PWW feed.	275
Table 5.4	Summary of environmental and performance related parameters for reactors operated during 35-41 d of steady-state condition of stressed phase I operation with PWW feed.	276

Table 5.5	Summary of solids characteristics estimated during stressed phase I operation with PWW feed.	278
Table 5.6	Aerobic sludge biomass activity test results carried out towards the end of stressed phase I operation with PWW feed.	279
Table 5.7	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.	281
Table 5.8	Photographic images of aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.	282
Table 5.9	Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.	283
Table 5.10	Filament index of aerobic sludge biomass estimated during stressed phase I operation with PWW feed.	283
Table 5.11	PS, PN and EPS contents in aerobic sludge biomass operated under stressed phase I operation with PWW feed.	284
Table 5.12	Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase I operation with PWW feed.	286
Table 5.13	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase I operation with PWW feed.	288
Table 5.14	Summary of environmental and performance related parameters for reactors operated during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.	295
Table 5.15	Summary of environmental and performance related parameters for reactors operated during 66-72 d of steady-state condition of stressed phase II operation with PWW+Cu(II) feed.	298
Table 5.16	Summary of solids characteristics estimated during stressed phase II operation with PWW+Cu(II) feed.	300
Table 5.17	Aerobic sludge biomass activity test results carried out towards the end of stressed phase II operation with PWW+Cu(II) feed.	302
Table 5.18	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.	305
Table 5.19	Photographic images of aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.	306
Table 5.20	Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.	307
Table 5.21	Filament index of aerobic sludge biomass estimated during stressed phase II operation with PWW+Cu(II) feed.	307
Table 5.22	PS, PN and EPS contents in aerobic sludge biomass operated under stressed phase II operation with PWW+Cu(II) feed.	308
Table 5.23	Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase II operation with PWW+Cu(II) feed.	311
Table 5.24	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase II operation with PWW+Cu(II) feed.	313

Table 5.25	Summary of environmental and performance related parameters for reactors operated during 73-120 d of recovery phase operation along with data for stressed phase I and II operations.	321
Table 5.26	Summary of environmental and performance related parameters for reactors operated during 114-120 d of steady-state condition of recovery phase operation along with data for stressed phase I and II operations.	324
Table 5.27	Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase I and II operations. . . .	326
Table 5.28	Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase I and II operations.	329
Table 5.29	Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.	331
Table 5.30	Photographic images of aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.	332
Table 5.31	Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.	334
Table 5.32	Filament index of aerobic sludge biomass estimated during stressed phase I, II and recovery phase operations.	335
Table 5.33	PS, PN and EPS contents in aerobic sludge biomass estimated during recovery phase operation along with data for stressed phase I and II operations.	337
Table 5.34	Summary of enzyme activities of aerobic sludge biomass assessed during recovery phase along with data for stressed phase I and II operations.	339
Table 5.35	Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase I and II operations. . . .	343
Table 6.1	Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with Cu(II) in feed.	354
Table 6.2	Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with AMX in feed.	355
Table 6.3	Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with CPS in feed.	356
Table 6.4	Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with PWW in feed.	357

Nomenclature

$\mu_{\max}$	Maximum specific growth rate
2,4-D	2, 4-dichloro phenoxy acetic acid
2-CP	2-chlorophenol
3D-EEM	Three dimensional excitation emission matrix
4-CP	4-chlorophenol
A/O	Anoxic oxic processes
A/O-MBR	Anoxic/oxic membrane bioreactor
A2O	Anaerobic-anoxic-aerobic
ADMBR	Aerobic dynamic membrane bioreactor
AFLP	Amplified fragment length polymorphism
AgNPs	Silver nanoparticles
AMP	Ampicillin
AMX	Amoxicillin IV
AOB	Ammonia-oxidizing bacteria
ARB	Antibiotic resistant bacteria
ARDRA	Amplified ribosomal DNA restriction analysis
ARGs	Antibiotic-resistant genes
ASBA	Aerobic sludge biomass activity
ASP	Activated sludge process
AUR	Ammonium uptake rate
AZI	Azithromycin
BOD	Biochemical oxygen demand
CAS	Conventional activated sludge
CeO ₂ NPs	Cerium dioxide nanoparticles
CETP	Common effluent treatment plant
CIP	Ciprofloxacin
COD	Chemical oxygen demand
CPCB	Central Pollution Control Board
CPS	Chlorpyrifos
CSTR	Continuous stirred tank reactor
CuONPs	Copper oxide nanoparticles
d	Day(s)
DGGE	Denaturing gradient gel electrophoresis
DHA	Dehydrogenase activity
DPAO	Denitrifying phosphate accumulating organisms
DWW	Domestic wastewater
E _{Catalase}	Catalase activity
E _{Dehydrogenase}	Dehydrogenase activity
EDX	Energy-dispersive X-ray
EGSB	Expanded granular sludge bed
EHT	Electron high tension
ENVIS	Environmental Information System
E _{Protease}	Protease activity
EPS	Extracellular polymeric substances
EPWW	Electroplating wastewater

ERY	Erythromycin
ETS	Electronic transport systems
E_{Urease}	Urease activity
FAS	Ferrous ammonium sulfate
FESEM	Field emission scanning electron microscope
FI	Filament index
Fig.	Figure
FISH	Fluorescent in-situ hybridization
FLX	Fluoxetine
FQs	Fluoroquinolones
FTIR	Fourier transform infrared spectroscopy
GPR	Growth phase reactor
h	Hour
HPLC	High performance liquid chromatograph
HRT	Hydraulic retention time
ISV	Interface settling velocity
K_s	Half saturation substrate concentration
LDH	Lactate dehydrogenase
LR	Laboratory grade
Ltd.	Limited
MBR	Membrane bioreactor
MCRT	Mean cell retention time
MHA	Micro aerobic hydrolysis acidification
min.	Minutes
MLD	Million litre per day
MLSS	Mixed liquid suspended solid
MLVSS	Mixed liquid volatile suspended solid
MR	Mother reactor
MSM	Minimal salts medium
MT	Metric ton
MWTP	Municipal wastewater treatment plant
nm	Nanometer
NOB	Nitrite oxidizing bacteria
OMWW	Olive mill wastewater
OUR	Oxygen uptake rate
PAO	Phosphate accumulating organism
PCR	Polymerase chain reaction
PN	Protein
POME	Palm oil mill effluent
PPCPs	Pharmaceuticals and personal care products
PS	Polysaccharide
PWW	Piggery wastewater
R_{amx0}	Control reactor fed with 0 mg/L of amoxicillin
R_{amx500}	Reactor fed with 500 mg/L of amoxicillin
R_{amx1000}	Reactor fed with 1000 mg/L of amoxicillin
R_{amx2000}	Reactor fed with 2000 mg/L of amoxicillin
RBC	Rotating biological contactor
R_c	Control reactor with no piggery wastewater in the feed
R_{cps0}	Control reactor fed with 0 mg/L of chlorpyrifos
R_{cps50}	Reactor fed with 50 mg/L of chlorpyrifos
R_{cps100}	Reactor fed with 100 mg/L of chlorpyrifos

R_{cps200}	Reactor fed with 200 mg/L of chlorpyrifos
R_{Cu0}	Control reactor fed with 0 mg/L of copper
R_{Cu10}	Reactor fed with 10 mg/L of copper
R_{Cu20}	Reactor fed with 20 mg/L of copper
R_{Cu40}	Reactor fed with 40 mg/L of copper
R_{Cu80}	Reactor fed with 80 mg/L of copper
R_s	Reactor fed with screened piggery wastewater
R_{sCu40}	Reactor fed with screened piggery wastewater and 40 mg/L of copper
R_{sCu80}	Reactor fed with screened piggery wastewater and 80 mg/L of copper
RFLP	Restriction fragment length polymorphism
R_r	Reactor fed with raw piggery wastewater
R_{rCu40}	Reactor fed with raw piggery wastewater and 40 mg/L of copper
R_{rCu80}	Reactor fed with raw piggery wastewater and 80 mg/L of copper
ROS	Reactive oxygen species
RPM	Revolutions per minute
SAOR	Specific ammonia oxidizing rates
SAUR	Specific ammonia utilization rate
SBBR	Sequencing batch biofilm reactor
SBR	Sequencing batch reactor
SFBR	Single fed and sequencing batch reactor;
SMBR	Sequencing batch membrane bioreactor
SMP	Soluble microbial product
SNIRR	Specific nitrite reducing rates
SNOR	Specific nitrite oxidizing rates
SNRR	Specific nitrate reducing rates
SOUR	Specific oxygen uptake rate
SRB	Sulphate reducing bacteria
SRT	Solid retention time
SRUSB	Sulfate reducing upflow sludge bed
SSCP	Single stranded DNA conformation polymorphism
SSFCWS	Subsurface flow constructed wetlands
STP	Sewage treatment plant
SVI	Sludge volume index
SWW	Slaughterhouse wastewater
TC	Tetracycline
TCP	3,5,6-trichloro-2-pyridinol
TCS	Triclosan
TDS	Total dissolved solid
TGGE	Temperature gradient gel electrophoresis
TOC	Total organic carbon
T-RFLP	Terminal restriction fragment length polymorphism
TSS	Total suspended solids
TWW	Textile wastewater
UASB	Up-flow anaerobic sludge blanket
VFA	Volatile fatty acids
V_{max}	Maximum bio-degradation rate
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant
ZVI	Zero-valent iron



Abstract

The biological processes are widely used for domestic and industrial wastewater treatment to remove the organic matter before its discharge to the natural environment. Various human and industrial activities add undesirable organic and inorganic pollutants (such as heavy metals, pharmaceuticals, agrochemicals, and others) to wastewater, which find their way to the sewage treatment plants. Microorganisms play an essential role in the biological process of sewage treatment. It degrades organic matter present in sewage and utilizes the energy for synthesis of new cells. The undesirable pollutants in wastewater are likely to impart detrimental effects to microorganisms resulting in hindrance in treatment efficiency or sometimes even shut down the operation. Therefore, studies are required to evaluate the behavior, characteristics, and activity of aerobic sludge biomass in presence of undesirable pollutants — such as a heavy metal, an antibiotic, a pesticide, and piggery wastewater. The three fold objective of this experimental investigation are to (i) assess impact of stress imposed by undesirable pollutant on aerobic sludge biomass, (ii) assess the recovery potential of stressed aerobic sludge biomass after discontinuation of stress-causing undesirable pollutant, and (iii) identify the tools and techniques suitable for field application in assessing the health status of aerobic sludge biomass.

The seed biomass has been collected from a facultative lagoon of the sewage treatment plant of IIT Guwahati, Assam, India. It has been used to cultivate aerobic sludge biomass in a growth phase reactor of 1 L capacity for 30 d and thereafter maintained in a mother reactor of 6 L capacity from 31th d onward till the end of the investigation. The experiments have been carried out in batch reactors with a capacity of 1 L by added sufficient amount of aerobic sludge biomass from the mother reactor. The reactors have been fed with stress-causing pollutant along with carbon source. The reactors have been operated at ambient conditions without pH and temperature control for two cycles per day with fill phase of 10-20 min, react phase of 670-690 min, settling phase of 10-20 min, decant phase of 5-10 min, and wash phase of 10-20 min with a total time period of 12 h. The reactors have been labeled as R_{xxyy} , where R stands for reactor; xx stands for stress-causing pollutant [e.g., copper (Cu), amoxicillin IV (AMX), chlorpyrifos (CPS), and screened and raw piggery wastewater (s and r)]; and yy stands for concentration (mg/L) of stress-causing pollutant. For example, R_{Cu10} indicates that reactor has been fed with 10 mg/L of Cu(II), while R_{Cu0} indicates 0 mg/L of Cu(II) in the feed and this reactor is termed as control reactor. The reactor operation has been carried out in two phases. In the first phase, reactors have been fed with varying concentrations of stress-causing pollutants along with the carbon source and operated till steady-state performance has been observed — this phase of operation has been termed as *stressed phase operation*. Thereafter addition of stress-causing pollutants in the feed has been discontinued and reactors have been fed with only carbon source and operated till steady-state

performance has been observed — this phase of operation has been termed as *recovery phase operation*. The duration of both the phases has been chosen based on the steady-state chemical oxygen demand (COD) removal efficiencies. The initial and effluent sample analysis have been carried out to assess environmental related parameters (room temperature, initial pH, and initial concentration of stress-causing pollutant), reactor performance related parameters (effluent pH, residual concentration of stress-causing pollutant, COD, and alkalinity), biomass related general parameters [mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI), interface settling velocity (ISV), and aerobic sludge biomass activity (ASBA)] and microbiological parameters [microscopic and photographic observations, filament index (FI); cell viability test; polysaccharides (PS), protein (PN), extracellular polymeric substances (EPS) contents; enzyme activity test viz. catalase (E_{Catalase}), dehydrogenase ($E_{\text{Dehydrogenase}}$), protease (E_{Protease}), urease (E_{Urease}), and amplified ribosomal DNA restriction analysis (ARDRA)].

The impact of copper metal [Cu(II)] stress on aerobic sludge biomass has been studied with four different Cu(II) concentrations (10, 20, 40, and 80 mg/L). The reactors have been operated for 0-25 d with Cu(II) in the feed (termed as stressed phase operation) and then another 29 d without Cu(II) in the feed (termed as recovery phase operation). COD removal of Cu(II) fed reactors have decreased with increase in Cu(II) doses. The reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 75%, 62%, 49%, and 39% of COD removal, respectively, compared to 91% for control reactor R_{Cu0} during steady-state condition of stressed phase operation. The Cu(II) retention by aerobic sludge biomass of reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 42%, 26%, 10%, and 3%, respectively, at steady-state condition. In addition, presence of Cu(II) in feed has majorly affected SVI and ISV for aerobic sludge biomasses. The Cu(II) exposure to the biomasses has resulted in disintegration of flocs structure, and a significant reduction in filaments has been observed. ASBA and enzyme activities have been drastically inhibited due to Cu(II) stress. The presence of Cu(II) in the feed to the reactor biomasses have caused significant reduction in microbial community structure similarities between control and Cu(II) fed reactors in the range of 38-71%. During recovery phase operation, reactors R_{Cu10} and R_{Cu20} have shown improvement in COD removals with no recovery symptoms observed for reactor R_{Cu40} and R_{Cu80} biomasses. The reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 82%, 72%, 59%, and 41% of COD removal, respectively, in comparison to 91% for control reactor R_{Cu0} during the steady-state condition of recovery phase operation. A low SVI and high ISV with good COD removal have been obtained for reactor R_{Cu10} and R_{Cu20} biomasses. The increased ASBA values have indicated recovery for reactor R_{Cu10} and R_{Cu20} biomasses, whereas a much slower recovery have been observed for R_{Cu40} and R_{Cu80} biomasses. All four enzymes have shown partial recovery for reactor R_{Cu10} and R_{Cu20} biomasses, whereas E_{Catalase} and E_{Urease} have not recovered at all for reactor R_{Cu40} and R_{Cu80} biomasses. The reactor R_{Cu10} and R_{Cu20} biomasses have shown recovery in microbial community structure with similarity in the range of 80-86%. On the other hand, reactor R_{Cu40} and R_{Cu80} biomasses have shown slow and partial recovery in the microbial community

structure with similarity in the range of 61-76%. The reactor performance, aerobic biomass activity, and biomass morphology have been affected due to stress caused by Cu(II) resulting in incomplete recovery of biomass. Cu(II) doses at 40 and 80 mg/L have caused severe impact on reactor performance and biomass morphology leading irreversible changes.

The addition of amoxicillin IV (AMX) stress to the reactors has influenced the performance, flocs, and microbial community structure along with aerobic sludge biomass activity. The reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have been fed with 0, 500, 1000, and 2000 mg/L of AMX, respectively. The reactors have been operated for 43 d with AMX in the feed — termed as stressed phase operation and then another 35 d without AMX in the feed — termed as recovery phase operation. The COD removal for reactors R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ have been 74%, 38%, and 23%, respectively, whereas control reactor R_{amx0} has shown 93% of COD removal during the steady-state condition of stressed phase operation. AMX has encouraged the increase in filamentous growth, and morphological studies have suggested that AMX has transformed the biomass from denser flocs to weaker filamentous form compared to the control reactor biomass. The ASBA values have decreased with increased AMX concentration in the feed. The microscopic examination has revealed abundance of filamentous biomass during stressed phase operation. The photographic observation has indicated floc sizes between 1.0-7.0 mm for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses. The cell viability has decreased to 17% for reactor $R_{amx2000}$ biomass, and PS, PN, and EPS secretion by aerobic sludge biomasses have increased in response to AMX stress. The AMX stress to aerobic sludge biomass has severely impacted $E_{Catalase}$ (51-85% inhibition) as compared to other enzyme activities. ARDRA has revealed 52-80% similarity in microbial community structure in response to 500-2000 mg/L of AMX in feed as compared to control reactor. During the recovery phase operation, all the reactors have shown COD removal of almost 93% at the steady-state condition. Also, reactors have maintained filamentous biomass with slightly increased SVI. ISV has deteriorated due to abundance of filaments in all reactors during the recovery phase operation. The active portion in aerobic biomass has also increased upto 64% for reactor $R_{amx2000}$ biomass in the recovery phase operation. The discontinuation of AMX has allowed the aerobic sludge biomasses to regain their organic degradation capability. All four enzyme activities have also shown improvement and have indicated almost similar enzyme activity values compared to control reactor R_{amx0} biomass. Stressed reactor biomasses have regained their microbial community structure (68-94% similarity) compared to control reactor biomass during recovery phase operation, indicating almost complete recovery after discontinuation of AMX in the feed. However, increase in filamentous biomass during stressed phase operation needs further in-depth investigation.

The presence of chlorpyrifos (CPS) stress has affected the treatment efficiency of CPS fed reactors. The reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} have been fed with 0, 50, 100, and 200 mg/L of CPS, respectively. The reactors have been operated for 34 d with CPS in the feed — termed as stressed phase operation and then another 42 d without CPS in the

feed — termed as recovery phase operation. The COD removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 72%, 39%, and 18%, respectively, whereas control reactor R_{cps0} has shown 91% of COD removal during the steady-state condition of stressed phase operation. The biomass growth especially filamentous part in CPS fed reactors has been constrained and influenced a bit by CPS doses. The ASBA values and cell viability have appeared to have decreased with increased CPS concentration in the feed. PS, PN, and EPS contents in reactor biomasses fed with CPS have varied slightly. $E_{Dehydrogenase}$ and E_{Urease} have significantly been affected due to CPS stress. ARDRA has established the relation between control and CPS fed reactors and has confirmed the microbial community changes (42-79% similarity as compared to control reactor) during stressed phase operation. During recovery phase operation, all reactors have shown enhanced COD removal (more than 88% during steady-state condition). The reactors fed with CPS during stressed phase operation have recovered in terms of filamentous part of the biomass and have shown almost similar results as the control reactor during recovery phase operation. SVI has increased for all reactors due to abundance of filamentous biomass, which also appeared to be a reason for decreased ISV. The discontinuation of CPS has allowed the aerobic sludge biomass to regain its organic degradation capability and cell viability. As soon as CPS has been discontinued from the feed during the recovery phase operation, the enzyme activity of aerobic sludge biomass has returned to its original levels. ARDRA has revealed regain in microbial community structure of reactor R_{cps50} , R_{cps100} and R_{cps200} biomasses and has shown 78-93% similarity compared to control reactor R_{cps0} biomass. Overall, it has indicated complete recovery of aerobic sludge biomasses after the discontinuation of CPS stress in the feed.

The addition of piggery wastewater (PWW) stress has affected the treatment efficiency of PWW fed reactors. The reactors R_c , R_s and R_r have been fed with Lactogen 3, screened PWW, and raw PWW, respectively, and operated for 41 d — termed as stressed phase I operation. The adverse effect of PWW on COD removal has been observed. At steady-state condition, the COD removal for PWW fed reactors (R_s and R_r) have been ~76% and 65%, respectively, while the COD removal for control reactor R_c has been ~95%. Alkalinity for reactors R_s and R_r have drastically increased due to addition of PWW. Significant changes have been observed in SVI and ISV. It could be seen that reactor R_s and R_r biomasses have lower SVI values (93 mL/g and 89 mL/g) as compared to control reactor R_c biomass (127 mL/g). On the other hand, PWW fed reactors have shown higher ISVs (3.05 m/h for R_s and 3.16 m/h for R_r) compared to control reactor R_c biomass has exhibited reduced ISV (2.43 m/h). Decreased ASBA values have indicated deterioration in bio-degradation capabilities of biomass. The morphology of flocs have transformed from denser to pinpoint flocs in reactor R_s and R_r biomasses. The active portion of biomass has decreased in the reactor biomasses exposed to PWW stress. PS, PN, and ESP contents have shown minor changes for PWW stressed reactors compared to the control reactor. The PWW stress to aerobic sludge biomass has indicated severe impact on E_{Urease} (11-73% inhibition) as compared to other enzyme activities, which has reflected the changes in biomass characteristics due to imposed

stresses. The ARDRA result has indicated minor changes in the microbial community of reactor R_s and R_r biomasses (84-92% of similarity) when compared to control reactor R_c biomass. Aerobic sludge biomasses of reactor R_s and R_r have then been equally divided for the next phase of study. The reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been fed with Lactogen 3, screened PWW+40 mg/L of Cu(II), screened PWW+80 mg/L of Cu(II), raw PWW+40 mg/L of Cu(II), and raw PWW+80 mg/L of Cu(II), respectively, and operated for another 31 d — termed as stressed phase II operation. The addition of Cu(II) (40 and 80 mg/L) along with PWW during stressed phase II operation has a significant impact on performance-related parameters. The reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have shown a drastic reduction in COD removal (8-29%), whereas control reactor has exhibited 94% of COD removal. The biomass growth has been further inhibited due to addition of Cu(II). The PWW+Cu(II) fed reactor biomasses have exhibited minimum variation in SVI (73.4-76.0 mL/g) when compared to control reactor R_c biomass (111.6 mL/g). In addition, ISV has been observed to be higher for PWW+Cu(II) fed reactor biomasses (3.36-3.46 m/h) than control reactor R_c biomass (2.22 m/h). The ASBA and cell viability have further decreased due to the combined stresses of PWW+Cu(II). The microscopic examination has revealed disintegration of flocs and disappearance of filaments. In addition, PS, PN, and EPS secretion has been enhanced in response to PWW+Cu(II) stress. Aerobic sludge biomass has indicated severe inhibition of $E_{Protease}$ and E_{Urease} in the range of 69-91% and 77-93%, respectively, compared to other enzyme activities due to PWW+Cu(II) stress in the feed. ARDRA has revealed a drastic shift in microbial community structure due to addition of PWW+Cu(II). The similarity in microbial community structure between control and PWW+Cu(II) fed reactor biomasses have been observed in the range of 29-63%. The PWW+Cu(II) feed has been discontinued in reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} and fed with Lactogen 3 and operated for another 48 d — termed as recovery phase operation. When PWW+Cu(II) has been discontinued in the feed, the steady-state COD removal for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as 78.0%, 63.7%, 57.1%, and 49.2%, respectively. The reactor R_{sCu40} and R_{rCu40} biomasses have exhibited increased biomass growth as compared to reactor R_{sCu80} and R_{rCu80} biomasses. Increased floc size and reappearance of filaments has indicated partial recovery of aerobic sludge biomass. Similarly, ASBA and cell viability have shown increase in their respective values suggesting recovery of biomass. PS, PN, and EPS contents in aerobic sludge biomass have been observed to be stabilized during recovery phase operation. $E_{Catalase}$, $E_{Dehydrogenase}$, and $E_{Protease}$ have shown significant recovery for the reactor biomasses exposed to lower doses of Cu(II), while slight improvement has been observed for the reactor biomasses exposed to higher doses of Cu(II). E_{Urease} has not shown any improvement during recovery phase operation. ARDRA results have suggested 47-86% of similarity between control reactor and other reactors after discontinuation of PWW+Cu(II). These results have indicated only partial recovery of aerobic sludge biomasses, while complete recovery appeared to be non-achievable.

This investigation has indicated that solids analysis (MLSS and MLVSS) of biomasses did not provide an alert in degrading health status of sludge biomasses. SVI and ISV of biomasses have been adversely impacted in the presence of Cu(II) and PWW+Cu(II) stresses in the feed, but have marginally affected in the presence of AMX and CPS stresses. It clearly rules out application of SVI and ISV tests as an indicator for deterioration in biomass health. The microscopic examination has revealed floc structure and shape of biomass, which has significantly changed in presence of Cu(II) and PWW+Cu(II) stressed. On the other hand, AMX stress has encouraged increase in filamentous biomass while CPS exposure has a minor impact on floc morphology. However, microscopic examination is a basic technique and may be used as preliminary test to understand the flocs structure. ASBA has also indicated impacts of Cu(II)/AMX/CPS/PWW+Cu(II) stresses during stressed phase operation, revealing deterioration in organic degradation capability of aerobic sludge biomass. Therefore, ASBA seems to have potential to be used as an indicator for aerobic sludge biomass health status. In addition, cell viability has also shown significant variations in aerobic sludge biomass in response to Cu(II)/AMX/CPS/PWW+Cu(II) stresses. As a result, cell viability test might be utilized as an alerting tool by the treatment plant operators. Moreover, it has appeared that E_{Catalase} and $E_{\text{Dehydrogenase}}$ have been considerably affected in presence of stress-causing pollutants, and these two enzyme activities have potential to be utilized as a warning indicator for biomass quality. The microbial community structure has also shown changes in aerobic sludge biomass, when exposed to Cu(II)/AMX/CPS/PWW+Cu(II) stresses. Therefore, basic techniques such as microscopic examination and ASBA test have appeared to have potential to be used as preliminary testing for getting an alert on biomass health status. In addition, cell viability tests, enzyme activity assays (E_{Catalase} and $E_{\text{Dehydrogenase}}$), and ARDRA might be used as confirmation tests for evaluation of aerobic sludge biomass quality with the help of skilled manpower and laboratory facilities available in nearby institutions of research and higher learning.

Keywords: Aerobic sludge biomass; Copper; Amoxicillin; Chlorpyrifos, Piggery wastewater; COD; SVI; ISV; Aerobic sludge biomass activity; Biomass morphology; Enzyme activity; Cell viability; Filamentous biomass; ARDRA.

Introduction and Literature Review

1.1 Prologue

Rapid urbanization and population growth have increased water demand ([Lewandowski and Boltz, 2011](#)). An increase in water demand has led to an increase in water pollution in the form of sewage, industrial effluents, and runoff from land-based activities ([Jawed, 1996](#)). Almost 80% of the water supplied for domestic use comes back as wastewater ([Gautam et al., 2017](#)). In most cases, untreated wastewater is let out, which either sinks into the ground as a potential pollutant of groundwater or is discharged into the natural drainage system causing pollution in downstream areas. Total sewage generation in Class I and Class II towns in India are 35558 and 2697 MLD, respectively, and total sewage treatment capacity of Class I and Class II towns are 11554 and 234 MLD, respectively, which is 32% and 8%, respectively, of the sewage generation ([Central Pollution Control Board, 2013](#)).

The conventional biological treatment technologies have been and will continue to be the most preferred one for removing degradable substances ([Jawed, 1996](#)), mostly organic from the sewage as it contains 99% water and 1% solid (70% organic and 30% inorganic) contents ([Mara, 2003](#)). The conventional systems are initially designed to remove carbonaceous organic compounds and ammonia, which is harmful to the receiving water bodies ([Seviour and Blackall, 1999](#)). Due to human and industrial activities, many pollutants like heavy metals, pharmaceuticals, agrochemicals, nanoparticles, and phenolic compounds are also discharged into the sewage ([McGrath et al., 1995](#); [Carballa et al., 2004](#); [Zorita et al., 2009](#); [Prabhasankar et al., 2016](#); [Lamba et al., 2020](#)). The occurrence of toxic pollutants in sewage finds its way to the sewage treatment plant ([Tebbutt, 1998](#)) and may upset the treatment plant due to its inherent toxicity. The diverse information is available in literature related to toxic pollutants and their impact on the wastewater treatment processes, without throwing much light on the microbiological aspect of the biological treatment processes and therefore, there is a need to explore the same. The emphasis has always been on reactor

performances in the presence of several pollutants in wastewater, while biomass also plays an important role, but has not been taken into consideration in the research yet.

1.2 Thesis organization

This thesis has been written to present detailed information on the background, objectives, scope, methodology, outcome, and limitations of the research carried out along with some suggestion for the future work based on the experience gained while conducting the studies. In the present research, the emphasis has been laid on the aerobic sludge biomass stressed with toxic compounds and analyzing the results with available literature as those obtained from the experimental studies carried out. This thesis has been organized in seven chapters. The on-going [Chapter 1](#) present a brief discussion about organic matters and its removal from wastewater by biological processes. It also provides information on aerobic and anaerobic processes, the role of microorganisms, and different reactors used in wastewater treatment for organic removal. The source of various pollutants and its impact on treatment efficacy in different reactor setup are explained in this chapter, along with the objective and research methodology of the current study based on the lacunae in the literature. The aerobic sludge biomass exposed to copper (Cu(II)) metal ion is described in [Chapter 2](#). [Chapter 3](#) deals with the aerobic sludge biomass behavior in the presence of amoxicillin (AMX). [Chapter 4](#) focuses on the tolerance of aerobic sludge biomass for chlorpyrifos loading. [Chapter 5](#) deals with the treatment capability of laboratory-grown aerobic sludge biomass treating piggery wastewater along with Cu(II). The tools and techniques that may be used to test the aerobic sludge biomass quality on-site laboratory are covered in [Chapter 6](#). [Chapter 7](#) summarizes key findings of this work along with the limitations for further studies.

1.3 General aspects

The aim of wastewater treatment is generally to allow sewage and industrial effluents to be disposed off without threat to human health or damage to the receiving water bodies ([Grady et al., 2011](#)). The characteristic of wastewater is the main parameter for the selection and design of appropriate wastewater treatment process and the selection of effective treatment technologies ([Orhon, 2009](#)). The wastewater generated from the kitchen, bathing, laundry, urine, and night soil is considered as domestic wastewater or sewage. Besides this, sewage also contains waterborne pathogenic organisms of cholera, jaundice, typhoid, dysentery, and gastroenteritis ([Reynolds et al., 2002](#)), which originate from the night soil of already infected persons ([Dohare and Kawale, 2014](#)). The sewage generally contains biodegradable organics as a major component. The overall objectives of the biological treatment of wastewater are to (i) transform dissolved and particulate biodegradable constituents into acceptable end products, (ii) capture and incorporate suspended and non-settleable colloidal organic solids into biological flocs or biofilm, (iii) transform or remove nutrients such as nitrogen and phosphorus, and (iv) in some cases, remove specific trace organic constituents and compounds ([Metcalf and Eddy, 2003](#)).

1.4 Domestic wastewater composition

Domestic wastewater is a combination of human and animal excreta (feces and urine) and greywater resulting from washing, bathing, and cooking (Bitton, 2005; Mara, 2003; Tjadraatmadja and Diaper, 2006). The chemical characteristics of untreated domestic wastewater are presented in Table 1.1. The organic matter in domestic wastewater is readily biodegradable and composed mainly of proteins (40-60%), carbohydrates (25-50%), fats and oils (10%), urea derived from urine, and a large number of traces of pesticides, surfactants, phenols, antibiotics, heavy metals and priority pollutants (Moriyama et al., 1989; Frølund et al., 1995; Comber and Gunn, 1996; Szilveszter et al., 2010; Mara, 2003).

Table 1.1 Typical composition of untreated domestic wastewater [Source: Metcalf and Eddy (2003)].

Parameters	Concentration (mg/L)		
	High	Medium	Low
Chemical oxygen demand (COD)	800	430	250
Biochemical oxygen demand (BOD ₅)	350	190	110
Nitrogen (total as N)	70	40	20
Phosphorus (total as P)	12	7	4
Total suspended solids (TSS)	400	210	120
Total dissolved solids (TDS)	860	500	270
pH*	8	7.5	7
Oil and grease	100	90	50

* Unit is not applicable

1.4.1 Organic matter

Organic matter or compounds are derived from material that was once alive (i.e., plants and animals). Organic compounds in water are usually complex, non-polar molecules that do not dissolve well in water. It often provides huge amounts of energy to animals and microorganisms (Spellman, 2003). Organic matter in wastewater is regarded as a pollutant before the treatment process. The biological wastewater treatment process depends on microorganisms that degrade organic compounds present in the waste stream (Spellman, 2003). The organic compounds are used as food by the microorganisms. The organic matter is composed of carbon, hydrogen, oxygen, nitrogen, phosphorus, and many additional elements in much smaller amounts (Gray, 2004; Li and Chróst, 2006; Woodard and Curran, 2006).

The biodegradability of organic matter can be divided into (von Sperling, 2007a): *Rapidly biodegradable*: This fraction is usually in a soluble form and consists of relatively simple molecules that can be directly consumed by the heterotrophic bacteria of activated sludge (Henze et al., 1997). *Slowly biodegradable*: This fraction is usually in a particulate form, although soluble organic matter may be present. The slowly-biodegradable matter consists of relatively complex molecules that are not directly used by the bacteria. For this to occur, the conversion into a soluble fraction is necessary through the action of extracellular enzymes.

1.5 Biological treatment options for organic wastewater

Physical, chemical, and biological processes or a combination of these processes have been used to remove organic compounds from the wastewater. The biological approach has been paid much attention due to its economic and ecologic superiority. The complex and toxic organic matters can be treated using biological processes which are cost-effective, safe for environment and more efficient when compared to physicochemical processes, which are expensive and generate toxic byproducts (Jiang et al., 2016; Krishnan et al., 2017; Mirbolooki et al., 2017). Biological treatment of domestic wastewater is a process whereby organic substances are used as nutrition by bacteria and other microorganisms (Gray, 2004; Wang et al., 2009). The biological treatment processes suitable for wastewater containing organic matter are shown in Fig. 1.1. Almost any organic material can be used as food by bacteria,

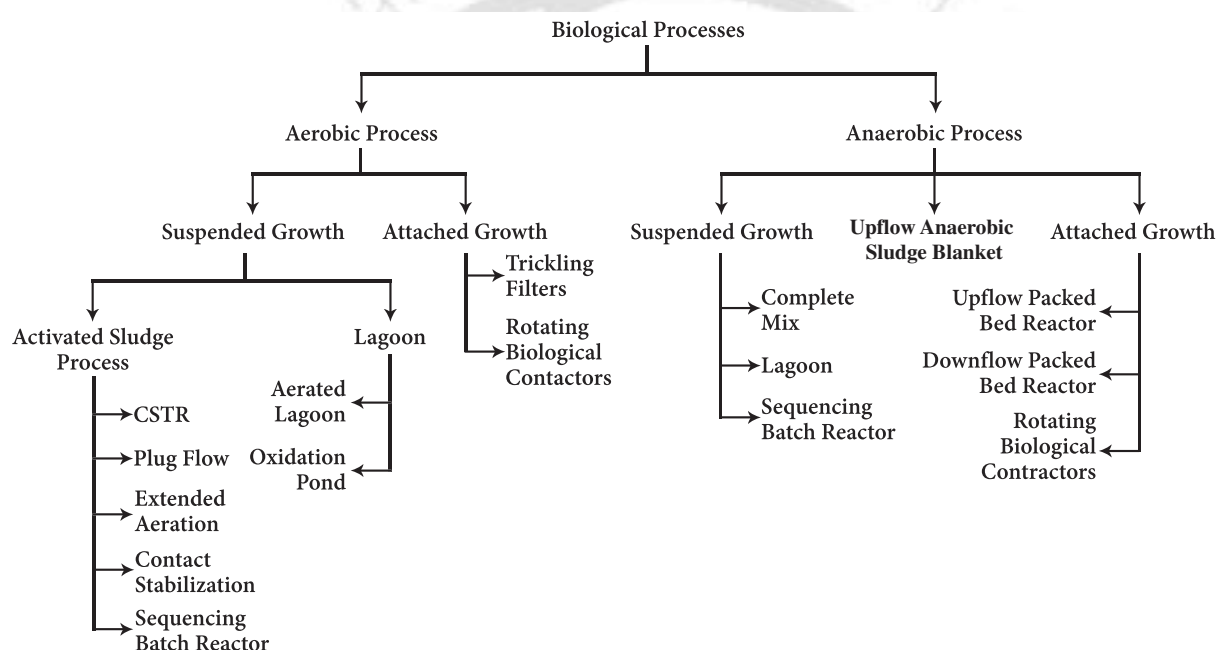


Fig. 1.1 Biological wastewater treatment processes for organic matter removal from wastewater [Source: Metcalf and Eddy (2003)].

fungi, ciliates, rotifers, or other organisms. The cellular enzymes catabolize the complex organic molecules, and synthesize new cell protoplasm (Haandel and Lubbe, 2012; Orhon, 2009). Oxygen is required for microbial metabolism either in dissolved or anion (nitrate and sulfate) forms, which acts as an electron acceptor for aerobic or anoxic systems. Microbial metabolism is a decrease in the quantity of organic pollutants and an increase in the number of microorganisms, water, carbon dioxide, and other byproducts. In anaerobic systems, the electron acceptor is the substances other than oxygen (Orhon, 2009; Wiesmann et al., 2007). (Cervantes et al., 2006). Several types and combinations of biological treatment systems are used in the field of environmental engineering. The oxidation or reduction of organic matter is conducted in reactors that can be classified as aerobic or anaerobic, suspended or attached-growth, with or without mechanical mixing.

1.5.1 Aerobic bio-degradation

In aerobic bio-degradation, microorganisms require oxygen to convert food into energy. Fig. 1.2 shows the pathway of aerobic degradation of organic matter. They can produce oxygenase and peroxidase enzymes that help in the organic pollutant oxidation. A considerable number of bacteria and fungi generally possess the capability to release non-special oxidase and degrade organic pollutants (Elshorbagy and Chowdhury, 2013). The

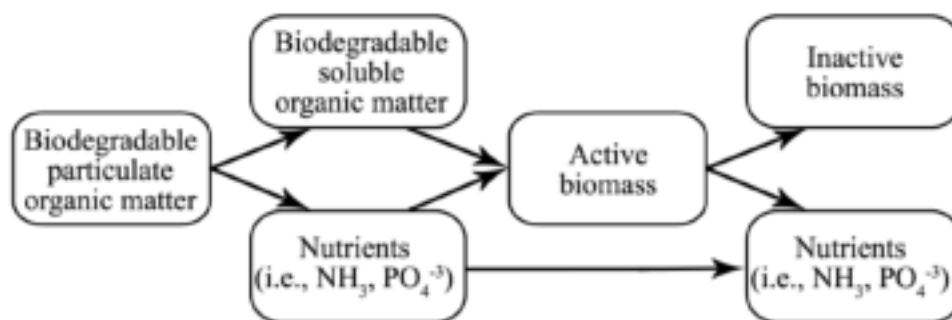


Fig. 1.2 Schematic representation of organic removal by aerobic process [Source: Grady et al. (2011)].

aerobic systems are typically used for wastewater with biodegradable chemical oxygen demand (COD) concentrations less than 1000 mg/L (Grady et al., 2011). The process for bio-degradation of organic matter be either suspended or attached-growth system. In suspended growth process, microorganisms are maintained in suspension by various mixing methods (Gray, 2004). In contrast, microorganisms are attached to inert packing media such as sand, gravel, rock, or a wide range of synthetic materials in the attached-growth system (Wang et al., 2009).

1.5.1.1 Aerobic suspended growth system

In suspended growth systems, the wastewater is continuously mixed to make the concentrations of all constituents uniform throughout the reactor and equal to the effluent concentrations. This system comprises, aggregates of microorganisms, generally growing as flocs in intimate contact with the wastewater (Haandel and Lubbe, 2012). A mechanical mixer or sparger in addition to manage mixing, also help the contact between microorganisms and wastewater. Different suspended growth biological reactors for wastewater treatment are described below.

Activated sludge process (ASP)

In the activated sludge process (ASP), biologically active sludge and wastewater are mixed in an aeration tank and kept in contact until the desired treatment efficiency is achieved. ASP is used for the treatment of both municipal and industrial wastewater. In ASP, microorganisms convert organic material of wastewater to microbial biomass and CO₂ in the aeration tank. Organic nitrogen is transformed into ammonium ion or nitrate, and organic phosphorus is converted to orthophosphate (Haandel and Lubbe, 2012; Manahan, 2001). New bacterial cells are formed during bio-degradation process of organic matter (Gray, 2004). A fraction of

solids is recycled to the head of the aeration tank to maintain the sludge concentration, also known as returned sludge that comes into contact with raw sewage. The remaining fraction of sludge is discarded (Gray, 2004), as shown in Fig. 1.3. Combining a high concentration of

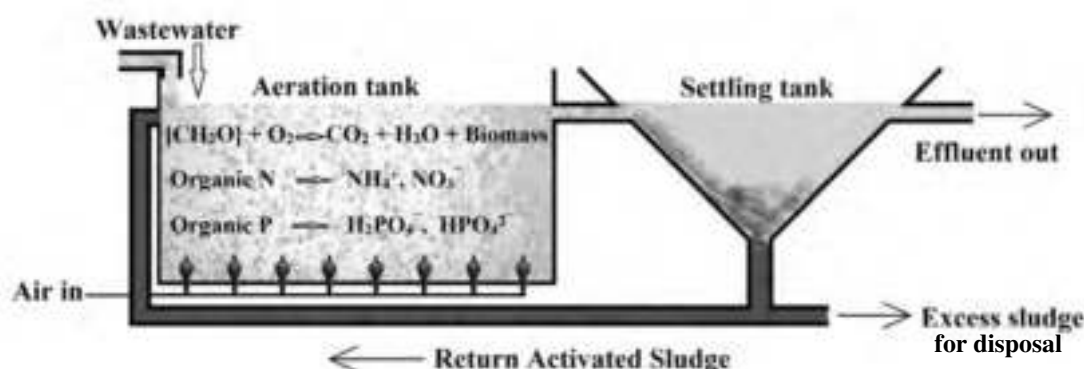


Fig. 1.3 Schematic diagram of activated sludge process [Source: Manahan (2001)].

hungry cells in the return sludge and an abundant food source in the influent waste provides optimum conditions for rapid utilization of organic matter. ASP provides two pathways to remove biochemical oxygen demand (BOD), as illustrated schematically in Fig. 1.4. BOD can be removed by (i) oxidation of organic matter to provide energy for the metabolic processes of the microorganisms, and (ii) synthesis and incorporation of the organic matter into cell mass. In the first pathway, carbon is removed in the gaseous form as CO_2 (Manahan, 2001). The portion of the carbon converted to CO_2 is released into the atmosphere and does not

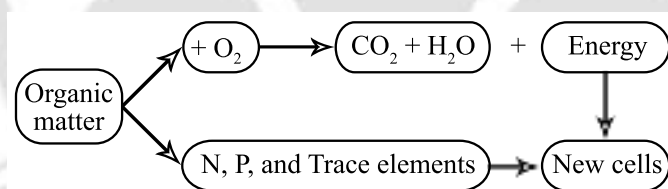


Fig. 1.4 Pathway of BOD removal by microorganism [Source: Manahan (2001)].

cause any disposal problem. There are various types of biological wastewater treatment reactors treating both domestic and industrial wastewater. ASP is one of the most widely used suspended growth treatment processes. Based on the modifications in ASP, other suspended systems have come up which are explained below.

Continuous stirred tank reactor (CSTR)

It is the most widely used reactor setup. The primary settled wastewater and recycled activated sludge are typically introduced at influent points in the aeration tank. The organic load on the aeration tank, mixed liquor suspended solids (MLSS), and the oxygen demand is uniform throughout the tank length (Haandel and Lubbe, 2012; Wiesmann et al., 2007). The liquid volume is constant, and the mixing is sufficient to make uniform concentrations of all constituents throughout the reactor (von Sperling, 2007b). Consequently, these reactors are also called completely mixed activated sludge, as shown in Fig. 1.5(a).

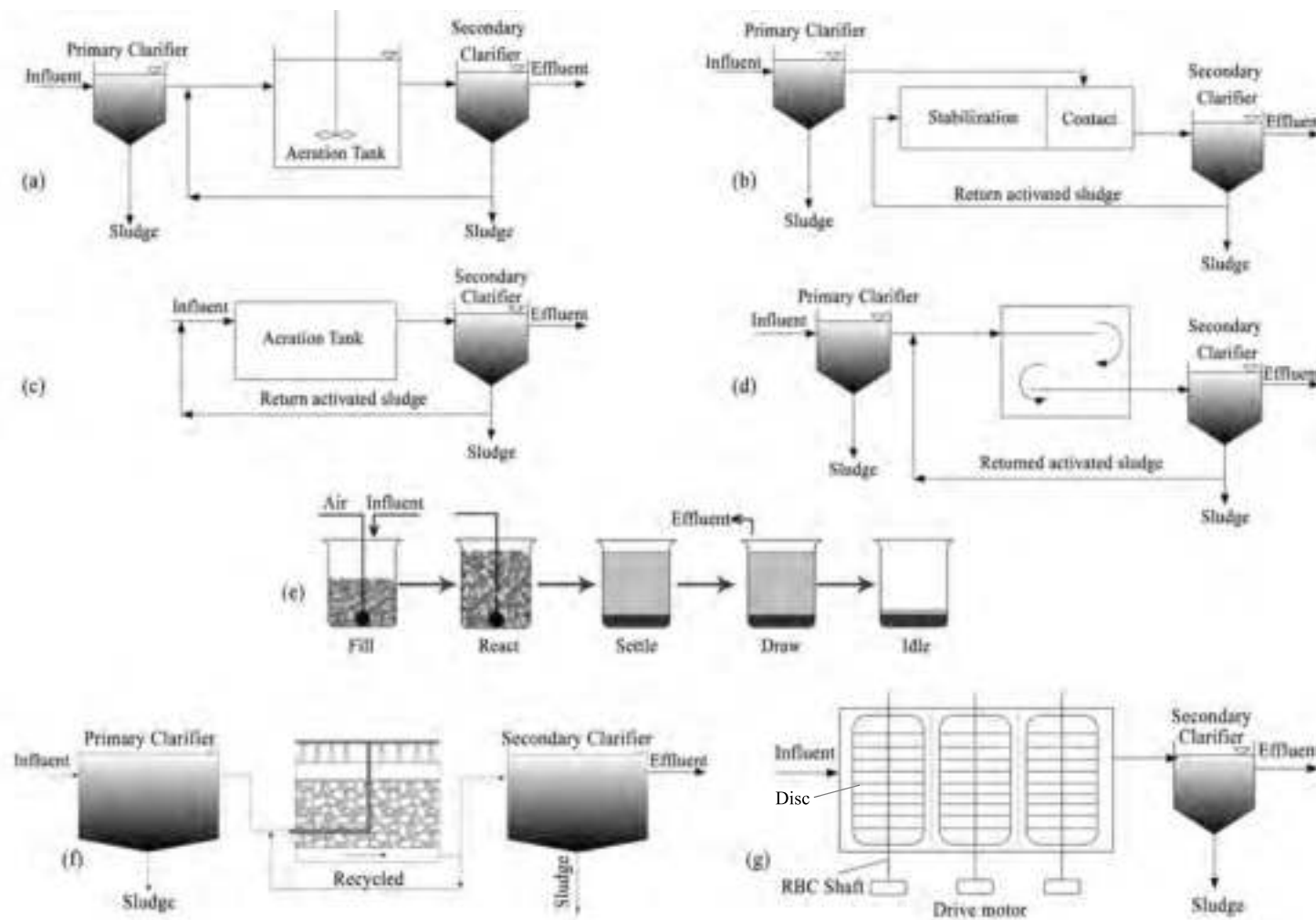


Fig. 1.5 Aerobic suspended growth biological reactors (a) Continuous stirred tank reactor (CSTR), (b) Contact-stabilization, (c) Extended aeration, (d) Plug-flow reactor, (e) Sequencing batch reactor; and aerobic attached-growth reactors (f) Trickling filter, (g) Rotating biological contactor [Source: [Metcalf and Eddy \(2003\)](#)].

Contact-stabilization

The raw wastewater is mixed with returned sludge in a relatively small contact tank in which rapid process of organic matter uptake by hungry microbes take place (Wang et al., 2009). After that sludge and wastewater then move to the stabilization tank, where microbial metabolism occurs (Cheremisinoff, 2003; Woodard and Curran, 2006). As compared with ASP, the aeration tank is also relatively small because only the sludge and bulk liquid in the clarifier proceeds to the aeration tank (Wang et al., 2009) as shown in Fig. 1.5(b). The advantage of contact-stabilization is a lower investment due to small tanks (Riffat, 2012). This modification is convenient only on wastes that are rapidly degradable (high proportion of relatively simple and dissolved organics) (Eckenfelder and O'Connor, 1961). The operation of contact-stabilization process is more complicated than CSTR, and it has too little or no nitrification capability.

Extended aeration

The design objective of this modification of ASP has to lower the costs for waste sludge handling and disposal, at a sacrifice of higher capital costs due to larger tankage and more land area per unit of biochemical oxygen demand (BOD) loading (von Sperling, 2007b). There may be savings in costs resulting from a lower level of intensity required from the operators. However, there is an increase in the amount of oxygen needed per Kg of BOD in the influent due to the increased amount of oxidation (larger surface area provide aeration via the surface of the aeration tank). Typical design values for hydraulic retention time (HRT), food/microorganism ratio (F/M), solids retention time (SRT), and volumetric loading rate (V_L) are 18-24 h, 0.05-0.15, 20-30 d, and 0.16-0.4 kg BOD₅/m³ d, respectively, with BOD removal efficiency of 75-90% (Riffat, 2012; Wise and Trantol, 1994). The reactor is operated in the endogenous decay phase, as evidenced by low F/M ratio. The idea is that the cells remain in the system by continuous recycling with cell mass decreasing due to endogenous respiration (Cheremisinoff, 2003; Wang et al., 2009). The extended aeration process requires high aeration energy and a relatively large aeration tank. It is most adaptable to a small plant (Wise and Trantol, 1994). The schematic diagram is shown in Fig. 1.5(c).

Plug-flow reactor

In plug-flow systems, the tank shape is long and narrow, with a length to width ratio of at least 12:1 (Cervantes et al., 2006). Both returned activated sludge, and feed is added at the beginning of the tank (Henze et al., 2008), as shown in Fig. 1.5(d). The main characteristic of a plug-flow configuration is a high ratio of organic loading to the mixed liquor at the beginning of the tank. There is little longitudinal mixing in a plug-flow tank except for that which is caused by diffused aeration. As the liquor flows through its length (Riffat, 2012), the substrate is used up. The lack of longitudinal mixing reduces the ability to handle shock loads (Haandel and Lubbe, 2012). There is a little dilution of the inflow so that microorganisms may be affected by the pollutant's toxicity.

Sequencing batch reactor (SBR)

Most activated sludge plants perform two fundamental aspects of ASP in two tanks, one for aeration and one for solids separation. SBR allows both operations to be carried out in one tank (Dionisi, 2017), as shown in Fig. 1.5(e). The tank is first charged with wastewater and seeded with activated sludge, and is aerated until the BOD is satisfied. The aeration can be achieved by either a mechanical mixer or compressed air. After that, aeration is stopped to allow the mixed liquor to settle, and the clear supernatant is decanted. Raw wastewater and seed (if necessary) are added, and the cycle repeats itself (Wang et al., 2009). A full range of treatment objectives can be achieved by controlling HRT, sludge age and organic loading rate (OLR) in SBR. In an SBR system, all reactions take place in a single tank, which reduces the land requirement for its installation.

1.5.1.2 Aerobic attached-growth system

In attached-growth processes, organic matters are converted by microorganisms that are grown and attached to an inert packing material. Packing materials include rock, gravel, slag, sand, redwood, and a wide range of plastic and other synthetic materials. Some of the aerobic attached-growth systems are discussed below:

Trickling filter

The trickling filter is the most widely encountered aerobic attached-growth biological treatment process used to remove organic matter from the wastewater. It consists of a highly porous medium bed to which organisms are attached and form a slime layer through which wastewater is percolated (Haandel and Lubbe, 2012; Pepper et al., 2011), as shown in Fig. 1.5(f). The bed medium usually consists of rock or plastic packing material (Spellman, 2003). The tank depth ranges from 0.9 to 2.5 m for stone media trickling filters. These provide durable and chemically inert surfaces for growth of biofilm. Media size ranges from 50 to 100 mm and porosities of 40-50% (Riffat, 2012). The organic matter present in the wastewater is adsorbed on to the biological slime layer (Wang et al., 2009) and degraded by aerobic microorganisms. The thickness of the slime layer increases as microorganisms grow resulting in the oxygen depletion to full depth of the slime layer. Thus, an anaerobic environment is established near the surface of the bed medium.

In addition, due to increment in slime layer thickness, the organic matter is degraded before it reaches the microorganisms near the surface of the medium, causing microbial death and washed off by the flowing liquid (Wiesmann et al., 2007). A new slime layer grows in its place. This phenomenon is referred to as sloughing. Uniform distribution of the wastewater and uniform packing of the reactor with solid substances is highly essential for both high loading and removal rate (von Sperling, 2007b; Wiesmann et al., 2007).

Rotating biological contactors (RBCs)

Rotating biological contactors consist of a series of parallel discs made from highly resilient plastic attached to a metal shaft. The metal shaft is connected to both end of the basin that

contains the wastewater to be treated (Salvato et al., 2003). As the discs rotate, only a portion of the discs assembly is immersed in the wastewater (Spellman, 2003), as shown in Fig. 1.5(g). The diameter, thickness and spacing between the media discs are 2-4 m, about 10 mm and 30 to 40 mm, respectively (Riffat, 2012). The attached microbes absorb organic material and other nutrients from the wastewater while the discs are immersed. As portion of each disc rotates out of the wastewater, it comes into contact with air, thus supplying oxygen for microbial assimilation of the organic substances (Henze et al., 2008). RBCs can be arranged in a single basin with multiple rotating disc assemblies or multiple rotating disc assemblies in two or more basins.

1.5.2 Anaerobic bio-degradation

In anaerobic processes, neither oxygen nor nitrate is present. These processes are carried out by a large group of microorganism, which generally lives in a symbiotic relationship (Henze et al., 1997). In the anaerobic process, insoluble organic pollutant breaks down into soluble substance (amino acids and sugars), and acid-forming microorganisms converts amino acids and sugars into organic acid, which is further converted into CO_2 and CH_4 by methane forming bacteria (Elshorbagy and Chowdhury, 2013). Fig. 1.6 shows the pathway of anaerobic degradation of organic matter. The anaerobic systems can be applied to treat wastewater having COD concentration range of 2000-20000 mg/L (Kolarik and Priestley,

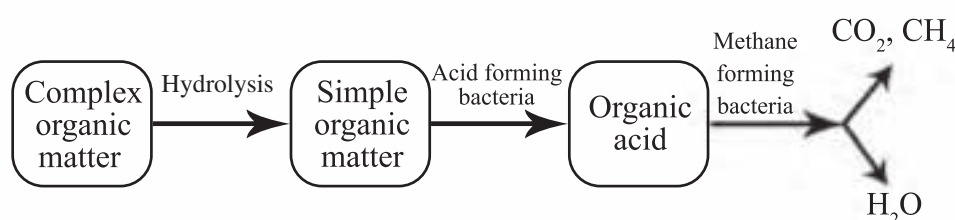


Fig. 1.6 Schematic representation of organic removal by anaerobic process [Source: Wang et al. (2009)].

1996). The effluent quality generally do not meet discharge standards, thereby requiring aerobic polishing. There are two types of anaerobic wastewater treatment systems: attached and suspended growth, as shown in Fig. 1.1.

1.5.2.1 Anaerobic suspended growth system

Suspended growth systems are those in which anaerobic microorganisms feed upon the organic content of wastewater both suspended in a vessel that contains no managed support medium for microorganism attachment. Major anaerobic biological reactors used for wastewater treatment are described below.

Complete-mix anaerobic reactor (CMAR)

Complete mix reactor is a tank with a mixing system. The CMAR without sludge recycling is more suitable for waste with a high concentration of solids or extremely high dissolved organic concentrations. This system is also applicable in a situation where thickening of

effluent solid is difficult. The HRT and SRT are equal and in the range of 15-30 d. The schematic diagram is shown in Fig. 1.7(a).

Contact anaerobic reactor (CAR)

The system is similar to CMAR except that the reactor biomass is separated in the clarifier provided downstream. A portion of the settled solids is recycled having a HRT of 6 to 12 h. The schematic diagram is shown in Fig. 1.7(b). Gravity separation is the most common approach for solids separation and thickening before sludge recycle.

Anaerobic sequencing batch reactor (ASBR)

Anaerobic sequencing batch reactor is considered as a suspended growth process with reaction and solid-liquid separation in the same vessel, as shown in Fig. 1.7(c). The operation of ASBR consists of four steps: (i) feed, (ii) react, (iii) settle, and (iv) decant (Riffat, 2012). During

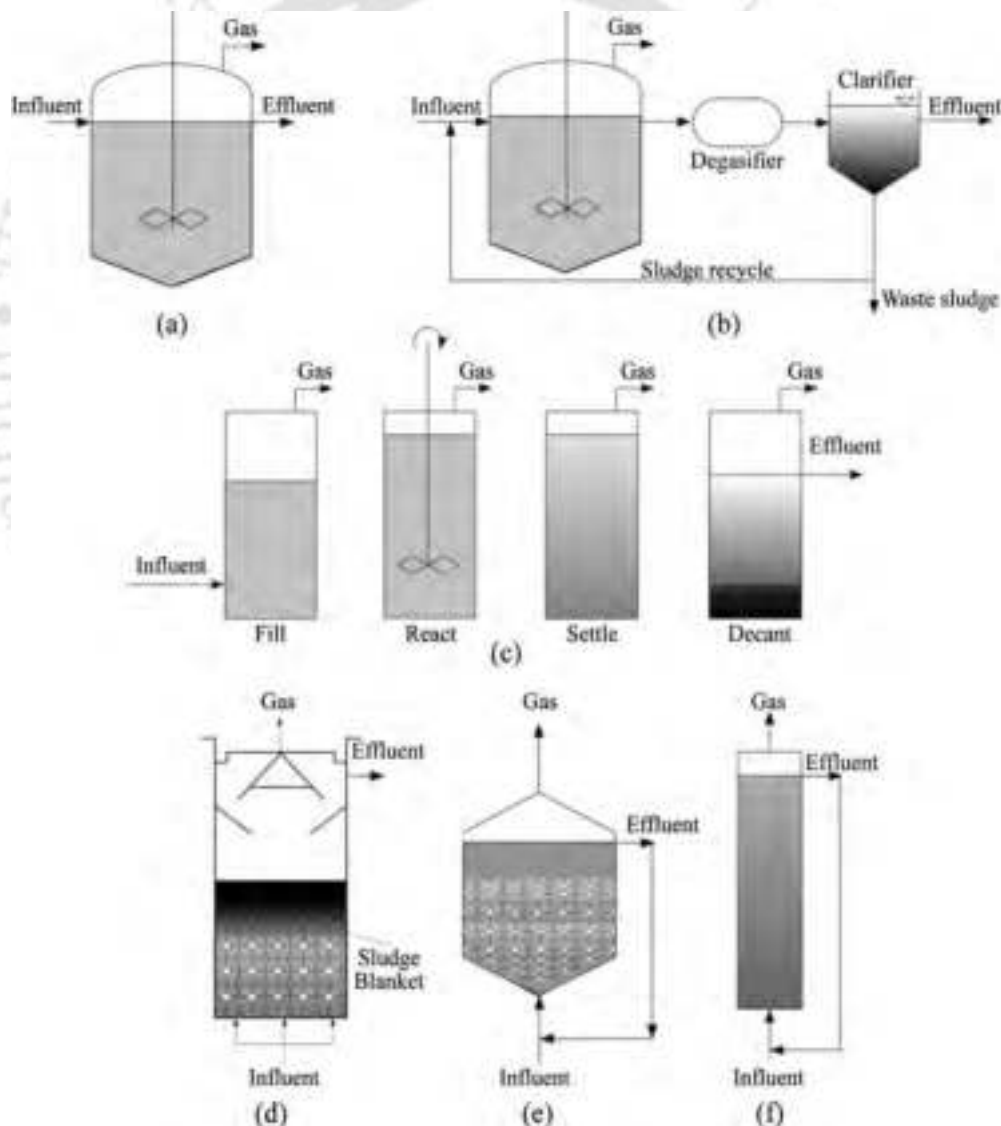


Fig. 1.7 Anaerobic suspended growth biological reactors (a) Complete mix anaerobic reactor, (b) Contact anaerobic reactor, (c) Anaerobic sequencing batch reactor, (d) Upflow anaerobic sludge blanket; and attached-growth biological reactors (e) Anaerobic packed-bed reactor, (f) Anaerobic fluidized bed reactor [Source: Metcalf and Eddy (2003)].

the react period, intermittent mixing for a few minutes in each hour is applied to provide uniform distribution of substrate and solids.

Upflow anaerobic sludge blanket (UASB)

Upflow anaerobic sludge blanket system is one of the most advanced high-rate anaerobic wastewater treatment systems. This systems is efficient at removing 80-90% of COD from wastewater with HRT of 8-10 h. The distinguishing characteristic of the UASB system is the granular bead, one to two millimeters in diameter, containing the anaerobic microbes. These active beads are developed within the anaerobic reactor (Chernicharo, 2015). As wastewater is induced to flow up through the anaerobic sludge blanket, as shown in Fig. 1.7(d), hydrodynamic drag causes the blanket to be fluidized or expanded (Gray, 2004). Under anaerobic conditions, microorganisms metabolize the organic matter and utilize the energy for its growth. Those species that tend, for whatever reason, to form into solid bead-like structures will become incorporated into solids that are too heavy to be carried up and over the effluent weir. Those that do not tend to form into relatively heavy solids will be carried up over the weir and out of the system. The wastewater enters the reactor at the bottom and is distributed across the entire cross-section, flows the upward through the sludge blanket. Organic matter is degraded in the sludge blanket, after which the liquid effluent is discharged from the top (Riffat, 2012). Gas is collected in the hoods and removed. Liquid/solid separation takes place in the settler section.

1.5.2.2 Anaerobic attached-growth system

Anaerobic packed-bed reactor (APBR)

An anaerobic packed-bed reactor consists of a vessel filled with a stationary solid medium to which microbes are attached. The wastewater flows through the vessel and comes into contact with the microbes (Wiesmann et al., 2007). An anaerobic packed-bed reactor can be operated in either the upflow or downflow mode, and effluent can be used to even out variations in raw wastewater flow rate, as shown in Fig. 1.7(e). The most common packing materials are stones and plastic medium of different shapes (Metcalf and Eddy, 2003). The advantage of plastic media includes high surface-to-volume ratio, structural strength, and its inertness.

Anaerobic fluidized-bed reactor (AFBR)

In a fluidized-bed reactor, the media used are typically much heavier (e.g., sand), and the upflow velocity of wastewater is significantly higher (to fluidize the heavier media) (Metcalf and Eddy, 2003). The schematic diagram is shown in Fig. 1.7(f). The raw wastewater enters through the bottom of reactor and flows up through the solid media. The upflow velocity serves to fluidize the bed, allowing water and organic molecule to take a tortuous path through the bed. The organic matter and other solid material attached to microorganisms grown on sand within the reactor (Riffat, 2012). The up-flow velocity of the wastewater helps to keep the microbial film from growing too thick and from bridging between grains to

form blockages to flow. The treated water flows upwards, and collective gas devices collect the gas generated during the operation from the top.

1.6 Microorganisms present in activated sludge

All organisms in a biological treatment plant must necessarily have their origin from the outside. They come from wastewater, air, soil, surrounding, or animals that live close to the plant (seagulls, flies, rats, etc.) (Birkett and Lester, 1999). The microorganisms play a significant role in the biological wastewater treatment process. Microbes degrade the organic matter present in wastewater and utilize the released energy for their growth. The commonly found microorganisms in treatment plants are discussed below.

1.6.1 Algae

This group of microorganisms is photoautotrophs and may be either unicellular or multicellular. Because of the chlorophyll contained in most species, they produce oxygen through photosynthesis (Davis, 2010).

1.6.2 Archaea

They are single-cell organisms with no organized nucleus. However, at the molecular level, they are quite different in the composition of their membranes, cell walls, and RNA. Archaea are often found in environments of extreme temperature, pH, or salinity. Some archaea can grow at 113°C (Wang et al., 2009).

1.6.3 Bacteria

Bacteria occur in large numbers in activated sludge plants. The main task of the bacteria is the primary transformation and degradation of dissolved organic matter. Furthermore, they contribute to the degradation of suspended organic matter by producing extracellular enzymes (exo-enzymes). The content of bacteria in activated sludge may range between 10^{10} - 10^{12} CFU/mL (Henze et al., 1997). Bacteria, particularly the Gram-negative bacteria, constitute the major component of activated sludge.

1.6.4 Protozoa

Protozoa are single-cell organisms that can reproduce by binary fission (dividing in two). Most protozoa are free-living and solitary, but some do form colonies. Most protozoa are strict aerobe, but some, including amoebae and flagellates, can survive in anaerobic conditions. They are desirable in wastewater effluent because they act as polishers by consuming the bacteria. Ciliated protozoa are the most prestigious groups of protozoa in the ASP. Protozoa adds weight to flocs and improve their settleability (Gerardi, 2006).

1.6.5 Fungi

Fungi are multicellular, non-photosynthetic, heterotrophic organisms (Birkett and Lester, 1999; Davis, 2010). Activated sludge does not usually favor the growth of fungi, although some fungal filaments are observed in activated sludge flocs. Fungi may grow abundantly

under specific conditions of low pH, toxicity, and nitrogen-deficient wastes (Bitton, 2005; Gerardi, 2006).

1.6.6 Metazoa

Metazoa are multicellular microscopic animals with sizes varying from 100 to 500 μm . They help in removing freely suspended bacteria (i.e., nonflocculated bacteria) and other small particles from the wastewater (Bitton, 2005). They consume free-swimming bacteria and floc particles, thus contributing to the clarity of the effluent.

1.7 Significance of microorganism

The main role of microbes is to degrade organic matter present in wastewater. In simple terms, the successful operation of ASP is dependent on four major characteristics of the biomass. These are (i) the ability of the flocs to adsorb substrates, (ii) the assimilation and oxidation of organic matter, (iii) the oxidation of nitrogen, and (iv) maintenance of good flocculation to permit efficient settling of sludge (Birkett and Lester, 1999).

1.7.1 Formation of flocs

The flocs size distribution, structure, and density are the most important physical parameters governing the biosolid-liquid separation by gravity settling (Jin et al., 2003; Wilén et al., 2003b). It is a critical operation in the activated sludge process. Settling and compaction ability of activated sludge is crucial to the overall performance and efficiency of the treatment process as well as the quality of the treated wastewater. The particles in activated sludge may be divided into two basic fractions; flocs and primary particles. Flocs are typically 25-1000 μm in diameter and primary particles, such as bacteria and other flocs components, are approximately 0.5-5.0 μm in diameter (Wilén et al., 2003a). The morphology of activated sludge flocs contribute to the effectiveness of biomass separation and the quality of treated effluent (Jin et al., 2003; Liwarska-Bizukoja et al., 2015). The flocs are microbial aggregates containing microorganisms (Hou et al., 2015a). Flocs formation is packaging of a large and diverse population of bacteria in numerous flocs particles (Gerardi, 2006). These bacteria produce three necessary cellular components that enable them to stick together or agglutinate, as shown in Fig. 1.8. These cellular components are (i) pili or fibrils, (ii) sticky polysaccharides,

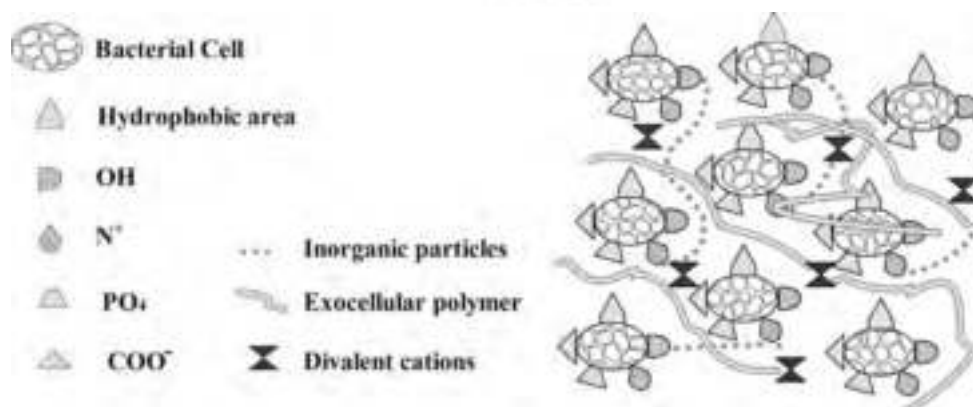


Fig. 1.8 Schematic presentation of an activated sludge floc [Source: Urbain et al. (1993)].

and (iii) poly- β -hydroxybutyrate (PHB) or starch granules. The glycocalyx or sticky coating outside the bacterial cell wall contributes to flocs formation by sticking cells together (Talaro and Talaro, 2002). Filamentous organisms also play a significant role in floc formation. They provide an internal backbone or network of strength to the floc particles and enable the particle to resist against shearing action and increase in size (Henze et al., 2008). The increase in flocs size provides a more significant number and microbial diversity resulting in improved treatment efficiency. The sludge settling problems can be generally related to inadequate bio-flocculation and an improper balance between flocs-forming and filamentous bacteria. The dispersed growth results from incomplete bio-flocculation, leaving a high proportion of individual bacterial cells. Because poor settling, dispersed cells are carried into the effluent, resulting in poor effluent quality (Bitton, 2005).

1.7.2 Enzymes action

A large and diverse population of bacteria and enzymes are necessary to degrade the large quantity and variety of substrates that enter a biological treatment unit. The microorganisms metabolize the organic matter into low molecular weight compounds by hydrolytic enzymes (Nybroe et al., 1992; Yu et al., 2007). The enzymes are placed into two groups depending on where they perform their biochemical reactions. *Endoenzymes* (intracellular enzymes) act within the cell wall, and *exoenzymes* (extracellular enzymes) cross the cell membrane to act in the cell's immediate environment (Koren and Bisesi, 2002; Szilveszter et al., 2010; Pepper et al., 2011). The amount of extracellular enzymes in the bulk solution of activated sludge is negligible, indicating that almost all extracellular enzymes are immobilized in flocs (Szilveszter et al., 2010). The enzymes are a special group of complex proteins found in all living organisms that act as catalysts of biochemical reactions and accelerate the rate of reactions (Pepper et al., 2011). There are two types of reaction that takes place. One is catabolic reactions, which result in a decrease in sludge production; for example, the degradation of stored food in bacterial cells. Second is anabolic reactions, which result in an increase in sludge production; for example, the degradation of organic matters into new bacterial cells (Manahan, 2000). The different enzyme classes and their functions are presented in Table 1.2. Hydrolases are very important in the degradation of organic matter

Table 1.2 Class of enzymes and their roles [Source: Gerardi (2006)].

Enzyme class	Function
Hydrolase	Adds water and breaks large molecules into smaller molecules
Isomerase	Rearranges atoms on a molecule
Ligase	Joins two molecules together
Lipase	Degrades lipids
Lyase	Removes chemical group from a molecule without adding water
Oxidoreductase	Oxidizes one molecule while it reduces another molecule
Peptidase	Degrades peptide bonds
Phosphatase	Transfers phosphate group from molecule to another
Sucrase	Degrades the sugar sucrose

that enter wastewater treatment plants. These enzymes permit the solubilization of complex molecules into simple molecules that can be absorbed by bacterial cells. The decrease in enzymatic activity can occur in enzymes when a change in the structure or electrical charge of the active site occurs. This change is known as denaturation and usually occurs when hydrogen bonds or disulfide bonds ($-S-S-$) are disrupted (Bánfalvi, 2011). Denaturation may be temporary or permanent and may occur due to elevated temperatures, depressed or elevated pH values, and inhibitory or toxic wastes including heavy metals and oxidizing agents such as chlorine and hydrogen peroxide (H_2O_2) (Manahan, 2001). Bacteria degrade first the highly soluble and simple substrates, then the insoluble and complex substrates. The ability of any biological treatment unit to degrade a substrate is dependent upon a diverse population of bacteria and enzymes (Gerardi, 2006).

1.7.3 Microbial community

The knowledge of the microbial composition of activated sludge is important for understanding the mechanism of biological treatment processes (Wagner et al., 2002). The diversity in the microbial community structure helps to degrade a variety of organic pollutants present in wastewater. The microbial community of activated sludge is a mixed population of microorganisms containing many bacteria, protozoa, fungi, viruses, and algae. They are directly involved in the effectiveness of the treatment process in terms of organic matter and nutrient removal (El Moussaoui et al., 2018). Nitrification and phosphate removal is the key properties of the microbial community, which protects natural water bodies (Tiwari et al., 2017). Phylum *Proteobacteria*, β -*Proteobacteria* and *Bacteroidetes* are dominant bacterial species in activated sludge (Hesham et al., 2011). It is known that bacterial community structures in different municipal wastewater treatment plants (MWTPs) are quite different because of the differences in wastewater composition and operational condition. Occurrence of toxic pollutants in wastewater may affect the microbial diversity of activated sludge and lead to the disappearance of intolerant species from the treatment plant. Zheng et al. (2016a) have reported that the abundances of some genera decrease with the increase of chlortetracycline (CTC) concentration, such as *Nitrospira*, *Hyphomicrobium*, *Azospirillum*. However, the abundance of the genera *Flavobacterium*, *Azonexus*, and *Thioalbus* has increased with the increase of CTC concentration. It is reported that 100-200 mg/L of titanium dioxide nanoparticles (TiO_2 NPs) reduce the microbial diversity in activated sludge. The abundance of denitrifying bacteria significantly has decreased due to the inhibitory effect of TiO_2 (Li et al., 2014).

Molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), terminal restriction fragment length polymorphism (T-RFLP), single-stranded DNA conformation polymorphism (SSCP), fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and amplified ribosomal DNA restriction analysis (ARDRA) are available to explore the microbial community structure in activated sludge (Akbar et al., 2014; Das and Dash, 2015; Schwieger and Tebbe, 1998; Fernández et al.,

2008; Błaszczuk et al., 2011; Yang et al., 2011). Among these techniques, ARDRA is one of the most useful for studying bacterial phylogeny. It is highly cost-effective and less time-consuming. This technique can be performed in any standard laboratory conditions with regular molecular biology facilities (Das and Dash, 2015). This technique is a modification of the RFLP technique, which is applied to the 16S rRNA gene sub-unit of bacteria (Alves et al., 2005; Ventura et al., 2000).

In this technique, the bacterial 16S rRNA gene is amplified by polymerase chain reaction (PCR). Subsequently, the amplified product is digested with suitable restriction enzymes to yield products of various sizes (Massol-Deya et al., 1995). Thus, based upon the banding pattern of the restriction products, the phylogenetic relationship among the strains can be derived (Das and Dash, 2015). Mostly tetra-cutter restriction enzymes are used for this technique (Heyndrickx et al., 1996). However, to obtain a statistically significant result, at least three restriction enzymes are supposed to be used during this procedure (Das and Dash, 2015). Analysis of the pattern of bacteria is used to obtain the clusters of related bacteria, represented by the formation of a dendrogram (Błaszczuk et al., 2011). The tree drawn from this approach indicates the relationship of the organisms based on the restriction pattern obtained from the respective 16S rRNA genes. Related organisms are supposed to provide the same restriction pattern after digestion with the restriction enzyme (Gich et al., 2000).

A tetra-cutter restriction enzyme digests the 16S rRNA gene at particular sites to provide a unique restriction pattern, considered a signature of that organism (Das and Dash, 2015). Thus, the restriction pattern of one organism can be compared with the restriction pattern of another to deduce the phylogenetic relationship between them (Yang et al., 2012).

1.8 Source of heavy metals, pharmaceuticals, and pesticides in sewage

The treatment and safe disposal of sewage generated from urban areas in developing and under-developed countries is a matter of serious concern. Hence, the Urban Local Bodies (ULBs) have geared to tackle it on a priority basis for the last couple of decades. This has led to the design and construction of newer sewage treatment plants either as a centralized or decentralized facility, depending upon area-specific suitability. A large fraction of water coming out from households, hospitals, industries, commercial complexes, hotels, etc., become sewage after use. Various activities from above sources release different pollutants and discharge it to the nearby drain that finds its way to the sewage treatment plant. Fig. 1.9 explains the various sources of heavy metals, pharmaceuticals, and pesticides to the sewage. The electroplating activities can be considered as a small scale industrial activity (may also be termed as a cottage industry) which is a primary source of heavy metals. Similarly, wholesale stockiest of drugs (such as water-soluble/insoluble antibiotics amongst others) and pesticides/insecticides may fall under small scale commercial category. Many small scale industries/commercial outlets may not be able to comply with the pollution control norms mainly due to lack of awareness, financial resources, technical expertise and, therefore, find it easy to dump the wastes/spillages to the sewer networks either by ignorance or knowingly.

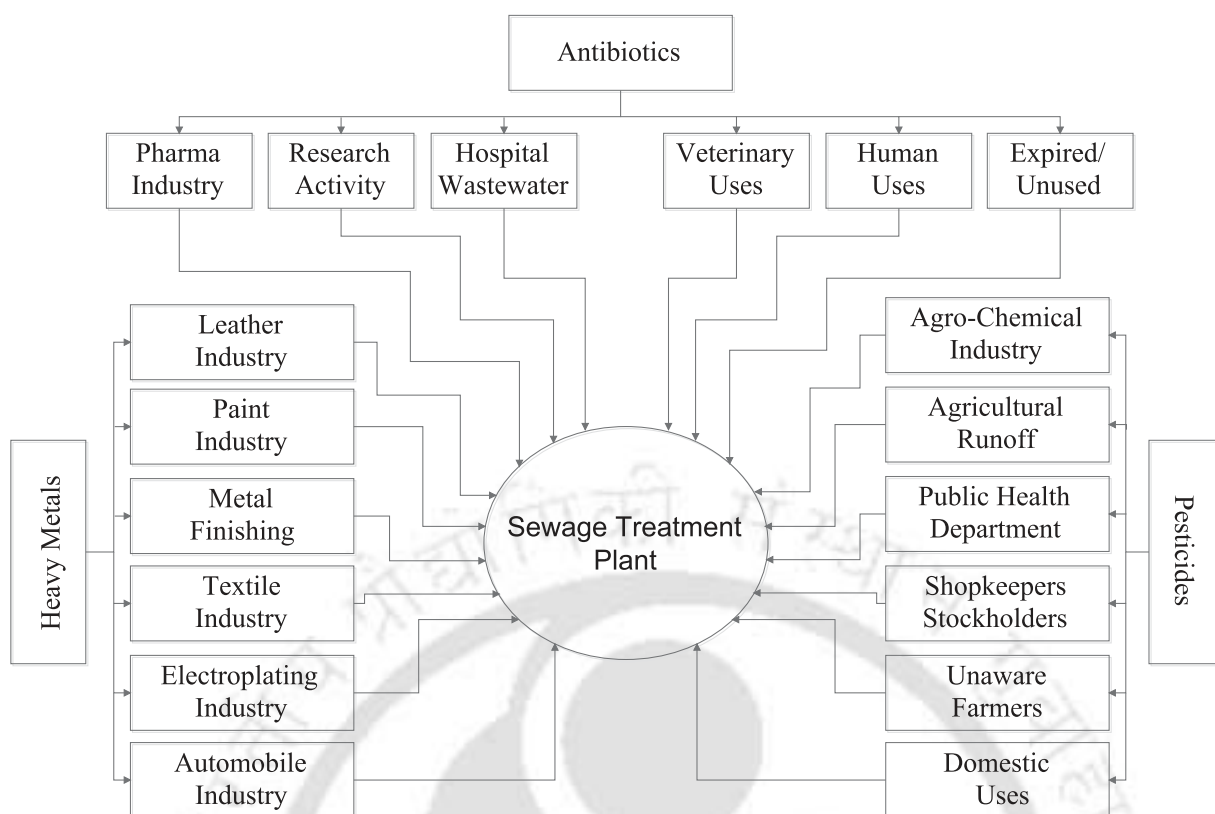


Fig. 1.9 Source of toxic pollutants to sewage treatment plant [Sources: [Bound and Voulvoulis \(2005\)](#); [Pamukoglu and Kargi \(2007a\)](#); [Tiwari et al. \(2017\)](#); [Dar et al. \(2019\)](#)].

The primary sources of heavy metals, pesticides, and pharmaceuticals and their concentration in sewage are discussed below in brief.

1.8.1 Heavy metals

Electroplating industries have a long history in India. With the increased demand for non-corrosive artistic looking consumer items, there has been a remarkable growth in electroplating units. The growth of these units is mainly urban-oriented ([Edelman, 1997](#)). More than 3000 authorized and unauthorized electroplating units operate in different industrial pockets in Delhi ([Singh, 2014](#)). In the plating process, the items to be plated are placed (as the cathode) in an electrolytic bath containing metal salts. A low voltage direct current is passed through the electrolyte; wherein metal ions are plated onto the cathode ([Schlesinger and Paunovic, 2010](#); [Sebayang and Hasan, 2012](#)). The plated items are washed with water and are dried in an oven. Several chemicals are used, such as numerous acids, alkalis, inorganics, and organics ([Tarvin, 1956](#)), depending upon the surface properties of items to be electroplated. Large quantities of metal salts are lost during the normal rinsing operations, accidental wastage through pipeline breakages, cracks in tanks, negligent operations, or the willful dumping of contaminated processing solutions ([Förstner and Wittmann, 1979](#)).

In electroplating units, the primary source of waste is identified as drag out losses, concentrated liquid wastes, spent acid bath, spent alkali baths, and spent passivation dip and rinse water ([Babu et al., 2009](#); [Tarvin, 1956](#)). The electroplating wastewater has a low

pH in the range of 3.0-5.0 and contains several metals in soluble forms. Due to a lack of proper disposal facilities and environmental awareness, small-scale electroplating industries are located in unauthorized zone and discharge effluents in drains. The wastewater samples collected from drains from the industrial belt in Delhi have indicated presence of nickel, total chromium, hexavalent chromium, copper, zinc, lead, and cadmium in the range of 1.7-105, 0.25-108, 0.01-60, 0.6-30, 0.02-9, 0.01-1.2, and 0.01-0.14 mg/L, respectively (Singh, 2014). In another study, Singh and Ram (2016) have observed presence of chromium, nickel, lead, zinc, and copper in the range of 21.5-47, 72-243, 5.3-9.7, 97-731, and 8.4-20.7 mg/L in electroplating industrial effluents (drain) in Chandigarh and nine districts of Haryana (namely Faridabad, Gurgaon, Yamunanagar, Karnal, Jagadhri, Panchkula, Panipat, Ambala, and Sirsa). Since the electroplating industries are small scale and operating from a small area, it may not have adequate storage space for safekeeping of the mother/spent bath liquor. Therefore, it is easier to dump the same to the sewer networks as the sewer network might be accessible easily. Tyagi (2013) has reported that there are 400 registered electroplating units in the state of Tamilnadu (located in Thiruvallur, Kancheepuram, Krishnagiri, Madurai, and Coimbatore). There are 80 electroplating units located in a mixed residential area in Madurai, and 58 units are under operation. These units are tiny and small, with inadequate space to provide an effluent treatment plant. The untreated wastewater is discharged into the public sewer. Major auto manufacturers and engineering units are located in Pune, Nasik, and Aurangabad. The electroplating activity is generally outsourced, resulting in the unplanned growth of these units. More than 500 units operating are not registered, and 90% of these units are small scale.

Similarly, there are 400 plating units in Gujarat. Most of the units are operating in residential areas with inadequate industrial infrastructure facilities. These are tiny units having no space for treating effluent. There are about 1000 electroplating units in Ludhiana connected to a common effluent treatment plant (CETP). The effluent is conveyed through tankers to CETP. The inferior working practices due to the unskilled and untrained workforce, insufficient working space, and improper layout for to wastage of chemicals, water, and increased effluent volume, thereby lead to contamination in sewage. A survey carried out by Central Pollution Control Board (CPCB) in Bengaluru city suggest that effluent generated from the electroplating industries found in the range of 20 L/d to 1500 L/d in case of industries sending their effluent to CETP, whereas in case of units having their individual effluent treatment plant (ETP), generating effluent in the range of 12000 to 29000 L/d (Central Pollution Control Board, 2014). In raw effluent, the concentration of total chromium, copper, nickel, iron, manganese and zinc have been observed in the range of 0.454-930, 0.412-19.3, 0.2-133, 0.9-111, 18-4.38 and 34.7-67 mg/L, respectively. Based on the study, the estimated effluent quantity is worked out to be around 16520 L/d (assuming average effluent generation is around 295 L/d). In raw effluent of another industrial area, the concentration of zinc, total chromium, copper, nickel, iron, and manganese have been estimated in the range of 0.551-1499, 0.1-778, 0.102-2474, 0.053-70.7, 0.471-383, and 0.05-188 mg/L, respectively.

The estimated effluent quantity is worked out to be around 3750 L/d (assuming average effluent generation is around 250 L/d) based on the study. Most of the units have been found storing their effluent in underground storage tanks. Due to the acidic nature of effluent, it may cause damages in the storage tanks and eventually percolate or may leach. The discharge or bypass of effluent into nearby underground drain/storm water drain may not be ruled out.

1.8.2 Pharmaceuticals

In recent years, increasing attention has been directed towards the discharge, presence, and potential effects of pharmaceuticals in the environment. Antibiotics are an essential group of pharmaceuticals used in health care for humans, animals, aquaculture, and agriculture to treat and prevent bacterial infections (Diwan et al., 2010). The pharmaceutical substances are a complex and synthetic chemical formulations that tend to react within the environment (Kurunthachalam, 2012). India has a USD 3.1 billion pharmaceutical industry, with a growth rate of 14% every year. After North America and Europe, India has the third-largest pharmaceutical industry in the world. The current market size for pharmaceuticals in India is USD 41 billion which is expected to reach USD 130 billion by 2030 (Sarwal et al., 2021). The consumption of antibiotic drugs has increased by 35% from 2000 to 2010. India has been the largest consumer of antibiotics in 2010 with 12.9×10^9 units (Van Boeckel et al., 2014). Worldwide, in 2010, at least 63,200 tons of antibiotics have been consumed in livestock-likely to be more than all human consumption. By 2030, this figure is projected to rise by two-thirds, to 105,600 tons, to meet the demands of a projected 8.5 billion human population (Van Boeckel et al., 2014).

Kurunthachalam (2012) has suggested the routes through which pharmaceutical chemicals can enter in the environment such as (i) from small scale pharmaceutical industries; (ii) improper and direct disposal by humans of unused/expired drugs into the trash and through the excretion of urine or feces; (iii) release from hospital waste; (iv) disposal by pharmacies; (v) veterinary use as a medicine; (vi) dairy waste disposal; (vii) household water/sewage; (viii) leaching from defective landfills; (ix) release from aquaculture; (x) release from molecular farming/pest control drugs; (xi) disposal of euthanized/medicated animal carcasses, etc. In India, hospital standards are reportedly inconsistent. Drug prescription pattern is irrational (Global Antibiotic Resistance Partnership (GARP), 2011). It would be a subject of interest to assess pharmaceuticals' concentration in the Indian wastewater (Singh et al., 2014).

In England, less than 53% of patients have been reported to complete their treatment. Disposal of pharmaceuticals via toilet or sink is generally lower in European countries than in the United States, with amounts of 28 to 53.8% reported in the United States (Hester and Harrison, 2015). The expiry date does not direct that the medicine will lose its potency entirely and will no longer be active or become harmless after the expiry date. Many of them retain 90% of potency for up to the next five years after the declared expiry date (Pomerantz, 2004). In certain medicines like antibiotics, using long expired medicines can contribute to

increase antibiotic resistance and treatment failures ([Mani and Thawani, 2019](#)). Being a massive consumer of medications, regulatory guidelines for safe disposal of medicines by consumers lack in India, and medicine takeback programs are not functional/effective as in the other countries.

National Formulary of India (2011) specifies guidelines for the disposal of medicines. Most people are not aware of and do not follow these guidelines ([Sonowal et al., 2017](#)). Common worldwide practice for unused medicines is to flush in the sink or dispose of in garbage has been reported by [Tong et al. \(2011\)](#). Food and Drug Agency (FDA) USA recommends that the most effective way to eliminate the potential harm of medicines is to flushing down to the toilet ([Patil and Patil, 2013](#)). The number of unused pharmaceuticals dispose of via the toilet or sink in Europe has been reported to be 14% in Germany, 3% in the Netherlands, 10.8% in Norway, 0% in Sweden, and 11.5% in the United Kingdom ([Hester and Harrison, 2015](#)).

The studies indicate that 33% of the total volume of drugs sold in Germany and 25% of those sold in Austria are disposed of through household waste or domestic wastewater. Disposing unwanted medicines in the trash and flushing down the sink have been 50.5% in India ([Lagishetty et al., 2015](#)). [Fick et al. \(2009\)](#) have reported that the ciprofloxacin, enoxacin, cetirizine, terbinafine, and citalopram have been detected at more than 1 µg/L in several wells in Hyderabad. Very high concentrations of ciprofloxacin (14 mg/L) and cetirizine (2.1 mg/L) have been found in the treatment plant's effluent, together with high concentrations of seven additional pharmaceuticals. Ciprofloxacin (up to 6.5 mg/L), cetirizine (up to 1.2 mg/L), norfloxacin (up to 0.52 mg/L), and enoxacin (up to 0.16 mg/L) have also detected in the two lakes, which clearly shows that the investigated area has additional environmental sources of insufficiently treated pharmaceutical waste ([Fick et al., 2009](#)).

Antibiotics might also be added to the environment from pharmaceutical plants due to the dumping of unused antibiotics ([Diwan et al., 2009, 2010](#)). The hospitals and research laboratory/centers also contribute a massive amount of pharmaceuticals to the environment. The total number of hospitals maintained by the Indian Government and their bed capacity, including rural and urban areas, are 37,725 and 7,39,024, respectively ([Press Information Bureau, 2018](#)). The total number of private hospitals and nursing homes in India is around 32,048 ([Ministry of Health and Family Welfare, 2017](#)). There are 6,844 drug manufacturing industries and 9,00,000 retail stores across India, distributing medicines ([Ayub and Mustafa, 2017](#)). Hospitals are the primary sources of pharmaceutical compounds. They include active principles of drugs and their metabolites, chemicals, heavy metals, disinfectants and sterilants, etc. ([Binh et al., 2018](#); [Verlicchi et al., 2010](#)). Most hospitals are in a common practice of co-treatment of the hospital and urban wastewater at an MWTP because of the land unavailability in an urban area. Apart from pharmaceutical industries, wastewater generated from other sources such as hospitals, poultry farms, and aquaculture find its way to the sewage treatment plant with a high concentration of organic matter and pharmaceuticals.

1.8.3 Pesticides

Farmers use pesticides to control insects, rodents, and other organisms to protect the crops and preserve food materials (Sharma et al., 2013; Mohanty et al., 2013). Pesticides have played a significant role in achieving maximum crop production. However, maximum usage and accumulation of pesticide residues are highly detrimental to aquatic and other ecosystems (Agarwal et al., 2015). Intense pest control activities, often based on heavy pesticide use, promote hazardous waste build-up in many pesticide handling phases. Due to a lack of education and guidance in the proper management of small quantities of pesticide-related waste, hazardous chemicals are often left lying around in rural and urban areas (Damalas et al., 2008).

India is the fourth-largest producer of agrochemicals globally, after the United States, Japan, and China (Koli and Bhardwaj, 2018). The demand for chlorpyrifos (CPS), one of the major pesticides, has been reported as 8694 MT, and consumption reported as 5795.78 MT in 2010-16 in India. About 150 industrial units are manufacturing pesticides (technical) and about 500 industrial units are engaged in formulations in the country (GCPC-ENVIS, 2015). The total production of pesticides in India has been reported as 2,12,700 MT until September 2018 (National Data Sharing and Accessibility Policy (NDSAP), 2017). Pesticides are categorized by their lifetime of effectiveness as non-persistent (lasting several days to about 12 weeks) and moderately persistent (lasting 1 to 18 months) (Koren and Bisesi, 2002). The concentrations of the pesticide continue to increase in the soil with time due to continuous application. Where leaching occurs, the pesticide can move into surface or underground water supplies. CPS has been detected in seawater and marine sediments, drinking water wells, river water, and soil (Mutua et al., 2015).

The half-life of CPS ranges from 7 to 120 d (Wang et al., 2017a). The half-life of CPS in aerobic aquatic systems and urban stream sediments is 22-51 d and 20-24 d, respectively (Yu et al., 2019b). The organophosphate insecticides are very toxic in nature and may vary in human. Even 120 mg of parathion has been known to kill an adult human, and a dose of 2 mg is enough to killed a child (Manahan, 2001). The pesticides are in concentrated form or even in diluted form may constitute severe hazards. The farmers of India, who lack a technical understanding of pesticides, their uses, and safety aspects, are vulnerable to misguidance, which increases the chance of unnecessary and inappropriate disposal of pesticides (Bondori et al., 2019; Koli and Bhardwaj, 2018). Large-scale pesticide manufacturers can afford sophisticated recovery, treatment, and cleanup techniques. However, small-scale pesticide users, single farms, or small application businesses struggle with pesticide-waste problems, including contaminated soils and disposal of unused products and equipment rinse water (Bondori et al., 2019). Mohanty et al. (2013) have reported that nearly half of the farmers dispose of leftover pesticide by burying, whereas one-third of the farmers dispose of leftover pesticide in the field. Agarwal et al. (2015) have reported varied residual pesticide concentration in water across India, ranging ng/L to µg/L level.

1.9 Impact of heavy metals, pharmaceuticals, and pesticides

Urban centers in high-income countries have households, commercial complexes, hospitals, kitchen and terrace gardens, parks, and manufacturing hubs, which discharge their wastewater to sewers. The centralized sewerage system closely interlinks all of them. The release of toxic contaminants from any of the above sources finds its way in the sewage network. However, a centralized sewerage system is inappropriate in low and middle-income countries because of its dense population (Williams et al., 2019). There is an advantage of the centralized sewage collection system over the decentralized system regarding toxic waste discharge. The discharge of toxic wastewater to the centralized system gets easily diluted due to high volume sewage flow in the centralized system and mixed large volume of sewage in the aeration tank. However, the decentralized system has a different and lower flow rates depending on the season and time (day/night), and wastewater treatment capacity is lower than the centralized system. India is a populous lower-middle-income country where centralized sewage connectivity is not much in practice. Sewage consists of a mixture of biotic and abiotic materials from humans, animals, and chemical by-products from industries (Le et al., 2018). The domestic wastewater treatment plant is designed to treat the organic matter coming from the households. However, it fails to remove even trace concentrations of anthropogenic organic chemicals (Zumstein and Helbling, 2019). The successful plant operation relies on the rapid bio-degradation of organic matter and solid-liquid separation (Szilveszter et al., 2010). Unfortunately, bio-degradation and separation are not always met due to the intermittent toxic pollutants in wastewater that directly or indirectly affect the plant operation. Many toxic substances present in sewage may exert inhibitory effects on microorganism resulting in the impairment of the enzymatic system of the living cell or direct damage to the cell structure (Henze et al., 2008).

The toxic effect on microorganisms is revealed by difficulty in nutrients uptake and a reduced cell growth or cell death. These toxic compounds are known as stress-causing agents and exposure duration of stress-causing agent to microorganisms can be termed as the *stressed phase*. Stress-causing agents include heavy metals, phenolic compounds, nanoparticles, pesticides, active pharmaceutical compounds, endocrine disruptors, etc. that inter-react with microorganisms and affect their metabolisms, cellular activities, and growth rates. Antibiotics exert antimicrobial effects on the biosynthesis of peptidoglycan in bacterial cell walls, which cause morphological and cytoplasmic membrane damage and further affect the normal microbial function resulting in the COD inhibition (Liu et al., 2018a). Heavy metals and pesticides can inhibit microbial activities due to its toxicity. Various stress-causing agents found in sewage and their level of impact on aerobic sludge biomass and treatment plant performance are discussed below in brief.

1.9.1 Heavy metals

Heavy metals (such as copper, zinc, lead, mercury, chromium, cadmium, iron, nickel, and cobalt) are extensively used in industries such as automobile, metal finishing, leather tanning,

electroplating, petroleum, textile-dyeing, etc. (Pamukoglu and Kargi, 2007b; Li et al., 2011; Yan et al., 2015). Small-scale industrial effluent containing a high concentration of heavy metals unintentionally find its way to the sewerage system (Brown and Lester, 1979; Sterritt and Lester, 1981). Metals such as nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), and lead (Pb) are considered toxic and can affect the biological processes (Hartz et al., 1985). The impact of heavy metals on various parameters of biological processes is presented in Table 1.3. The wastewater treatment plants are a logical focal point for the control of heavy metals discharged to the aquatic environment (Nelson et al., 1981). Biological removal of metals is critical because their overall retention depends on absorption or accumulation by the activated sludge biomass (Rudd et al., 1984). Biological wastewater treatment systems are sensitive to heavy metals that may upset the bio-degradation process. The trace amounts of metals are required for the growth and metabolism of microorganisms; however, metal beyond a specific concentrations can inhibit microbial activities. Dilek and Yetis (1992) have reported that heavy metals up to 10 mg/L of Ni(II), 50 mg/L of Cr(VI), and 10 mg/L of Cu(II), have no significant impact on treatment efficiency. Moreover, these heavy metals have stimulated the bio-kinetic parameters, maximum specific growth rate, and half-substrate saturation coefficient. The presence of Cu(II) and Cd(II) can significantly affect specific oxygen uptake rate (sOUR), microbial activities, COD, and metals removal efficiencies (Ong et al., 2005). Microbial cells protect themselves from metal toxicity by synthesizing the extracellular polymeric substance (EPS). It can be stimulated by 1 mg/L of Cu(II) (to protect the cells), while higher concentrations of Cu(II) can inhibit EPS production (Wen et al., 2016). Cabrero et al. (1998) have demonstrated the impact of different concentrations (1-20 mg/L) of Zn(II) and Cu(II) and combination of both metals on bio-kinetic parameters and have observed Cu(II) as more toxic than Zn(II) with a severe detrimental effect beyond 10 mg/L. However, a concentration of 10 mg/L and higher has caused severe upsets for the system. Combined effects of Cu(II) and Zn (II) on the activated sludge growth kinetics have indicated that these two heavy metals act neither synergistically nor antagonistically (Cabrero et al., 1998). There is no impact of metal combinations on acclimatized activated sludge as compared with the single metals. Combined effect of Ni(II) (5-10 mg/L) and Cr(VI) (1-50 mg/L) on bio-kinetic constants, i.e., the maximum specific growth rate (μ_m), and half-saturation substrate concentration (K_s) have been found to be stimulatory (Dilek et al., 1998). Cr(VI) up to 25 mg/L can increase protein (PN) and polysaccharide (PS) contents in EPS. The addition of Cr(VI) has resulted in a deterioration in COD removal, nitrification, and microbial activity. The nitrifying bacteria has tolerance to Cr(VI) as compared to organics-degrading bacteria (Zheng et al., 2016c).

The Cu(II) (>10.9 mg/L) can alter the microbial community structure of nitrifiers and lower their activity. *Zoogloea*, *Thauera*, and *Dechloromonas* can survive Cu(II) exposure and *Nitrospira* and *Nitrosomonas* are predominant nitrifiers. The nitrifying bacterial community have changed through increasing abundance and shifting to Cu(II)-tolerant species (Ouyang et al., 2016). Toxicity order of short term (24 h) impact of different concentrations of Cd(II)

Table 1.3 Summary of studied parameters to evaluate impact of different heavy metals on biological reactors.

Reactor type	Volume (L)	COD (mg/L)	Pollutants	Concentration (mg/L)	HRT (h)	SRT (d)	Parameters studied	Reference
SBR	6	760	Pb (II)	0-10	12	15	Water-quality parameters and microbial community structure	Liu et al. (2019)
SBR	7.7	360	Cu(II) & TC	0-40 & 20	12	20	SBR performance, enzymatic activity, ROS, LDH, and microbial community	Deng et al. (2019)
SBR	7.7	360	Cu(II) & TC	0-50 & 0-50	-	-	SBR performance, microbial activities, FTIR spectra of EPS, and microbial community	Wang et al. (2018)
Serum vials	0.25	-	Cu(II), Mn(II) & Zn(II)	10, 20 & 10	-	-	Short and long-term effects of trace elements on nitrification-anammox, and microbial community structure	Li et al. (2018a)
Anammox biofilter	1	-	Zn(II)	1-20	3.5	-	Reactor performance of anammox process, EPS and SMP contents, microbial community	Zhang et al. (2018c)
SBR	3	-	Ni(II)	0.1-10	-	10	Flocculability, settleability, dewaterability and surface properties of activated sludge	Yang et al. (2017)
SBR	-	500	CuO NPs & Cu(II)	0-50 & 0-40	12	15	Reactor performance, removal of CuO NPs and Cu(II), bacterial viability assay	Zhang et al. (2017)
TPSLB	3	-	Cr(VI)	300	116	-	Most effective solid adsorbent, desorption efficiency, microbial culture, mechanism of Cr(VI) removal	Lytras et al. (2017)
ADMBR	10	433	Cd(II)	0-10	8	14	Performance of ADMBRs, formation of the dynamic membrane, microbial community	Huang et al. (2017)
SBR	4	600	Cr(VI)	0-50	6	-	SBR performance, EPS profiling	Zheng et al. (2016c)
Anoxic-aerobic SBBR	7.7	1300	Cd(II)	0-50	-	-	SBBR performance, SAOR, SNOR, SNRR, and sOUR, PS, PN, EPS, 3D-EEM, microbial community	Wang et al. (2016)
SBR	5	500	Cu(II)	10-50	12	-	Bacterial metabolic activity, bacterial community, nitrifying bacterial community	Ouyang et al. (2016)
A/O-MBR	9.45	300	Cu(II)	0-10	-	-	Reactor performance and sludge characteristics	Wen et al. (2016)
A2O process	50	-	Cu(II)	10-40	1.5-7	-	Activated sludge performance and microbial diversity	Sun et al. (2016)
Internal-loop airlift reactor	6.8	-	ROX & Cu(II)	0-500	26	-	Individual toxicity, responses of AOB and NOB, combined toxicity of ROX and Cu(II)	Guo et al. (2016)
Anoxic-aerobic SBR	5.7	1643	Cu(II)	0-50	12	-	SBR performance, sOUR, SAOR, SNOR and SNRR, 3D-EEM, microbial community	Wang et al. (2015b)
SBR	5.7	1643	Cr(VI)	10-30	12	-	Reactor performance, settleability of granular sludge, sOUR, SAOR, SNOR, and SNRR, morphology and microbial community of granular sludge	Wang et al. (2015c)
SBR	3	300	Cd(II)	1-10	-	-	Viability and settling property, short & long-term effect on biological nitrogen and phosphorus	Chen et al. (2014b)
SBR	4	250	Ni(II)	5-40	12	6	Performance of SBR, sOUR, effects of Ni(II) on the electronic transport systems (ETS) and bio-activities	Wang et al. (2013)
SBR	4	650-820	Cu(II)	0-40	24	-	COD removal efficiency, settling and dewatering properties of activated sludge	Li et al. (2011)

3D-EEM: Three-dimensional excitation-emission matrix; A/O-MBR: Anoxic/oxic membrane bioreactor; A2O: Anaerobic-anoxic-aerobic; ADMBR: Aerobic dynamic membrane bioreactor; AOB: Ammonia-oxidizing bacteria; CuO NPs: Copper oxide nanoparticles; EPS: Extrapolymetric substance; SMP: Soluble microbial product; ETS: Electronic transport systems; FTIR: Fourier-transform infrared spectroscopy; LDH: Lactate dehydrogenase; NOB: Nitrite-oxidizing bacteria; PN: Protein; PS: Polysaccharide; ROS: Reactive oxygen species; ROX: Roxithromycin; SAOR: Specific ammonia-oxidizing rates; SBR: Sequencing batch reactor; SNOR: Specific nitrite-oxidizing rates; SNRR: Specific nitrate-reducing rates; sOUR: Specific oxygen uptake rates; TC: Tetracycline; TPSLB: Two-phase solid-liquid bioreactor

(5-110 mg/L), Cu(II) (5-50 mg/L), Cr(VI) (40-2000 mg/L), Pb(II) (150-500 mg/L), and Zn(II) (5-500 mg/L) on the microbial community of activated sludge has been $\text{Cd(II)} > \text{Cu(II)} > \text{Pb(II)} > \text{Zn(II)} > \text{Cr(VI)}$. Ciliated protozoa such as *Trochilia minuta* and *Chilodonella uncinata* have showed highest sensitivity to all the studied metals, while *Opercularia coarctata* and *Opercularia minima*, have been the most tolerant species (Madoni et al., 1996). The heavy metals affect both nitrifying and heterotrophic bacteria in the activated sludge by decreasing the ammonium uptake rate (AUR) and specific oxygen uptake rate (sOUR) activities (Madoni et al., 1999).

Cu(II) (1 mg/L), Cr(VI) (0.5 mg/L), and Zn(II) (1 mg/L) have affected the adsorption rate of organic matters and COD adsorption capacity of the activated sludge. The metal-laden wastewater at sub-lethal levels have affected the performance to different extents, depending on HRT. Metal ions have acted as a strong competitor against the organic compounds for active sites on the bio-floc instead of acting as a toxic microbial inhibitor, thus hampering organic adsorption and affecting the COD removal efficiency under shorter HRT (Chua et al., 1999). The impact of seven different heavy metals and their combinations (Pb(II), Ni(II), Cd(II), Pb(II)+Ni(II), Ni(II)+Cd(II), Pb(II)+Cd(II), and Pb(II)+Ni(II)+Cd(II)) ranging between 0-40 mg/L have been examined in an SBR. The inhibition order on specific ammonia utilization rate (SAUR) has been $\text{Ni(II)} > \text{Cd(II)} > \text{Pb(II)}$. No significant inhibition in the nitrification reaction of the activated sludge has been observed even when 40 mg/L of Pb(II) is added. Besides, no synergistic effect is observed when different heavy metals are simultaneously added in varying concentrations, and the overall inhibition effect is dependent on the highly toxic heavy metal. The specific adsorption capacity of activated sludge increased as the heavy metal concentration has increased or the mixed liquid volatile suspended solid (MLVSS) has decreased (You et al., 2009).

Metal toxicity is determined by the reduction in activity levels of variables such as the specific oxygen uptake rate and mass fraction of active cells present in the reactor (Oviedo et al., 2002). The sudden metal exposure to the activated sludge adversely affect sludge bio-activity, leading to a decline in the removal efficiency of metals and flocs disintegration. The IC_{50} is defined as the inhibitor concentration that decrease the bio-transformation of a substrate at a specific concentration by 50%. Metals IC_{50} have confirmed that Cu(II) possessed the highest toxicity towards sOUR, dehydrogenase activity (DHA) and COD removal in the following orders: $\text{Cu(II)} > \text{Cd(II)} > \text{Cr(VI)} > \text{Co(II)}$; $\text{Cu(II)} > \text{Cd(II)} > \text{Co(II)} = \text{Cr(VI)}$ and $\text{Cu(II)} > \text{Cd(II)} > \text{Cr(VI)} > \text{Co(II)}$, respectively (El Bestawy et al., 2013). The toxic effects of the metals Cd(II), Zn(II) and Cu(II) on microbial activity in the activated sludge process has been reported. The results have permitted the establishment of the following toxicity sequence: $\text{Cd(II)} > \text{Cu(II)} > \text{Zn(II)}$ (Oviedo et al., 2002).

Filamentous organisms are always present in non-bulking sludge; rather, the level of filamentous organisms varies with environmental conditions (Sezgin et al., 1978). The filamentous microorganisms are essential for strong and dense flocs structure; however, overgrowth causes bulking and decreased settling and dewatering capacity. They can grow

excessively and consumes food faster than floc-farming microorganisms under low dissolved oxygen (DO) levels. When many filamentous microorganisms grow out of the floc's into the bulk medium, their bridging interferes with the nearby flocs to produce a slowly settling, poorly compacting, and bulking sludge (Sezgin et al., 1978). Filamentous organisms are susceptible to heavy metals exposure. Even a trace concentration might be lethal for them. The sludge volume index (SVI) is the most commonly used parameter to obtain a quantitative measure of the settleability of an activated sludge sample (Mohlman, 1934). The SVI indicates the ability of microorganisms to separate from the wastewater during settling. The SVI is of operational importance since it reflects changes in the biomass settleability. Any increase of SVI with no increase of MLSS concentration indicates that the solids settling characteristics are changing, and a plant upset likely to occur (Liu and Liptak, 1999). It is reported that heavy metal reduces the filament population (Mcdermott et al., 1963), which result in lower SVI with increased interface settling velocity (ISV). Sezgin et al. (1978) have reported a relationship between filamentous microorganisms and SVI in an activated sludge process at different DO concentrations. Pinpoint flocs and overgrowth of filaments result in high SVI (above 200 mL/g) and decreased ISV, whereas strong and dense flocs give optimum SVI (below 150 mL/g) with increased SVI (Neufeld, 1976). An activated sludge that settles very rapidly (SVI of less than approximately 70 mL/g) can leave behind a turbid supernatant (Palm et al., 1980). It can be seen that ISV and SVI are related to each other.

Heavy metal causes the disappearance of filaments from the system and form highly stable pinpoint flocs (Neufeld, 1976). It has been reported that effluent suspended solids (SS) values decrease as SVI increase. Operation at high SVI is not considered desirable, as it can lead to high effluent SS levels as a result of inferior effluent quality (Parker, 1983). In a batch process, 50-60% of metal has been retained by sludge, whereas 80-85% of influent copper (50 mg/L) has been retained in continuous ASP by sludge. The metal retained by the flocs is due to the some physical or chemical attraction, not because of the bacterial incorporation. Metals are rapidly removed from aqueous solutions by biological flocs (Cheng et al., 1975; Neufeld and Hermann, 1975). The chromium adsorbs on the activated sludge up to 93 mg/g (Moore et al., 1961). The influence of the shock loading of heavy metals on activated sludge results in a reduced SVI at the higher metals loading. However, it also results in the formation of highly stable pinpoint flocs, causing the loss of biomass significantly, which leads to deteriorated effluent quality. If severe and prolonged, deflocculation will lead to the ultimate system failure due to biomass washout (Neufeld, 1976).

The settling properties of sludge have been impaired by copper dose, resulting in lower settling velocity due to damaged bacterial cells (Yang et al., 2013). Before Cu(II) addition, the SVI for the activated sludge process has ranged 290-400 mL/g. It has significantly changed to SVI of 126 mL/g within 10 d of Cu(II) addition (20 mg/L) (Li et al., 2011). The addition of 40 mg/L of Cu(II) has slightly decreases SVI. However, an increase up to 80 mg/L further lead to a significant decrease in SVI (Wong, 1999). The SVI has changed (from 200 mL/g to 108 mL/g) with the addition of 16.05 mg/L of iron in the anoxic process (Yagci et al., 2015).

Ni(II) exposure at 1 and 10 mg/L decreases SVI; moreover, long-term exposure has increased the suspended solids (SS) concentration in effluent with low SVI (Yang et al., 2017).

The heavy metals can be added in two ways to the activated sludge: slug and continuous manner in the wastewater (Moore et al., 1961). In a study, 430 mg/L of chromium has been added for 30 min in a first slug dose followed by second slug dose for two minutes at 1,440 mg/L of chromium on the next day. The results of these doses have been reflected in a higher BOD in the effluent and ceasing of nitrification (Edwards and Nussberger, 1947). In another study, a slug dose of 500 mg/L of Cr(VI) for 4 h has dropped BOD and COD removal of activated sludge and continued to drop for about 32 h before starting to recover after 4 days (Moore et al., 1961).

The above mentioned studies have suggested the impact of various metals and their concentration on reactor performance in terms of COD removal, nitrification, and denitrification. The presence of heavy metals in wastewater can cause the microbial community shift due to its toxicity, which also significantly deteriorates the treatment plant efficiency.

Nanoparticles (NPs) are diverse in physical and chemical properties and their structures. They are smaller than 100 nm in size. They are classified in three different categories as natural, incidental, and engineered (Adhikari et al., 2019; Qiu et al., 2016b). NPs are widely used in pharmaceuticals and personal care products (PPCPs) and industrial products, including surface coatings, water filters, semiconductors, cosmetics, textiles, pigments, etc. (Sun et al., 2013; Miao et al., 2016; Yazdanbakhsh et al., 2019; Zheng et al., 2011). Gross usage and production of NPs have inevitably increased their release into the environment. The households and industries release their NPs containing wastewater to sewer systems, and eventually, it reaches to the wastewater treatment plants (Miao et al., 2016). Widely used silver nanoparticles (Ag NPs) are toxic to microorganisms due to its antimicrobial properties. Excessive use of Ag NPs in commercial products increases the possibility of Ag NPs in waste streams that can decrease biological wastewater treatment efficiency. NPs in wastewater could disturb activated sludge being absorbed onto bacterial cell surfaces and thereby affecting active biomass (Sun et al., 2013; Miao et al., 2016). The impact of nanoparticles on microorganisms of activated sludge is just similar to heavy metals.

The lower concentration of Ag NPs has no evident impact on the reactor performance parameters in terms of COD removal, nitrification and denitrification as well as microbial community structure (Liang et al., 2010; Sun et al., 2013; Yazdanbakhsh et al., 2019; Qiu et al., 2016b; Zhang et al., 2016c), whereas toxic effects has been observed at high concentration of NPs. Exposure of 1.0 mg/L Ag NPs can affect settling behavior and SVI (Qiu et al., 2016b). Ag NPs at 5 mg/L concentration may cause remarkable shifts in microbial community structures. A further increase in Ag NPs concentration at 10 mg/L results in a decrease in COD, nitrogen, and soluble orthophosphate (SOP) removal. *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria* can grow in the presence of Ag NPs; however, the nitrifying bacteria

population declines, resulting in low nitrogen removal. Ag NPs exposure has accelerated lactate dehydrogenase (LDH) release from bacterial cells of aerobic biomass and has inhibited protein and polysaccharide production (Zhang et al., 2016c). *Thiotrichales* are more sensitive to Ag NPs than other biofilm bacteria. At 200 mg /L of Ag NPs, the bacterial population reduces drastically (Sheng and Liu, 2011). The NPs toxicity to aerobic biomass is caused by the NPs interaction with the cell membrane, interfering membrane integrity. Long-term NPs exposure affects key enzyme activities (dehydrogenase and catalase) and shows toxic impact on the microbial community.

Copper oxide nanoparticles (CuO NPs) are more toxic than Ag NPs. Long-term CuO NPs (at 1 mg/L) exposure can cause a decrease in COD, ammonia, and phosphorus removal. It can inhibit nitrite oxidoreductase, ammonia monooxygenase, and exopolyphosphatase enzyme activities in activated sludge. Lower doses of CuO NPs exposure can shift the microbial community structure and 60 mg/L of CuO NPs increase the reactive oxygen species (ROS) and LDH production (Wang et al., 2017d). CuO NPs interact with the bacterial cell surface and damage the cell surface. At 50 mg/L of CuO NPs, flocs stability can decrease that cause dewaterability problem.

Higher doses of copper nanoparticles (Cu NPs) (30-50 mg/L) can affect aerobic sludge biomass's surface charge and hydrophobicity. More Cu^{2+} ions are released from Cu NPs at the higher concentrations that increase the surface charge and decrease sludge's hydrophobicity. This phenomenon damages the bacterial cells and deteriorates the flocculating ability due to dispersed and pinpoint flocs generation (Chen et al., 2014a). NPs toxicity on aerobic biomass depends on the exposure time. The short-term exposure (24 h) of CuO NPs can lead to a loss in cell viability and hence, in response, more EPS is produced (Miao et al., 2017). Also, CuO NPs (50 mg/L) can cause a significant decrease in microbial activities of rotatory biological contactors (Miao et al., 2016).

Nickel oxide nanoparticles (NiO NPs) (over 5 mg/L) can decrease COD and nutrient removals efficiencies by affecting the enzyme activities of microbial cells. Low concentration of cerium oxide nanoparticles (CeO NPs) has no significant impact on reactor performances (Hou et al., 2015b; Qiu et al., 2016a; You et al., 2015; Xu et al., 2016); however, it completely inhibits ammonia monooxygenase and nitrate reductase activities. Nitrite oxidoreductase activity has not affected, whereas LDH measurably has been affected at 1.0 mg/L of CeO NPs (Hou et al., 2015b).

SVI increases immediately after the exposure of Ag NPs and increase further with a rise in NPs concentration to 5.0 mg/L. The poor settleability of the sludge is likely to be due to deposit of the NPs in the activated sludge that alter EPS and the flocs structures (Qiu et al., 2016a). Similarly, in the case of ZnO NPs, SVI increase after addition of the NPs and reach a peak of over 900 mL/g on 108 d (Puay et al., 2015).

The NPs have shown a toxic effect on microbial cells of activated sludge, which depends upon NPs dose and exposure time. NPs can damage cellular membranes, permeability,

enzymes activities and change the microbial community of a biological system. Changes and reduction in bacterial community structure indicate that NPs reduce the ability of the treatment process to handle changes in the influent wastewater conditions. The occurrence of NPs in wastewater is a kind of threat to the biological processes.

1.9.2 Antibiotics

Rampant and unregulated antibiotic use in livestock, mariculture, poultry, animal farms, hospitals, and discharges from pharmaceutical industries contribute huge amounts to the environment, becoming a serious concern (Elmolla and Chaudhuri, 2011; Mutiyar and Mittal, 2014). Disposal of expired/unused antibiotics from pharmaceutical industries, residential (residences, dormitories, inns, hostels, and care facilities), stock/retail shop owners, and commercial facilities (including hospitals) are the main source of antibiotics to sewerage (Brown et al., 2006; Michael et al., 2013; Gothwal and Shashidhar, 2015). Antibiotics have been formulated to kill or inhibit the growth of targeted microbial organisms and persist in the host organism (Jendrzejska and Karwowska, 2018). The unregulated use of antibiotics in the environment develop antibiotic resistance in the microorganism (Vicent et al., 2013). The human or animal body consumes only 10-20% of antibiotics. The residual amount (80-90%) is excreted with urine and feces (Gothwal and Shashidhar, 2015; Tiwari et al., 2017). Antibiotics in sewage affect the treatment plant's efficiency by altering the microorganisms that exist in the treatment plant. Table 1.4 contain various parameters affected during wastewater treatment in presence of antibiotics. Effluent from treatment plants imparts a negative impact on aquatic organisms such as tadpoles of *Xenopus tropicalis* and zebrafish (*Danio rerio*) of receiving water bodies (Carlsson et al., 2009).

Antibiotics inhibit the biosynthesis of peptidoglycan in cell walls, which causes membrane damage, ultimately causes cell death. Damaged bacterial cells show slow activity, and decrease in COD removal (Liu et al., 2018b). Usually, antibiotics block the synthesis of cellular components, resulting in cellular malfunction, which leads to cell lysis. Microorganisms are very sensitive to the toxic materials, and it may inhibit cellular activity and metabolism. Antibiotics directly attack microorganisms and lead to the failure of the biological system. The microorganism develops resistance against antibiotics upon continuous exposure and is known as tolerant microbes. However, those microorganisms which could not grow due to the antimicrobial effect are known as intolerant microorganisms. The influent, effluent and sludge samples for three wastewater have contained 43 pharmaceuticals compounds (Jelic et al., 2011). In influent 32 and effluent 29, target compounds have been found up to the concentration from ng/L to mg/L. *Proteobacteria* (24.0%), *Bacteroidetes* (19.8%), and *Firmicutes* are dominant species in activated sludge survived in the presence of 21 pharmaceuticals at the concentration up to 100 ng/g (Muter et al., 2017). Similarly, *Proteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, and *Zoogloea* are dominant microbial community during the treatment of antibiotics, PPCPs containing wastewater. The occurrence of PPCPs has negatively affected granular quality (granules disintegration) by altering the optimum conditions for microbial growth. Microorganism tries to adapt to the new condition

Table 1.4 Parameters affected in presence of pharmaceutical compounds during wastewater treatment.

Reactor type	Volume (L)	COD (mg/L)	Pollutant	Concentration (mg/L)	HRT (h)	SRT (d)	Parameters studied	Reference
SBR	7.7	506	AMP	0-30	16	20	Reactor performance, sOUR, SAOR, SNOR, SNIRR, SNRR, enzyme activity, and microbial community analysis	Yu et al. (2019a)
Fed batch reactor	0.5	1500	TC	0.001- 0.50	-	-	Rector performance and microbial community analysis,	Islam and Gilbride (2019)
Conical Flask	0.3	-	TC	25	-	-	Performance and TC degradation capacity	Shao et al. (2019)
SBR	6	1000	SMZ	5	24	10	Performance, 3D-EEM, microbial community analysis	Li et al. (2019)
Sponge-MBR	8	-	CIP	0.02-0.20	8	20	Performance of Sponge-MBR, FESEM, fouling behavior	Nguyen et al. (2019)
SRUSB	0.5	498	SMX	0.05	5	-	Reactor performance, effects of HRT, Removal of SMX	Qiu et al. (2019)
SBR	3	-	CTC	0-10	11	12	Biological nutrient removal, viability of activated sludge, enzymes activities	Chen et al. (2019)
SBR	4	-	TC	0.5	7	20	Performance of aerobic granular sludge, microbial community, effect of TC on ARGs	Zhang et al. (2019)
SBR	0.625	3000	OTC	15	12	-	Reactor performance, adsorption and bio-degradation, microbial community, enzyme activity	Wang et al. (2019b)
SBR	0.366	-	LVX	0-128	11.3	-	Minimum inhibitory and minimum bactericidal concentration analysis, reactor performance, microbial community analysis	Hao et al. (2019)
SBR	12	550	SDN & TMP	0.005	48	14	Reactors performance, dynamics of microbial community	Kruglova et al. (2019)
SBR	1	800	SMX, TC, ERY	0-200	24	10	OUR, ARGs, endogenous respiration, pollutant removal	Pala-Ozkok et al. (2018)
SBBR	8.5	87	FF	0-35	-	-	Reactor performance, sOUR, SAOR and SNOR, SNRR, 3D-EEM, microbial community, analysis	Gao et al. (2018)
SBR	4.8	-	DCN, GMN, PEN, NFT & NIF	0-0.30	-	-	Reactor performance, enumeration of bacteria, isolation antibiotic-resistant bacteria (ARB)	Jendrzewska and Karwowska (2018)
SBR	1.7	3879	AZI	69.0	36	20	Reactor performance, AZI mass balance, ROS production and LDH release, microbial community analysis	Liu et al. (2018b)
UASB	2	-	SDM & SMZ	1-9	11	-	Reactor performance, microbial community structure,	Du et al. (2018)
SRUSB	1.08	-	CIP	1-5	6	25	CIP removal, changes in SRB communities, CIP bio-degradation products, and pathways	Jia et al. (2018)
SBR	2	-	TC	0-5	16.5	18	Biological nutrient removal, bio-degradation and mass balance of TC	Liu et al. (2018a)

3D-EEM: Three-dimensional excitation-emission matrix; AMP: Ampicillin; ARB: Antibiotic-resistant bacteria; ARGs: Antibiotic-resistant genes; AZI: Azithromycin; CIP: Ciprofloxacin; CTC: Chlortetracycline; DCN: Doxycycline; ERY: Erythromycin; FESEM: Field emission scanning electron microscope; FF: Florfenicol; GMN: Gentamicin; HRT: Hydraulic retention time; LDH: Lactate dehydrogenase; LVX: Levofloxacin; MBR: Membrane bioreactor; NFT: Nitrofurantoin; NIF: Nifampicin; OTC: Oxytetracycline; OUR: Oxygen utilization rate; PEN: Penicillin; ROS: Reactive oxygen species; SAOR: Specific ammonia-oxidizing rates; SBBR: Sequencing batch biofilm reactor; SBR: Sequencing batch reactor; SDM: Sulfadimethoxine; SDN: Sulfadiazine; SMX: Sulfamethoxazole; SMZ: Sulfamethazine; SNIRR: Specific nitrite-reducing rates; SNOR: Specific nitrite-oxidizing rates; SNRR: Specific nitrate-reducing rates; sOUR: Specific oxygen uptake rates; SRB: Sulfate-reducing bacteria; SRUSB: Sulfate-reducing upflow sludge bed; TC: Tetracycline; TMP: Trimethoprim; UASB: Up flow anaerobic sludge blanket

by modifying its cellular machinery and are then able to degrade the PPCPs. PPCPs are either removed by biosorption or bio-degradation (Zhao et al., 2015). In the biosorption process, the adsorbed compounds sometimes get released from the biomass in the next cycle. Fluoroquinolones ofloxacin [(OFX), norfloxacin (NFX), and ciprofloxacin (CFX)] at a concentration of 9 and 32 mM adsorb on the sludge surface, and gradually desorb from sludge surface into the reactor. Denitrification and phosphate accumulating organisms have inhibited, and *Proteobacteria* species have predominated in the reactor in the presence of fluoroquinolones.

Microorganisms try to protect themselves from the toxicity. Hence, they secrete an extra polymeric substance (EPS). This gel-like structure forms a protective layer to the outer cell surface. Tetracycline (TC) antibiotic exposure enhances EPS secretion to protect microbial cells from toxicity. TC imparts chemical changes in EPS components and damages the protection system of bacterial cells. TC intolerant bacteria are replaced by tolerant bacteria (Zhang et al., 2013). TC can also cause disintegration of granules and leads to nitrite accumulation and increased solids content in the effluent (Sheng et al., 2013). Similarly, short-term exposure of CFX (0.2 and 2 mg/L) can adversely affect sludge's viability and integrity. Long-term exposure reduces phosphorus and nitrogen removal by inhibiting nitrite reductase and polyphosphate kinase enzyme activities. Hence polyphosphate and denitrifying bacteria population decrease in reactors (Yi et al., 2017). The addition of benzothiazole (1-50 mg/L) has reduced volatile fatty acids (VFA) accumulation and has caused membrane fouling in an integrated anaerobic fluidized bed membrane bioreactor. However, *Clostridium sensu stricto* and *Trichococcus* have survived over benzothiazole doses (Li et al., 2017).

The exposure of fluoxetine (FLX) to aerobic granular sludge in SBR affects the removal of carbon, nutrients, and phosphate. The microbial community adapts to the continuous exposure of FLX, resulting in the improved phosphate removal efficiency (Moreira et al., 2015). The addition of oxytetracycline (OTC) (0-12 mg/L) has reduced nitrogen and COD removal efficiencies of anoxic-aerobic SBR. The specific oxygen uptake rate (sOUR), specific nitrate reduction rate (SNRR), specific ammonium oxidation rate (SAOR), and specific nitrite oxidation rate (SNOR) have decreased due to OTC toxicity; however, microbial cells try to protect it by increasing EPS contents. The microbes in the reactor become weakened due to OTC exposure. In contrast, few bacteria become predominant due to OTC toxicity (Wang et al., 2015a). Similarly, SAOR, sOUR, SNRR, and SNOR have decreased in the presence of florfenicol (FF) in a sequencing batch biofilm reactor (SBBR). The COD and nitrogen removals have been inhibited, and EPS production has increased at high FF concentrations. FF concentration has induced variations in the microbial community and has shown that the nitrification process is affected because of the decreased density of *Nitrosomonas* and *Nitrospira* (Gao et al., 2018).

At lower concentrations, microorganisms resist the toxicity and try to protect the cells; however, the protection system fails at a higher concentration. Anammox granules can resist the lower doses of amoxicillin (AMX), FF, and sulfamethazine (SMZ) toxicity and efficiently

perform nitrogen removal. Specific anammox activity (SAA) remain unaffected at higher doses of AMX, FF, and SMZ (Zhang et al., 2015). Lower chlortetracycline (CTC) concentration has no significant impact on the SBBR performance. In contrast, inhibition of nitrogen and COD removal have reduced at high CTC concentrations. Further, increased CTC doses cause an increase in EPS production. *Aequorivita*, *Buchnera*, *Flavobacterium*, *Azonexus*, and *Thioalbus* can survive at higher CTC doses (Zheng et al., 2016a). At 100-300 µg/L of antibiotics (doxycycline, gentamicin, penicillin, nitrofurantoin, and rifampicin) has slightly affected the COD removal, without effecting nitrogen or phosphorus concentrations. The most significant bacterial population has become resistant to penicillin and nitrofurantoin (Jendrzewska and Karwowska, 2018). The lower concentrations (5-50 µg/L) of TC and sulfamethoxazole (SMX) have no significant effect on COD and NH_4^+ -N removals, whereas, higher concentration of TC and SMX (10 mg/L) has drastic impact. TC and SMX stimulate EPS production and reduce *Proteobacteria* and *Bacteroidetes* diversity, while *Actinobacteria* percentages increase. Microbial diversity is maximum at lower concentrations, and it decreases at higher doses (Zhang et al., 2016b). The effect of OFX at concentrations of 1-15 mg/L on the microbial community can cause a reduction in COD, ammonia, and phosphorus removal efficiencies and an increase in EPS contents (Kong et al., 2017). Anammox bacteria could grow even in extreme conditions provided by OTC, sulfathiazole, Cu(II), and zinc. Anammox SSA has decreased in the presence of Cu(II) and Zn(II), along with antibiotics. SAA depends upon the concentration and exposure time of OTC and sulfathiazole concentration (Lotti et al., 2012).

A combination of different antibiotics is more toxic than single antibiotics. A mixture of azithromycin, NFX, SMX, and trimethoprim have caused granules disintegration, reduced nitrogen removal, and disappearance of intolerant microbial community structure. Long-term antibiotics loading has encouraged multi-drug resistance (Rodriguez-Sanchez et al., 2017). SMX and TC at 100 µg/L concentration have hardly affected the pollutant removals in anoxic/aerobic membrane bioreactor. The abundance of nitrifying bacteria has decreased with increased (up to 1000 µg/L) antibiotic concentration to the reactor (Zhu et al., 2017b). Microbial communities are susceptible to toxicity, and a lower concentration of antibiotics may cause the disappearance of intolerant species. Specific antibiotics aim for a specific type of bacteria; hence sometimes other bacteria grow, and target bacteria disappear from the system. New bacterial community appear in the bioreactor at 50 µg/L of SFX. Dominant classes have been γ -proteobacteria and β -proteobacteria; among them, *Thiotrix* sp. (γ -proteobacteria) has the resistance to SMX. *Actinobacteria*, *Chloroflexi*, *Chlorobi*, and *Sphingobacteria* classes are more vulnerable to SMX load and disappear from the system (Collado et al., 2013).

Short-term (24 h) addition of erythromycin (ERY) (10 mg/L) and OTC (10 mg/L) can completely inhibit the phosphate removal of an enhanced biological phosphorus removal (EBPR) system. Moreover, in the combined antibiotics test, neither synergistic nor antagonistic effect is detected between ERY and OTC (Hu et al., 2016). NFX, at a lower concentration (0-6 mg/L), did not reduce COD and NH_4^+ -N removals, whereas higher concentration (6-35 mg/L) has inhibited their removals and increased EPS production. SAOR, sOUR, SNOR, and

SNRR have started to decrease with increased NFX concentration (from 0 to 35 mg/L) (Zheng et al., 2016b).

The SVI displays a gradual increase in ampicillin concentration increment due to the quick increment of the EPS content under ampicillin stress (Yu et al., 2019a). The SVI do not change with 10 mg/L of chlortetracycline (Chen et al., 2019). The SVI at steady-states increase from 55 to 118 mL/g, with an increase in OTC concentration from 0 to 12 mg/L (Wang et al., 2015a). Antibiotics in wastewater affects the organic and nutrient removal process, microbial activity (SAOR, sOUR, SNOR, and SNRR), EPS production, enzyme activity, flocs stability, and cell viability, etc. The impact of antibiotics depends upon the antibiotic concentration. Lower doses of antibiotics do not severely impact microbial cells, whereas higher doses drastically inhibit cellular activity. The antibiotics are designed to kill bacteria; however, many microbial communities resist antibiotic action and become an antibiotic-resistant community. Microorganisms that are sensitive to antibiotics disappear from the biological process, and tolerant species remain in the system. As a result, a shift in microbial structure has been reported in the literature in the presence of antibiotics.

1.9.3 Pesticides

Pesticides are the most extensively used chemical in agricultural activities to protect the crops from insects and pests. An increase in pesticides and their metabolites concentration in soils may affect soil microbial community and their metabolic activities (Dar et al., 2019). The extreme usages and incorrect applications of pesticides make their appearance in the sewage system, which may impact the receiving water bodies. Runoff from agricultural land, kitchen garden and parks, effluent from small industries, and disposal by stockholders contribute pesticides in sewage. The aerobic biomass is more sensitive to chlorpyrifos (CPS) in sewage. The microbial cells metabolize the CPS and produce 3,5,6-trichloro-2-pyridinol (TCP), which is a secondary metabolite and more toxic than CPS. It exerts an antimicrobial effect due to its excellent solubility in water and inhibits the growth of CPS degrading bacterial community (Hou et al., 2018; Wang et al., 2017a). Influence of pesticide on various parameters of the biological processes is summarized in Table 1.5. Microbial cells degrade pesticides and utilize it as a secondary carbon source for their growth and maintenance. Microbial communities shift from peptone degradation to chlorophenols degradation after the gradual decrease in peptone (from 300 mg/L to 0), which allows the microorganism to degrade chlorophenols [4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP)] mixtures. The 5-chloro-2-hydroxymuconic semi-aldehyde (CHMS) is a degradation product of 4-CP that accumulates in the bacterial cells (Sahinkaya and Dilek, 2006). CPS, TCP, methyl parathion, and carbonfuran can also be used as sole carbon and energy sources for bacterium obtained from sludge (Xu et al., 2018).

Diuron, N-(3,4-dichlorophenyl)-N,N-dimethyl-urea, is extensively used in many crops. Diuron can be degraded in aerobic and anoxic conditions. Moreover, aerobic degradation is affected more than anoxic degradation due to presence of diuron. Under anoxic conditions,

Table 1.5 Parameters studied with various concentration of agrochemicals in different reactors.

Reactor type	Volume (L)	COD (mg/L)	Pollutants	Concentration (mg/L)	HRT (h)	SRT (d)	Parameters studied	Reference
Constructed wetland	-	53	CPS and TCP	0.10-0.50	8 d	-	Reactor performance, CPS and TCP removal	Yu et al. (2019b)
Conical flask	-	-	CPS	50	-	-	Isolation and characterization, antagonism and tolerance assays, degradation studies	Shabbir et al. (2018)
Conical flask	0.02	-	BFN and CPS	100	-	7	Inoculum concentration, temperature, pH, and initial concentration, bio-degradation studies	Zhang et al. (2018b)
Anaerobic bioreactor	7	70	CPS	0.8	-	-	Reactor performance	Hou et al. (2018)
SBR & SFBR	4 & 3	1000	2,4-D	20-300	48	10	Reactor performance, degradation of 2,4-D, specific carbon consumption rates	Elefsiniotis et al. (2017)
SBR	20	350	MCPA	20-75	24	40-60	Herbicide tolerance studies, intermediate compound	Chouhan et al. (2017)
Bench bioreactor	14	-	CPS	100-500	24-96	-	Volatilization/abiotic degradation, variable environmental conditions, adsorption on bacterial cells, kinetics of CPS bio-degradation, photo-degradation of CPS	Khalid and Hashmi (2016)
Constructed wetland	-	60-70	CPS	1	-	-	Microbial diversity	Zhang et al. (2016a)
Fenton-SBR	3	-	2,4-D & MCPA	180	12	30	Oxidation byproducts and pathway, ecotoxicity and biodegradability	Sanchis et al. (2013)
MBR	130	200	Azinphos-methyl Diazinon Malathion Phorate and CPS	5	-	-	COD, phosphorous and nitrogen analysis, potentiometric and microbiological analysis	Ghoshdastidar et al. (2012)
Anaerobic-Aerobic Reactor	2	430	triadimenol	1-25	8-32	-	Reactor performances and kinetic study	Shawaqfeh (2010)
SBR	2	1070	Isoproturon and 2,4-D	0-50 & 0-700	48	12-15	Kinetics study, biomass acclimation	Celis et al. (2008)

2,4-D:2, 4-dichloro phenoxy acetic acid; BFN: Bifenthrin; CPS: Chlorpyrifos; MBR: Membrane bioreactor; MCPA: 2-methyl-4-chlorophenoxyacetic acid; SBR: Sequencing batch reactor; SFBR: Single-fed and sequencing batch reactor; TCP: 3,5,6-trichloro-2-pyridinol

low concentrations of diuron accelerates its elimination after being acclimatized (Stasinakis et al., 2009). The pesticide concentration and exposure time are the main criteria on which pesticides toxicity depend. Isoproturon is not degraded during initial exposure in both aerobic and anaerobic systems but after acclimatization, it starts to degrade up to 500 mg/L (Celis et al., 2008). Exposure of 4-CP competitively inhibits bio-degradation of 2,4-DCP. In aerobic biomass, the 4-CP degrader microbial population fraction can be higher than 2,4-DCP degraders (Sahinkaya and Dilek, 2007b). Both 4-CP (at 53 mg/L) and 2,4-DCP (at 25 mg/L) can inhibit their own degradation. However, *Pseudomonas* sp. and *Pseudomonas stutzeri* are the dominant species that can degrade chlorophenols (Sahinkaya and Dilek, 2007a). 2-CP and 2,4-DCP induce a higher and stable inhibitory effect on microorganisms with time.

The dominant bacterial communities isolated from soil are *Pseudomonas*, *Agrobacterium*, and *Bacillus* that can degrade CPS at 25-200 mg/L and TCP at 25-100 mg/L (Maya et al., 2011). Moreover, *Serratia marcescens*, *Pseudomonas kilonensis*, *Bacillus pumilus*, *Klebsiella*, and *Achromobacter xylosoxidans* can degrade CPS up to 400 mg/L. In addition to that, further exposure of benzene, toluene, and xylene (at 100 mg/L) can decrease CPS degradation and microbial activity (Khalid et al., 2016). CPS and TCP can interfere with cytochrome C (Cyt C) content and electron transport system (ETS) activity of the sludge (Zhang et al., 2018a). Domestic sewage microorganism *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Enterobacter ludwigii* can grow in the presence of CPS and it binds to the cell surface and enters into intracellular components (Shabbir et al., 2018).

The impact of pesticides on aerobic biomass behavior is completely missing; however, pesticide interaction with isolated strains from different sludge has already been discussed in this section. CPS is widely used in agriculture worldwide and is detected in the runoff. Both CPS and TCP can cause serious environmental hazards and require removal before discharge into rivers and/or lakes (Yu et al., 2019b). The presence of agrochemical compounds in wastewater severely affect the biological process and exhibit the shift in microbial community structure. The bio-degradation product of CPS is TCP, which is more toxic to aerobic biomass than CPS due to its antimicrobial property; although pesticides can drastically affect the microbial community structure.

1.9.4 Real wastewater

The increasing trend towards constructing small-scale industrial clusters in the urban area increases the possibility of discharge of real-raw wastewater in the sewage line. The real wastewater contains toxic compounds that may affect treatment efficiency. The following literature discusses the impact of real wastewater on treatment efficiency on the treatment plant. A summary of the studied parameters has been presented in Table 1.6. Dairy wastewater has exhibited the overgrowth of filamentous bacteria frequently in SBR, which cause bulking problems. Anaerobic-micro-aerated feeding patterns help to suppress filamentous overgrowth and form aerobic granules while increasing suspended solids removal efficiency. Average removal efficiencies for COD, total nitrogen (TN), and total phosphorus (TP) have been 94%,

Table 1.6 Parameters studied during real wastewater treatment.

Reactor type	Volume (L)	COD (mg/L)	Type of wastewater	Pollutants (mg/L)	HRT (h)	SRT (d)	Parameters studied	Reference
SBR	30	3450±65	PWW	AMP, ERY, TET, KAN, and CIP	-	-	Reactor performance, ARB, zeta potential, and relative hydrophobicity	Wang et al. (2019a)
SMBR	8	3551±252	PWW	Sulfonamides, tetracyclines, and fluoroquinolone	-	-	Removal of bulk organic matter and nutrients, removal of antibiotics, biomass characteristics	Xu et al. (2019)
Anaerobic biofilter reactor	13.4	3000	Coal gasification	Phenol (0-800)	96	-	Reactor performance, methane production, microbial community analysis	Li et al. (2018b)
Activated sludge plant	208	1045	OMWW	Organic matter	10	-	Reactor performance, sOUR, microbial analysis	El Moussaoui et al. (2018)
Erlenmeyer flasks	0.25	75000	OMWWI	Phenol	-	30	Isolation and identification of bacterial strains, microbial consortium stability and process performance, bacterial community structure, phytotoxicity assessment	Arous et al. (2018)
AnMBR and AeMBR	4 & 2.5	2000	TWW	Real wastewater	48	60	Reactor performance, filtration characteristics of AnMBR and AeMBR, optical profilometer and SEM-EDS analyses, dominant bacterial consortia	Yurtsever et al. (2017)
SBR	2	140	TWW	Dye, COD, TDS	-	7	Absorption, COD, and TDS parameters	Mirbolooki et al. (2017)
EGSB	1.47	-	PWW	Amoxicillin (19.7-214.7)	20	-	Reactor performance, diversity and abundance of bacteria	Meng et al. (2017)
MBR	36.2	900	POME	Real wastewater	15	60	Reactor performance, EPS characterization, microbial community	Neoh et al. (2017)
SBBR	8.5	85	MWW	CTC (0-35)	-	-	Performance of SBBR, microbial activities in SBBR, 3D-EEM, microbial community analysis	Zheng et al. (2016a)
Column reactor	20	1250	SWW	COD, nitrate, nitrite, ammonia, phosphate	-	-	Reactor performance, 3D-EEM, Particle size distribution and ammonia oxidation ability	Liu et al. (2015)
SBR	10	11500	POME	COD	12	-	Performance of SBR, determination of growth kinetic	Lim and Vadivelu (2014)
SBR	3.5	1650-2450	TWW	COD	72-120	-	Reactor performance and kinetic study	Sathian et al. (2014)
SBR	10	1200	EPWW	CN-, Zn(II)	72-240	-	Effect of dilution rate of EPWW, Effect of HRT	Sirianuntapi-boon (2013)
SBR	155000	260	DWW	-	12	107	Organic matter and nutrients removal, biomass and microbial communities analysis	Fernandes et al. (2013)
SBR	3	228- 418	PWW	-	12	-	Effect of influent pH, activities of AOB and NOB, sludge concentration and settle ability	Yan et al. (2013)
UASB	18	3000-6000	PWW	-	-	-	Treatment efficiency , microbial community by PCR-DGGE profiling	Li et al. (2010)
SBR	9	5417	PWW	-	240	45	Effect of initial COD and SS concentrations, Bacteria inactivation and toxicity reduction, process applicability	Ben et al. (2009)

3D-EEM: Three-dimensional excitation-emission matrix; AMP: Ampicillin; AnMBR and AeMBR: Anaerobic and aerobic membrane bioreactor; AOB: Ammonia-oxidizing bacteria; CIP: Ciprofloxacin; CN⁻: Cyanide; CTC: Chlortetracycline; DWW: Domestic wastewater; EGSB: Expanded granular sludge bed; EPWW: Electroplating wastewater; ERY: Erythromycin; KAN: Kanamycin; MWW: Mariculture wastewater; NOB: Nitrite-oxidizing bacteria; OMWW: Olive mill wastewater; PCR-DGGE: Polymerase chain reaction-denaturing gradient gel electrophoresis; POME: Palm oil mill effluent; PWW: Piggery wastewater; SBBR: Sequencing batch biofilm reactor; SMBR: Sequencing-batch membrane bioreactor; sOUR: Soluble oxygen uptake rate; SWW: Slaughterhouse wastewater; TC: Tetracycline; TWW: Textile wastewater

95%, and 83%, respectively (Meunier et al., 2016). In another study, the decentralized system for urban wastewater treated through SBR has affected the performance and microbial diversity under limited aeration. The sludge bacterial flocs have formed an irregular arrangement with organisms attached, such as *Euglypha* sp. and pedunculate ciliates (Fernandes et al., 2013).

The palm oil mill effluent (POME) has been treated anaerobically at low pH using an SBR. The influent COD concentration has been gradually increased from 5000±500 mg/L to 11500±500 mg/L. The SBR has successfully removed 90% content of POME. The maximum specific growth rate ($\mu_{\max}$) is estimated to be 1.143 per d while the half-saturation constant (K_s) is determined to be 0.429 g/L. The decay coefficient (b_d) and biomass yield (Y) are 0.131/d, and 0.272 mg biomass/mg COD consumed, respectively (Lim and Vadivelu, 2014). POME is fed to MBR operating in an aerobic environment for 40 d, followed by 10 d without aeration and water pumping (influent stopped) to simulate failure event, and finally resumed to operational condition. It has resulted in altered microbial communities structure. The phyla *Proteobacteria* (19-23%), and *Chloroflexi* (11-13%) are dominant members in biofilm under operational condition while *Proteobacteria* (18%) and *Planctomycetes* (16%) are found to be dominant in biofilm under static and non-operational conditions (Neoh et al., 2017).

The aerobic granular sludge reactor has been used to treat slaughterhouse wastewater along with biological nutrient removal. COD (95%), ammonia (99%), and phosphate (84%) removal have been enhanced with sludge granulation. The granular size of 0.6-1.2 and 1.2-1.8 mm, accounting for 70%, has been beneficial for the growth of ammonia-oxidizing bacteria (AOB) and nitrate oxidizing bacteria (NOB), respectively. The SOUR of AOB and NOB have been 31.4 and 23.3 mg O₂/gMLSS h, respectively (Liu et al., 2015). Micro-aerobic hydrolysis-acidification (MHA) anoxic-oxic (A/O) processes have been developed to treat actual petrochemical wastewater. The main pollutants in the influent are benzene, ketone, alcohols, amine, nitrile, and phenols, which could be removed by MHA-A/O treatment. The dominant species in anoxic, and oxic reactors are *Anaerolineaceae* and *Sulfuritalea*, *Lactococcus* and *Blastocatella*, and *Saprospiraceae* uncultured and *Nitrosomonadaceae*, respectively (Yang et al., 2015). An anoxic/oxic membrane bioreactor (A/O-MBR) has excellent performance for the removal of COD and NH₄⁺-N from piggery wastewater. The non-VSS accumulation has led to poor settling capacity and high mixed liquor suspended solids (MLSS). The A/O-MBR also has smaller particle size flocs due to the lack of an organic carbon source. The increase in EPS contents and non-VSS accumulation has led to severe membrane fouling (Zhou et al., 2016). The aeration rate (0, 0.2, 0.4, and 1.0 L/min) has affected conventional activated sludge (CAS) and algal-bacterial symbiosis (ABS) systems treating domestic wastewater. The ABS has shown better removal of NH₄⁺-N (19%), TN (12%), and TP (47%) than the CAS system under lower aeration rate (0.2 L/min). The reduction in aeration rate could enhance algae growth but weaken hydraulic shear force, which contribute to the interactions between algae and sludge flocs and stability of the ABS system. In addition, algae growth has protected both AOB and NOB from damage (Tang et al., 2016). A biological

anaerobic/anaerobic/anoxic/membrane bioreactor (A3-MBR) has treated the effluent from the ethanol fermentation industry. The system has yielded a stable effluent concentration of COD (202 ± 23 mg/L), TN (62.1 ± 7.1 mg/L), ammonia (0.3 ± 0.13 mg/L) and TP (8.3 ± 0.9 mg/L). The microbial community structure in each reactor has been diverse. *Bacteroidia*, *Clostridia*, and *Synergistia* are the dominant microorganisms for organic degradation in the anaerobic units, and α -*Proteobacteria* and β -*Proteobacteria* are the dominant microbial populations in A3 and MBR; *Arcobacter* and *Thauera* are two representative genera in the anaerobic and the anoxic reactor, respectively (Zhu et al., 2017a).

A laboratory-scale MBR with intermittent aeration has been operated to treat the reverse osmosis concentrate derived from coal gasification wastewater. The intermittent aeration represents a slight effect on organic matter reduction but a significant effect on nitrite and nitrate reduction. The richness and diversity of microorganisms in MBR sludge are lower than the activated sludge. The most relative abundance of the genus in both sludge are unclassified *Roseibaca* (62%), and *Desulfarculaceae* (9%). High salinity and intermittent aeration has represented different influences on the denitrifying genus. The non-aeration phase has provided feasible DO conditions for denitrifying genera, ensuring denitrification (Jia et al., 2017).

A sequencing batch biofilm reactor (SBBR) has effectively treated saline mustard tuber wastewater (MTWW) with an average COD removal efficiency of 86% and total nitrogen removal efficiency of 86% at 30 g NaCl/L salinity during 100 d operation. The gradual increase in salinity from 10-30 g NaCl/L did not affect performance but has reduced microbial diversity. Proliferation of halophilic microbes have stabilized biological performance of SBBR in response to the rising salinity. Autotrophic AOB (*Nitrosomonas*) and AOA *Candidatus Nitrososphaera* both have contributed to ammonium oxidation. NOB has been significantly inhibited, and partial nitrification and denitrification (PND) have gradually contributed to nitrogen removal with an increased salinity (Wang et al., 2017b). The activated sludge system has treated olive mill wastewater (OMWW) through an increasing food to microorganism ratio (F/M). Removal efficiencies have been high with 95 and 93% removals for COD and phenolic compounds, respectively. Also, the flocs settleability shown by the SVI measurement has been optimal. The biomass has showed reasonable promptness in responding to the OMWW degradation rate through increased profitable growth, a stable physiological state, and a good settleability of the flocs (El Moussaoui et al., 2018).

1.9.5 Other factors and pollutants

Till now, literature survey has suggested the impact of heavy metals/pharmaceuticals/agrochemicals pollutant on the treatment performance of various reactors. Following literature reviews would describe the influence of various chemicals (i.e., phenolic compounds, nanoparticles, and salinity) and operational condition (DO, organic/nutrient loading rate and aeration rate) on the reactor performance and aerobic sludge biomass characteristics. Numerous studies have been conducted by various researchers to study the impact of

pollutant on reactor performance and its biomass and is presented in [Table 1.7](#). In activated sludge systems, the aeration process consumes most of the energy. The energy cost can be dramatically reduced by decreasing the operating DO concentration. However, low DO may lead to incomplete nitrification and poor settling performance of activated sludge flocs. Long-term low DO conditions produce larger flocs and more EPS per unit sludge, which produce a more extensive anoxic area and lead to the low oxygen diffusion performance in flocs. The low DO concentration (1.1 mg O₂/L) has a negative effect on sludge settleability, leading to the proliferation of filamentous bacteria (*Thiothrix* sp., Type 021N and Type 1851) ([Martins et al., 2003](#)).

Temperature is of concern for its effects on microbial activity and substrate conversion rate. The majority of biological treatment systems operate in the mesophilic temperature range, growing best in the temperature range of 20-40°C ([Gray, 2004](#)). The higher temperatures result in increased biological activity that, in turn, increases the rate of substrate removal. The increased metabolism at higher temperatures can also lead to problems of oxygen limitations in the aerobic system. As the temperature rises, the proteins in enzymes begin to breakdown by a process termed denaturation, adversely affecting bacterial metabolism ([McKinney, 2004](#)). The variation in F/M ratio (0.03-1.0) has no significant influence on flocs size and morphology. There is no clear relationship between SRT and floc size. However, flocs at the lower SRTs (4-9 d) are irregular and variable in size than those at higher SRTs (16 and 20 d). The effluent suspended solids at lower SRTs is higher than that at higher SRTs ([Liao et al., 2006](#)).

Activated sludge is widely used to treat industrial wastewater, but its efficiency is affected by a variety of factors, including toxic substances such as tetrahydrofuran (THF). At THF concentrations higher than 160 mM, significant inhibition of five enzymes (catalase, dehydrogenase, urease, phosphatase, and protease) have been represented. As the concentration of THF increase in influent, diversity in the microbial community decrease, indicating its acute toxicity to microorganisms ([Yao et al., 2010](#)). Filamentous bulking is a complex problem in wastewater treatment plants, leading to worsening of the settling properties and the effluent quality. Filamentous bulking (SVI below 150 mL/g) has not been witnessed in SBRs under anoxic feeding pattern with a short fill time. In contrast, serious bulking (SVI>500 mL/g) has occurred under aerobic feeding pattern when fed with ethanol, propionate, acetate, and glucose, in which *Thiothrix* and *Sphaerotilus natans* have proliferated as dominant filaments. Compared to the glucose-fed reactor, relatively light bulking has been caused in the starch-fed reactor with growth of *Nostocoida limicola II*. In addition, flocs in the starch-fed reactor are more open and fluffy than flocs formed on readily biodegradable substrates ([Guo et al., 2014a](#)).

Understanding of the competition between floc-formers and filaments is critical to prevent filamentous bulking in practice. Short feeding under anoxic condition (fill time<10 min) has resulted in a well-settling sludge with SVI<100 mL/g. The sludge settleability has deteriorated (SVI>200 mL/g) and filamentous bulking has been observed when the substrate

Table 1.7 Summary of parameters studied for treatment of various toxic compounds.

Reactor type	Volume (L)	COD (mg/L)	Pollutants	Concentration (mg/L)	HRT (h)	SRT (d)	Parameter studied	Reference
SBBR	7	150	2,4,6-trichlorophenol	0-30	-	-	Removal performance of target pollutants, reactor performance, microbial community analysis	Tong et al. (2019)
SBR	3	48.93- 972	Caffeic acid, hydroxybenzoic acid, and protocatechuic acid	50-600	-	-	Quantification of target genes, dynamics of microbial communities	Muñoz-Palazon et al. (2019)
SBR	1	-	Ag-Fe NPs	0-100	-	-	Reactor performance, sOUR, FESEM, LDH and ROS and enzyme activity, and microbial community analysis	Yazdanbakhsh et al. (2019)
SBR	6	-	Phenol, ammonia nitrogen, and thiocyanate	400, 100 and 100	12	-	Reactor performance, Settling, and SVI, staining, FESEM, and CLSM, granular biomass activity test, EPS analysis	Tomar and Chakraborty (2018)
SBR	2.5	500	Triclosan	100	2	8	Reactor performance and mass balance	Orhon et al. (2018)
SBR	5	300	4-CP	50	8	20	Reactor performance, acute sludge toxicity, microbial diversity	Zhao et al. (2018)
SBR	2	-	2-CP	60	-	-	Nitrifying batch assays, nitrifying process,	Martínez-Jardines et al. (2018)
SBR	1.5	1000	Acid Red 14 dye and Ag NPs	20 & 5	-	-	Nuclear microscopy and Ag NPs distribution	Bento et al. (2017)
RBC	4.8	195	CuO NPs	0-50	-	-	CuO NP adsorption by biofilms and the toxic effects, physicochemical properties of EPS, microbial communities	Miao et al. (2017)
SBR	2.5	23.9	2-FP	12.4	28.8-120	-	Reactor performance, aerobic granules properties, bacterial community dynamics	Ramos et al. (2017)
SBR	3	92	BPA	0-12	16	-	Biomass characteristics, microbial characteristics of aerobic granules	Cyzdik-Kwiatkowska et al. (2017)
SBR	7.7	-	NiO NPs	0-60	-	-	SBR performance, microbial activity, enzymatic activity, biotoxicity of NiO NPs, microbial diversity	Wang et al. (2017c)
SBR	5	500	Ag NPs	0-10	10	15	Nutrient removal, morphology, structure and surface integrity of sludge, microbial community structure	Zhang et al. (2016c)
RBC	-	195	CuO NPs	0-50	-	-	Aggregation and stability of CuO NPs, microbial activities of biofilm, ROS and LDH production,	Miao et al. (2016)
SBR	1.5	1000	Acid red 14	0-60	12	15	Reactor performance, morphology and properties of sludge,	Franca et al. (2015)
SBBR	3	-	CeO ₂ NPs	0-50	-	-	Reactor performance, SEM analysis, Role of EPS, bacterial viability , toxicity of CeO ₂ NPs	Hou et al. (2015b)

2-CP: 2-chlorophenol; 2-FP: 2-fluorophenol; 4-CP: 4-chlorophenol; Ag-Fe NPs: Silver-Iron nanoparticles; BPA: Bisphenol A; C/N: Carbon/Nitrogen; CeO₂ NPs: Cerium dioxide nanoparticles CLSM: Confocal laser scanning microscopy; CuO NPs: Copper Oxide nanoparticles; EPS: Extrapolymetric substance; FESEM: Field emission scanning electron microscope; LDH: Lactate dehydrogenase; NiO NPs: Nickel oxide nanoparticles; RBC: Rotating biological contactor; ROS: Reactive oxygen species; SBBR: Sequencing batch biofilm reactor; SBR: Sequencing batch reactor; SEM: Scanning electron microscopy; sOUR: Soluble oxygen uptake rate

has been added in a limiting rate by prolonging the anoxic fill time up to 90 min. In contrast, sludge settleability in fully aerobic systems has been quite poor ($SVI > 500$ mL/g) despite the feeding length. Compared to the systems with an anoxic fill phase, more types and abundant filamentous bacteria have been identified in fully aerobic systems. The extensive filaments, including *Thiothrix nivea*, Type 021N, Type 1851, and *Microthrix parvicella* have proliferated in fully aerobic systems resulting in the bulking problem. The substrate gradients play an important role in the competition between filaments and floc-formers (Guo et al., 2014b).

It is clear from the literature review that operational parameters such organic loading rate, HRT, SRT, temperature and dissolved oxygen can damage the flocs morphology and chemical compounds stimulate the secretion of extra polymeric substance which affects the settling and SVI of the aerobic sludge biomass.

The impact of various contaminants on aerobic sludge biomass also depends on reactor type. In the case of CSTR, contaminants that enter the tank are immediately dispersed due to continuous mixing and uniformly distributed in the reactor, which ensures that influent concentration peaks and/or toxic loads are quickly diluted (von Sperling, 2007a). The uniform conditions maintain the biomass in a constant average physiological state (Grady et al., 2011). Moreover, attached-growth processes have successfully been applied for the treatment of relatively high-strength wastewater offering the advantage of biomass concentrations much higher than achieved in suspended-growth systems. Attached growth systems offer the opportunity for the spatial distribution of mixed microbial populations, thus further enhancing the efficiency of such systems and increasing their resilience against toxic contaminants (Cervantes et al., 2006). The performance of this reactor type is strongly influenced by the manner in which influent is added. The biofilms growing in trickling filters, where the substrate is supplied by diffusion, are capable of withstanding the periodic addition of contaminants. This is because of the contact time between the microorganisms and the toxic contaminants in trickling filters is relatively short. In SBR technology, the fill phase may be composed of static fill, mixed fill, as well as react fill. Under static fill, influent is introduced to the reactor under a non-mixed, non-aerated environment. Mixed fill phase provides mixing without aeration and react fill is accompanied by both mixing and aeration. Because the SBR reactor serves as an equalization basin during the fill phase, it can easily tolerate stress imparted by toxic contaminants without degradation in effluent quality. The microorganisms receiving the full impact of any toxic contaminant respond accordingly, and the microbial population undergoes a continual shifting. However, long-term continuous exposure of toxic contaminants significantly affects the microorganism.

1.10 Interaction of heavy metals, antibiotics, and pesticides with aerobic sludge biomass

All microorganisms need essential metals such as copper, iron, manganese, and zinc to maintain homeostasis and cell functioning. The most common way by which microorganisms deal with excess metal is to pump the metal ions out and restrict uptake simultaneously.

Besides, some microorganisms have mechanisms to sequester and immobilize metals, whereas others enhance the metal solubility in the environment (Roane et al., 2009). Metals cannot be degraded through biological, chemical, or physical means to an innocuous by-product. The chemical nature of metal can be changed through oxidation or reduction; the elemental nature of metal remains the same (Roane et al., 2009). Metals interact with microbial cells through biosorption and bio-accumulation processes. Microbial interaction with metals depends on the bio-availability of the metal. It usually consists of the free ionic species or species that can be readily transformed into the free ionic species in solution (Roane et al., 2009). Tabak et al. (2005) have suggested several ways of metal-microbial interaction, i.e., biosorption, bio-accumulation, bio-transformation, bio-leaching, and biomineralization, as shown in Fig. 1.10. The bio-accumulation consists of active and passive uptakes. In living microorganisms, some heavy metal ions will go through the cell membrane. Transport of heavy metals across microbial cell membranes, known as intracellular accumulation, may be mediated by the same mechanism used to convey metabolically important ions such as potassium, magnesium, and sodium (Javanbakht et al., 2014). This metal uptake is referred to as an active uptake or intracellular uptake (bio-accumulation). In the dead cells, the metal ions are attached to the extracellular structure of bacterial cells, and is referred to as passive uptake; this process is called biosorption. In Gram-negative bacteria, the cells consist of extracellular lipo-polysaccharides (LPS), cell wall, cell membrane, and cytoplasm contents. In Gram-positive bacteria, the cell is surrounded by a thick cell wall consisting of several charged functional groups such as carboxyl, hydroxyl, phosphate, amine, and amide. These functional groups play an important role in the retention of metal ions on the cell surface (Al-Gheethi et al., 2015). In general, the degree of metal-microbial interaction depends upon the affinity of metal for microbial cells (Sterritt and Lester, 1981). EPS adsorbed copper has accounted for a large proportion of the total copper accumulated in biofilms (Miao et al., 2016).

Antibiotics can be degraded by microorganisms or eliminated by sorption onto the sludge at WWTP. During the bio-degradation process, antibiotics can be degraded into by-

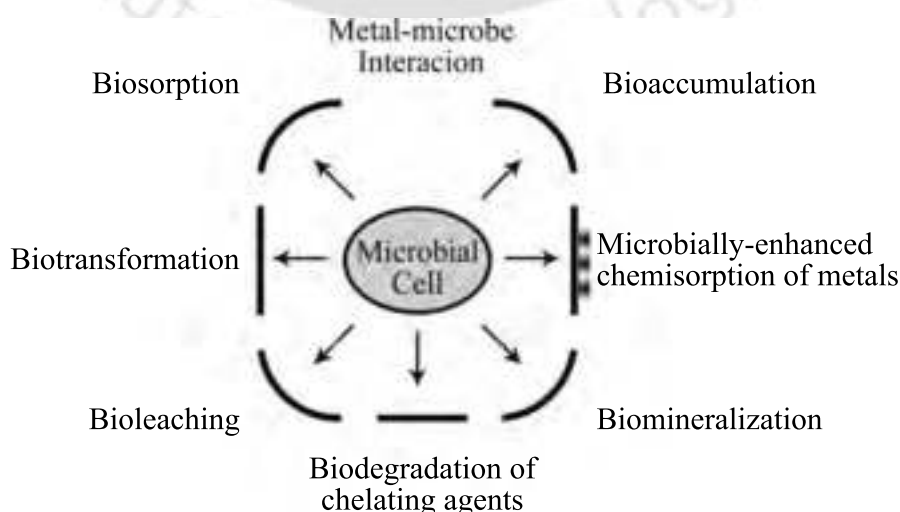


Fig. 1.10 Various types of metal-microbe interactions [Source: Tabak et al. (2005)].

products called transformation products (TPs), which are more polar than the main compound. Due to high polarity, resulting in low biodegradability, some transformation compounds cannot be eliminated efficiently (Grzešekowiak et al., 2018). Some of TPs can be more stable and toxic than the main compounds. Antibiotics have three main routes during biological degradation in WWTPs; it can (i) mineralize, (ii) transform to more hydrophobic compounds, and (iii) transform to more hydrophilic compounds. The fate of antibiotics in WWTP depends on the physicochemical properties of the compound and ongoing processes, as well as on environmental factors (climate, pH, redox condition, water, and sediment components). The interaction pathways of antibiotics with microorganisms at the wastewater treatment plant include adsorption, bio-degradation, and oxidation processes. In addition, other pathways, such as hydrolysis, photolysis, and volatilization, may be neglected due to their insignificant role for antibiotics reduction in wastewater treatment processes (Li and Zhang, 2010). The reason for the poor treatment of antibiotics depends on the four major points: (i) the relatively low concentration of antibiotics in the wastewater leads to lack of enzymes that are responsible for bio-degradation of antibiotics, (ii) inhibitory or toxic properties of antibiotics that can stop the microorganism activity responsible for bio-degradation, (iii) properties of antibiotics, and (iv) operational conditions of WWTP.

Bio-degradation of chlorpyrifos (CPS) is carried out both aerobically and anaerobically by the microbes through two major degradation pathways, i.e., catabolism and co-metabolism. The catabolic process involves the complete breakdown of complex organic compounds or its fragments. In contrast, the co-metabolic process leads to partial degradation of organic compounds without benefiting the organism (Dar et al., 2019). The accumulation of pesticides leads to the proliferation of pesticide degrading microbes (Shabbir et al., 2018). Microorganisms degrade pesticides by utilizing it as carbon sources and electron donors. However, its degradation depends on various environmental parameters and physiological, ecological, biochemical, and molecular aspects. CPS is hydrolyzed rapidly to its primary metabolite, 3,5,6-trichloropyridinol (TCP), other intermediary derivatives (Dar et al., 2019).

Ag NPs may pose a significant threat to the microbial community in wastewater treatment systems due to the adsorption and accumulation of nanoparticles in microbial aggregates (e.g., activated sludge flocs and biofilms) (Sun et al., 2013). The interaction pathway of Ag NPs with microbial cells is via sorption, as well as possible aggregation and sedimentation onto the sludge (Qiu et al., 2016b). Some NiO NPs are absorbed on the sludge surface or penetrate the cell membrane into the interior of microbial cells in the activated sludge. NiO NPs facilitates the increase in reactive oxygen species by disturbing the balance between the oxidation and anti-oxidation processes. The variation in lactate dehydrogenase demonstrates that NiO NPs could destroy the membrane and cause variations in the microbial morphology and physiological functions (Wang et al., 2017c). Some CuO NPs may be absorbed on the surface of activated sludge or penetrate the microbial membrane of the microbial cell, interior of activated sludge (Wang et al., 2017d). The large amounts of the NPs have been absorbed onto the activated sludge surfaces, along with EPS destruction

around the absorption site. Proteins are the most sensitive compounds damaged in the presence of nanoparticles, leading to the entry of nano-scale particles into the cell. NPs can penetrate cell membranes and subsequently affect the bacterial cell membrane integrity. The decrease of biomass activity has been attributed to endogenous respiration and biomass death during exposure experiments (Yazdanbakhsh et al., 2019).

Toxicity of metals/pesticides/antibiotics depends on the concentration, pH, HRT, SRT, biomass concentration, microbial community structure and environmental factor such as temperature and DO, etc. The reaction with proteins, cell wall or membrane, DNA modification, and inhibition of translations and transcription, etc., are the most common phenomenon through which metals/pesticide/antibiotics affect the microorganisms. The heavy metals are toxic to most microorganisms at specific concentrations and often cause serious upsets in biological waste treatment plants (Majid et al., 2010). The summary of various toxic influences of metals on the microbial cell is shown in Fig. 1.11. Metals bind to cellular ligands and displace essential metals from their regular binding sites (Roane et al., 2009). Metals also disrupt proteins by binding to sulfhydryl groups and disrupt nucleic acids by binding to phosphate or hydroxyl groups. As a result, protein and DNA conformation are changed, and the functions are impaired. It also affects oxidative phosphorylation and membrane permeability (Roane et al., 2009). Microorganisms often use specific transport pathways to bring essential metals across the cell membrane into the cytoplasm. Unfortunately, metals can also cross membranes via diffusion, non-specific uptake systems, or pathways designed for other metals. It has been suggested that metals block the enzyme systems or interfere with some essential cellular metabolites (Madoni et al., 1996). Metals are also known to coagulate and precipitate the proteins, many of which are denatured by this action and involved in the loss of cell membrane integrity. Cu(II) and Cr(VI) combine with thiol (R-SH) compounds by neutralizing the thiol (-SH) group within the cells (Roane et al., 2009). Among other divalent metal ions, Cu(II) has been found to cause unwinding and rewinding

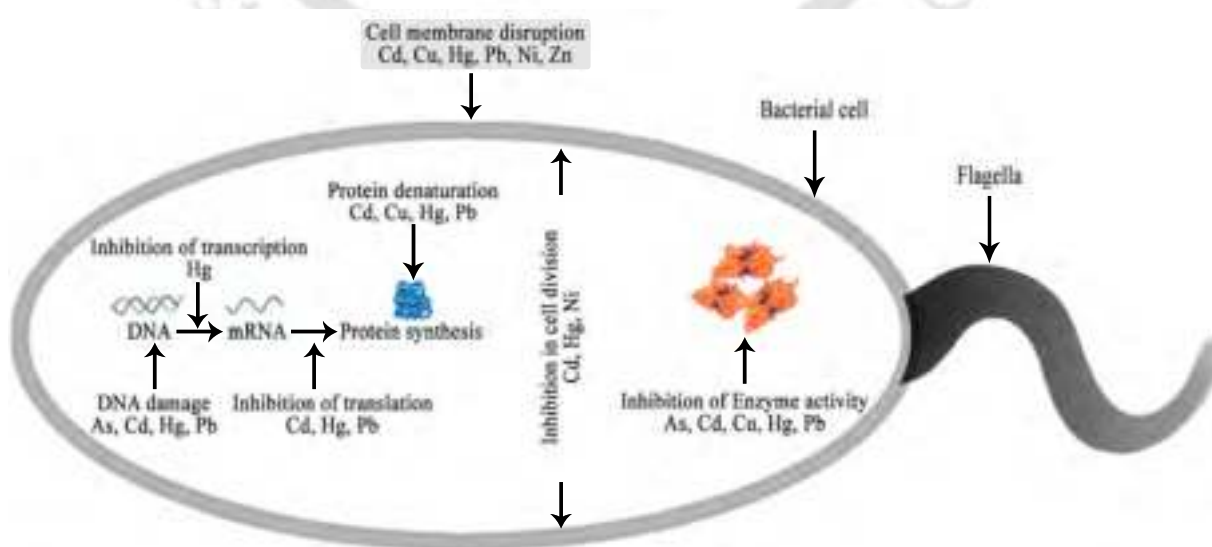


Fig. 1.11 A summary of toxic influences of metals on the microbial cell [Source: Roane et al. (2009)].

of DNA double helix depending on the experimental conditions. Cu(II) ions bind with a high affinity to DNA, which can lead to mutations and errors during transcription. Millimolar concentrations of Cu(II) ions induce DNA condensation, compaction, and cause DNA damage and strand breaks (Erxleben, 2018). Metals such as copper and iron can exist in different redox states. They may generate reactive oxygen species that damage proteins and nucleic acids (Roane et al., 2009). Many microorganisms demonstrate resistance to metals in water, soil, and industrial waste. The metals interact with essential cellular components through covalent and ionic bonding and can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA (Bruins et al., 2000). The metal-microbe interactions result in decreased growth, abnormal morphological changes, and biochemical processes inhibition in individual cell (Pepper et al., 2011).

The toxicity mechanism of nanoparticles on microorganisms is similar to heavy metals. For instance, due to their small size, Ag NPs can pass through cell membranes and accumulate in the cell, causing cell malfunction. Ag NPs can attach to the cell membrane and change membrane permeability, and cause cell death. Ag NPs can weaken cell membranes by damaging enzymes and also affect electron transport. Release of Ag^+ can also contribute to the antimicrobial effect that damage DNA, prevent cell replication (Dakal et al., 2016; Akter et al., 2018). The increased EPS production could provide a shelter to protect the microorganism against the toxicity of CuO NPs and prevent the oxygen from entering the inner regions of activated sludge (Wang et al., 2017d).

The mechanism of CPS action is not well explained, and literature is limited on this topic. The literature suggests that the aerobic sludge biomass is sensitive to the change in CPS concentration. The system's performance has affected mainly by 3,5,6-trichloro-2-pyridinol (TCP) accumulation. TCP is more toxic than CPS and has excellent solubility in water (81 mg/L). TCP has antimicrobial properties, which prevents the proliferation of CPS degrading microorganisms (Hou et al., 2018; Wang et al., 2017a).

Antibiotics are complex molecules that can have different functional groups within their chemical structure, which involve alteration of cell membranes, inhibition of cell wall, protein, and nucleic acid synthesis, and metabolic or anti-competitive antagonism (Grześkowiak et al., 2018). The enzymes involved in cell wall biosynthesis and the synthesis of nucleic acids and metabolites serve as a direct target for antibiotics (Egorov et al., 2018). Microorganisms have several following mechanisms to avoid the lethal actions of antibiotics; (i) production of enzymes inactivating it (e.g., hydrolysis by β -lactamase); (ii) changes in bacterial cell wall permeability; (iii) preventing the entrance of antibiotics; (iv) changes in antibiotic target sites; and (v) active transportation systems like efflux pumps in cell walls, which prevent the build-up of antibiotics inside cells (Grenni et al., 2018). The largest group of pharmaceutical drugs and its mode of action, is shown in Fig. 1.12. The class β -lactams consist of amoxicillin, ampicillin, carbenicillin, cloxacillin, penicillin, methicillin, cephalexin, cefprozil, cefuroxime, loracarbef, which have different side chains of β -lactam ring. It is used for a wide range of infections, including streptococcal infections. It is the most commonly

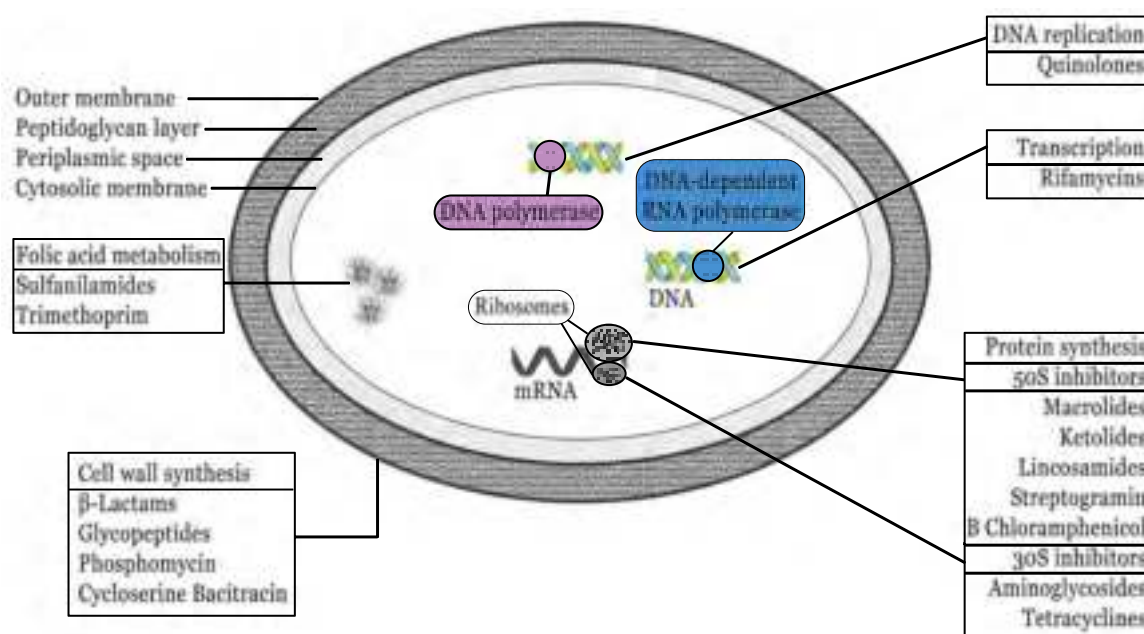


Fig. 1.12 Main classes of antimicrobial drugs, its targets, and mode of action on a bacterial cell [Source: Egorov et al. (2018)].

consumed class of antibiotics for human medicine in most countries. The action mechanism of β -lactams is to bind and inactivate enzymes required for bacterial cell wall synthesis resulting in defective cell walls formation. The activity of glycopeptides polymer units of the cell wall is inhibited by these antibiotics (Grześkowiak et al., 2018). Eventually, this leads to lysis of the cells and the death of the organism. Because β -lactam antibiotics are hydrolyzed in the ambient pH and temperature conditions, its concentrations in the environment are low despite its large consumption rate.

1.11 Summary

The domestic wastewater treatment systems are designed for sewage, which is not supposed to contain any toxic constituents. Sewage contains organic matter in the majority and various other compounds in trace amounts such as heavy metals, pharmaceuticals, etc. If improperly collected and treated, this sewage could harm the environment and human health. However, toxic pollutants (such as heavy metals/antibiotics/pesticides etc.) in higher concentrations are often encountered in domestic sewage of developing and under-developed countries. The efficient functioning of any biological wastewater treatment system depends on the wastewater characteristics and active aerobic biomass present in the aeration tank. Starting from scratch, environmental engineers usually follow the following major steps to establish a fully functional treatment plant for a community:

- * Design of treatment plant
- * Construction of treatment plant
- * Start-up and commissioning
- * Operation at steady state condition

✱ Handover functional treatment plant to the owner

In the first step, the design of biological wastewater treatment plants depends on the characteristics of wastewater and wastewater volume to be generated during its designed life. After completing the design process, the second step is construction of the treatment plant, which is a major task that may take 2-3 years. The construction of the Muharraaq Sewage Treatment Plant (STP) with a treatment capacity of 100,000 m³/d has been started in July 2012 and completed in June 2014 ([Verdict Media Limited, 2011](#)). The existing Chandler Airport Water Reclamation Facility has been upgraded and expanded during a period of 29 months of construction ([McCarthy Building Companies, 2015](#)). Construction, operation, and maintenance of a sewage treatment plant is an expensive proposition. CPCB estimates that the construction cost for an STP comes to Rs. 1 crore per MLD (approx. \$135000/MLD). Additional costs in an STP include provisions of power supply, installation of standby power generators, and employing technical manpower. Apart from these, the maintenance of a medium-sized STP (minimum 100 MLD) requires an amount between Rs. 70 lakh to Rs. 2 crores annually. After the successful construction of the treatment plant, the third step is to commission the plant with active biomass. The cultivation of biomass from scratch is a batch mode operation. During this step, either initial seed sludge is usually obtained from another treatment plant to save time or the biomass cultivated in-situ. The cultivation of a sufficient amount of aerobic biomass in the plant takes almost a year. Once enough biomass is cultivated in batch mode, the plant operation is shifted to a continuous mode of operation to obtain steady-state conditions with respect to plant performance.

The primary objectives of treatment plants are to treat the sewage to meet the effluent discharge standards. One of the challenges of sewage treatment is the changes in sewage characteristics. Some changes can be either short-term (seasonal, monthly, weekly, or daily) or long-term ([CPHEEO, 2013a](#)). Besides, heavy metals, pesticides, drugs, and pharmaceuticals also enter in sewage system via diverse routes and affect activated sludge microbes by damaging their cellular machinery resulting in malfunction of the plants. The changes in BOD, COD, MLSS, and MLVSS, and turbidity have been used to indicate the effects of toxic compounds on the treatment plant ([Neufeld and Hermann, 1975](#)). Sometimes the major portion of biomass is affected or inactivated. In that case, the wastewater treatment plant needs to be fully decanted, cleaned, and again new biomass needs to be cultivated in the plant. These processes may take approximately 12-18 months and also cost a lot of manpower and money. The untreated wastewater would be discharged directly to the receiving water body during this phase, which is a serious concern.

Machine/equipment/power shortage failures are common problems resulting in plant dysfunction ([CPHEEO, 2013b](#)), and it can be easily detected. The changes in wastewater composition is a hidden factor that directly attacks active biomass, causing plant failure, and is not easily distinguished by plant operators. Toxic pollutants in sewage directly affect the biomass and their impact could be acute or chronic. Acute toxicity is the result of either a single exposure or multiple exposures of toxic compounds in a short period of time. The

plant operator must identify the problem early because plant operation can be suspended within a short duration due to acute toxicity. Performance-related data does not serve the purpose in this situation. Chronic toxicity is resulted from the repeated or continuous addition of pollutants for the long-term. The plant operator gets sufficient time to understand the problem, and they may rely on performance-related data.

In India, the operation and management of the sewage treatment plants are outsourced (CPHEEO, 2013b). The contractors hire unskilled workers to cut down the cost of treatment. Unskilled and untrained operators cannot detect problems in the plant. The acute effect is more severe than the chronic effect because it does not provide sufficient time to respond to it. To estimate toxicity effects, the tools and techniques should be simple so that an unskilled worker can perform it. The techniques such as microscopy and tests determining enzyme activity and changes in microbial community have unique features, and performing these tests requires a well-established laboratory facility and skilled manpower. These tests provide qualitative and quantitative information about biomass, but it is also costly and tedious. Hence, there is a need to develop a technique that an unskilled worker can perform the test on the field.

Heavy metals are found in a treatment plant, which affects the operation and efficiency of the plant. Sooner or later, a plant operator finds a problem exerted by heavy metals and try to eliminate it. In spite of being discontinued in influent, the residual amount of heavy metals remains in the aeration tank for several days. After metal discontinuation, the adsorbed metals start to desorb from the sludge surface, and tolerant microorganism growth increases. If the effect of the heavy metals is less severe on aerobic biomass, the treatment plant will take time to regain its original treatment efficiency level. Recovery time depends on the intensity of impact caused by stress-causing agents on the biomass. If heavy metals cause severe inactivation of biomass, new biomass is required to start the treatment plant. This part of the studies is completely missing in the literature. Researchers have mainly focused on reactor performance parameters and metals, antibiotics, and pesticides removal profiles in various reactors. However, changes in sludge characteristics, biomass morphology, and activity in the presence of metals, antibiotics, and pesticides are missing in the literature.

1.12 Lacunae

Biomass is the heart of a biological process. Limited literature reports have discussed its behavior in the presence of metals, antibiotics, pesticides, etc. There are several studies mentioning the impact of heavy metals, antibiotics and pesticides on reactor performance but did not provide relevant information about the behavior of aerobic sludge biomass after being exposed to such pollutants. The aerobic sludge biomass is the most affected part of treatment plant after the exposure with toxic substances, reflected through poor settling with slow bio-degradation rate, inhibition of microbial activities, and growth reduction which may result in the deteriorated plant performance. Hence it is requisite to evaluate the aerobic sludge biomass characteristics and behavior when metals, antibiotics, and pesticides are

present in influent. This will provide an actual insight related to the impact of heavy metals, antibiotics, and pesticides on biomass.

However, termination of such toxic substances in influent does not resolve the problems that have developed due to their presence. The post-termination effects of metals, antibiotics, and pesticides are long-lasting that may not allow aerobic biomass to perform well, resulting in the hampered treatment plant efficiency. A vast literature is available on the studies related to the reactor operation and performance in the presence of heavy metals, antibiotics, and pesticides. However, the emphasis has not been given on with behavior of biomass during and after discontinuation of heavy metals, antibiotics, and pesticides exposure. This assessment can be useful to assess the recovering capacity, characteristics, and behavior of biomass after the termination of such pollutants in the influent. Also, the development of a quick and reliable tool is required to evaluate aerobic sludge biomass quality. The tools should be simple enough for an unskilled worker to perform on the field with a short training imparted. Apart from this, the following research gaps are identified based on literature:

- * There is limited information on aerobic sludge biomass characteristics, behavior, and morphology at different concentrations of heavy metals, antibiotics, and pesticides. A detailed evaluation is needed to understand the adverse impacts of such toxic pollutants on the flocs structure of aerobic sludge biomass, and its enzyme activities. Also literature is silent on the status of biomass if the toxic pollutants have been stopped. Most authors have investigated performance-related parameters in the presence of such toxic pollutants. In contrast, studies on the post termination effects on biomass are very much needed.
- * There are research studies mentioning the effects of heavy metals, antibiotics, and pesticides on biological wastewater treatment performance, and most studies were carried out at lower concentrations for short-term reactor operation. However, information is limited on the microbial community changes in activated sludge as the increase in concentrations of such toxic pollutants for a long-term operation, and the relationship among the performance, microbial activities, and microbial community structure is also missing.
- * Heavy metals, antibiotics, and pesticides undoubtedly affect the biological process and remain in the system even after its termination in the influent. The recovery of aerobic biomass depend on the extent of toxicity of heavy metals, antibiotics, and pesticides. The assessment of recovery capability of aerobic biomass is entirely missing after exposure.
- * Heavy metals, antibiotics, and pesticide exposures are responsible for slow organic degradation rates. However, the main reason, i.e., aerobic sludge biomass activities in response to different concentrations of heavy metals, antibiotics, and pesticides, is not addressed so far in published literature.
- * Currently, there is a lack of information on biomass behavior, EPS, and cell viability of

aerobic sludge biomass after being exposed to heavy metals, antibiotics, and pesticides. It is necessary to understand the connection between these parameters with different concentrations of heavy metals, antibiotics, and pesticides on biological processes.

- * Most authors have successfully treated synthetic piggery wastewater. A real piggery wastewater contains a huge amount of heavy metals and suspended solids. The piggery wastewater treatment in the presence of copper and suspended solids is still scarce, and the effect on raw and pre-treated piggery wastewater on biological process is lacking in the literature.
- * Most studies have been performed in a controlled environment. Therefore, studies must be performed in an uncontrolled or real environment similar to field conditions to check the impact of these pollutants on aerobic biomass and reactor performance.

1.13 Objectives of the study

The three fold objective of this experimental investigation are to (i) assess impact of stress imposed by undesirable pollutant on aerobic sludge biomass, (ii) assess the recovery potential of stressed aerobic sludge biomass after discontinuation of stress-causing undesirable pollutant, and (iii) identify the tools and techniques suitable for field application in assessing the health status of aerobic sludge biomass. This study mainly focuses to assess aerobic sludge biomass behavior, structural changes, and tolerance capacity when heavy metals, pesticides, antibiotics, and piggery wastewater are present in influent. This study also examines the recovery capacity of aerobic biomass after discontinuation of the heavy metals, pesticides, antibiotics, and piggery wastewater in the influent. The above objectives are further elaborated.

- * The impact of heavy metal (copper) on aerobic sludge biomass has been investigated by framing objectives as to (i) study the effect of copper on environmental, performance, biomass related parameters of the reactor; (ii) investigate the biomass morphology, floc structure, and filament index; (iii) determine aerobic sludge biomass activity and enzyme activity; (iv) estimate the microbial community shift due to copper stress; and (v) determine the recovering potential of aerobic sludge biomass after discontinuation of copper in influent.
- * The effect of antibiotic (amoxicillin) doses on aerobic sludge biomass has been evaluated, which aims to (i) investigate the effect of amoxicillin on organic removal and biomass growth; (ii) determine the biomass concentrations, sludge settling characteristics, morphology, and viability of aerobic sludge biomass in the presence of amoxicillin; (iii) estimate the influence of amoxicillin on the aerobic sludge biomass activity and enzyme activity; (iv) analyze the changes in protein (PN), polysaccharide (PS) and extracellular polymeric substances (EPS) contents due to amoxicillin in reactors; (v) estimate the microbial shift due to addition of amoxicillin to the reactors; and (vi) evaluate the response of aerobic sludge biomass in terms of organic removal, biomass

behavior and morphology, flocs composition, and microbial activity and community shift after discontinuation of amoxicillin in the feed.

- * The adverse effect of pesticide (chlorpyrifos) on aerobic sludge biomass has been studied with objectives to (i) investigate the effect of chlorpyrifos on environmental, and performance-related parameters of the reactor with different concentration of chlorpyrifos; (ii) analyze the influence of chlorpyrifos on the solids characteristics and settling, viability, and flocs structure; (iii) determine the effect of chlorpyrifos on aerobic sludge biomass activity and enzyme activity; (iv) estimate the changes in biomass morphology and PN, PS, and EPS contents due to chlorpyrifos in reactors; (v) investigate the influence of chlorpyrifos on the microbial community structure; (vi) study the variation in reactor performance and biomass characteristics after discontinuation of chlorpyrifos in influent along with microbial activity and structure; and (vii) determine the recovery potential of aerobic sludge biomass after discontinuation of pesticide in influent.
- * The capability of aerobic sludge biomass in treating actual wastewater (piggery wastewater) has been evaluated with focus to (i) estimate the impact of filtered and non-filtered piggery wastewater spiked with copper on aerobic sludge biomass, focusing on performance and biomass-related parameters of reactors; (ii) determine the extent of filtered and non-filtered piggery wastewater influence on solids and settling characteristics, flocs morphology, and viability of aerobic sludge biomass; (iii) determine aerobic sludge biomass activity and enzyme activity treating piggery wastewater spiked with copper; (iv) estimate PS, PN, and EPS content in aerobic sludge biomass treating piggery wastewater; (v) changes in the microbial community structure due to exposure of piggery wastewater spiked with copper; (vi) study the biomass behavior and reactor performance along with flocs structure, viability, biomass and enzyme activity, and shift in the microbial community after discontinuation of piggery wastewater spiked with copper in the influent; and (vii) recovery potential of aerobic sludge biomass impacted with actual wastewater.

The goal of this research is to give more emphasis on biomass and evaluate the impact of heavy metals/antibiotics/pesticides to aerobic sludge biomass and its influence on the reactor performance, microbial diversity, activity, morphology, and settling of the aerobic sludge biomass. This may also help in identifying suitable tools and techniques, which may find application for field monitoring of status of biomass in the actual treatment plant.

1.14 Research methodology

The seed sludge has been collected from IIT Guwahati and cultivated in a Growth Phase Reactor (GPR) and further grown in Mother Reactor (MR) (*Appendix-II*). The aerobic sludge biomasses have been exposed to different concentration of heavy metals/antibiotics/pesticides/piggery wastewater spiked with copper, and their impacts on aerobic sludge biomass have been studied in two phases, i.e., stressed phase (when biomass exposed to toxic

pollutants) and recovery phase (when exposure of toxic pollutants discontinued). Fig. 1.13 shows the overview of research methodology.

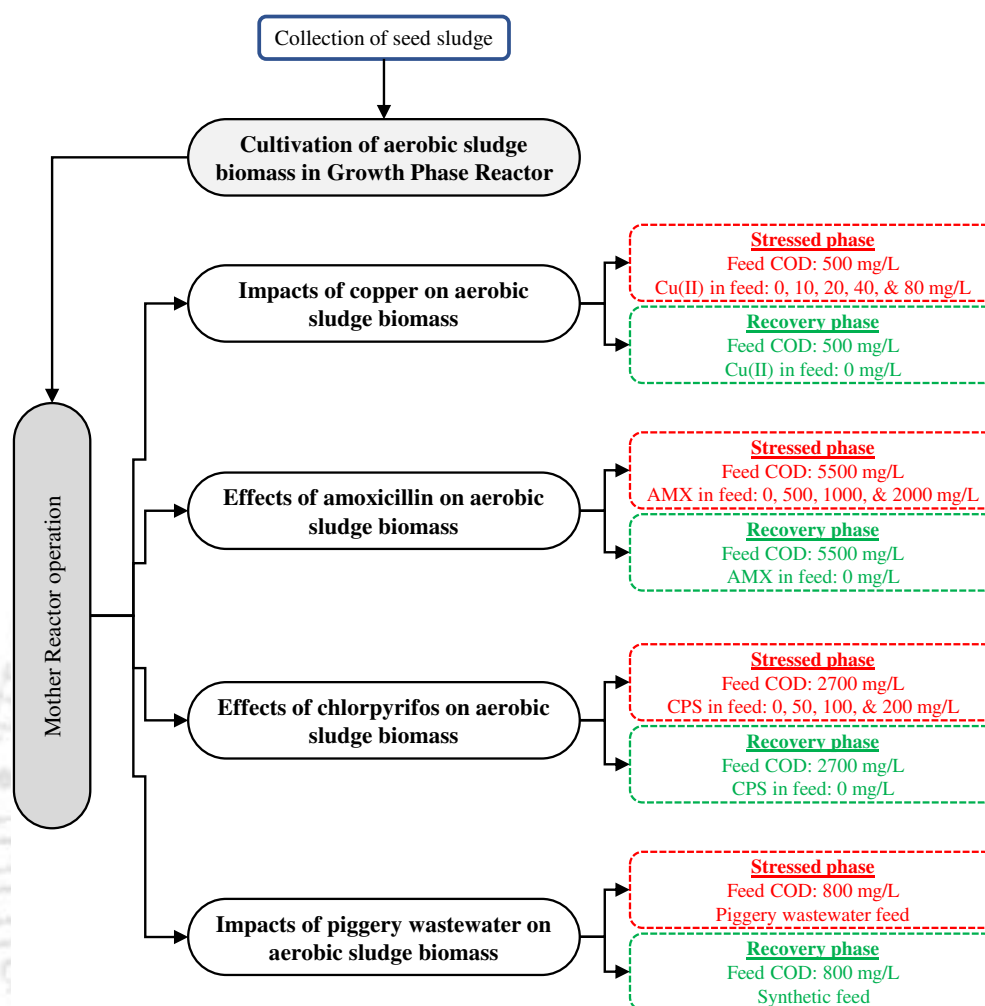


Fig. 1.13 Flow chart of research methodology.

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Impacts of Heavy Metal (Copper) Stress on Aerobic Sludge Biomass

The urban sewage is being contaminated with heavy metals due to its indiscriminate disposal. The metal contaminated sewage is likely to find its way to the sewage treatment plant (STP). This may cause irreversible changes in biomass morphology and microbial community as well and thereby affecting treatment performances. This chapter investigates the effects of heavy metal [Cu(II)] on treatment performance and biomass morphology. The impacts of heavy metal [Cu(II)] on biomass have been examined additionally through biomass related general and microbiological parameters.

2.1 Background

Heavy metals (such as copper, zinc, lead, mercury, chromium, cadmium, iron, nickel, and cobalt) are extensively used in industries such as automobile, metal finishing, leather tanning, electroplating, petroleum, textile-dyeing, etc. (Pamukoglu and Kargi, 2007a; Li et al., 2011; Yan et al., 2015). The effluents generated by these industrial activities contain a high concentration of heavy metals that unintentionally find their ways to the sewerage system (Brown and Lester, 1979). The wastewater samples collected from drains from the industrial belt in Delhi has indicated the presence of nickel, total chromium, hexavalent chromium, copper, zinc, lead, and cadmium in the range of 1.7-105, 0.25-108, 0.01-60, 0.6-30, 0.02-9, 0.01-1.2 and 0.01-0.14 mg/L respectively (Singh, 2014). In another study, Singh and Ram (2016) have observed the presence of chromium, nickel, lead, zinc, and copper in the range of 21.5-47, 72-243, 5.3-9.7, 97-731, and 8.4-20.7 mg/L in drains of Chandigarh and Haryana probably coming from electroplating units located within the city. A lower concentration of heavy metals serves as a micro-nutrient for microorganisms (Dai et al., 2016) in its cell growth. However, higher concentration causes inhibition or toxicity to cell growth, loss of flocs structure, microbial community structure changes, and decreases

treatment efficiency (Ong et al., 2004). Toxicity of heavy metal ions depends on its ionic species type, concentrations, and the organisms as well as the environmental conditions such as pH, temperature, dissolved oxygen (DO), presence of other metal ions, ionic strength along with the operating parameters such as sludge age and hydraulic residence time (HRT) (Pamukoglu and Kargi, 2007b; Stoveland and Lester, 1980).

Efforts have been made to study the effects of heavy metal toxicity on metal removal efficiency and microbial communities of activated sludge. Yan et al. (2015) have reported that continuous exposure of 20 mg/L of Cu(II) imposes an inhibitory effect on COD and ammonia removal in sequencing batch reactors (SBRs). Li et al. (2011) have investigated the effect of continuous exposure of Cu(II) on extracellular polymeric substances (EPS) of bulking activated sludge. According to Feng et al. (2016), the inhibitory effects of Cu(II) on substrate removal efficiency and dehydrogenase activity increase with increase in Cu(II) concentration. The study of enzyme activities can provide suggestive information regarding the biological process and may help to understand the bio-degradation of organic matter and toxic compounds. Yang et al. (2017) have investigated the effect of Ni(II) (0, 0.1, 1, and 10 mg/L) on the flocculability and settleability of activated sludge. Short-term exposure to Ni(II), even at a level of 10 mg/L, has little effect on sludge volume index (SVI) and effluent suspended solids. Su et al. (2016) have examined the responses of activities and communities of bacteria to Cr(VI) (1, 5, 8, 10, and 20 mg/L) in activated sludge. Cr(VI) concentration up to 8 mg/L has caused no visible effect on the COD degradation, whereas Cr(VI) loading of 10 and 20 mg/L concentrations have a slight inhibition on COD removal efficiency. After discontinuation of Cr(VI), the COD removal efficiency has appeared to recover slowly to its original level. Dai et al. (2016) have studied the impact of continuous exposure of Cu(II) at 20 mg/L concentration and shock dosage of Cu(II) 10, 20, and 40 mg/L on pollutant removal in activated sludge and have observed that COD and NH₃-N removal have been severely inhibited, which have failed to recover in the recovery phase. However, the sludge has shown tolerance to Cu(II) toxicity to some extent under shock dosages. The bio-transformation of organic matter or pollutant depends on the enzyme activity produced by activated sludge. The solid-liquid separation procedure is influenced significantly by the morphology of the activated sludge. The morphology of activated sludge flocs effectively contributes to biomass separation and quality of treated effluent (Jin et al., 2003; Liwarska-Bizukojc et al., 2015). Therefore, it is desired to study enzyme activity and flocs morphology of activated sludge. So far, most of the published literature has focused on bio-transformation, pollutant removal, and identification of organisms in the activated sludge in the presence of heavy metals.

These studies have revealed the impact of heavy metals on the performance of the activated sludge reactors; however, there is a lack of study, and no attention has been paid to correlate the performance of the reactors with biomass morphology. This research aims to assess the impact of Cu(II) on reactor performance, changes in biomass morphology, and its correlation with reactor performance. In addition, efforts have also been made to assess the recovery potential of aerobic sludge biomass when the metal is discontinued in the feed.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

All chemicals and reagents used in this study are of analytical or HPLC grade, manufactured by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals and reagents used in the study are given in *Appendix-I* (Table A-I.1). All the reagents and stock/standard solutions have been prepared using milli-Q water (Merck Millipore, Germany). The characteristics of milli-Q water used in this study are given in *Appendix-I* (Table A-I.2).

2.2.2 Aerobic sludge biomass

The aerobic sludge biomass withdrawn from the mother reactor (MR) has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in detail in *Appendix-II*.

2.2.3 Reactor selection

The emphasis of this study is to assess the impact on aerobic sludge biomass reactor performance and to observe the changes in biomass morphology due to presence of Cu(II) in the synthetic feed. The performance and morphology changes have to be assessed under four different fixed loads of Cu(II), while adequate organics are available for bio-degradation. Hence, the reactor has to be fed with fresh synthetic feed periodically to ensure the availability of organics. This may increase accumulated residuals of Cu(II) along with planned loads of Cu(II) with every fresh feed. Considering these requirements, it is planned to use batch reactor of 1 L capacity for this study. These reactors are planned to be operated in five different steps as shown in Fig. 2.1 and explained below which is similar to sequencing batch reactor (SBR):

- * **Fill** : The fill operation consists of adding the synthetic feed to the reactors containing settled active aerobic sludge biomass. The fill phase may be of 10-20 min duration.
- * **React** : In this step, biomass and synthetic feed is allowed interaction for biochemical

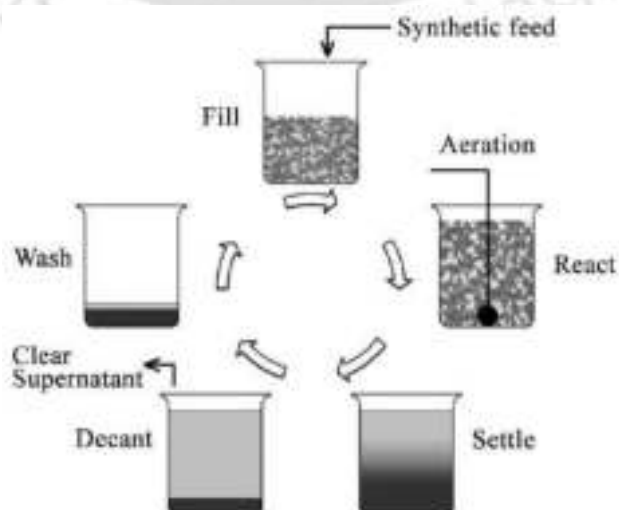


Fig. 2.1 Schematic sequence of operation of reactor.

reaction through aeration. The duration of this step may be 670-690 min.

- * **Settle** : Towards end of the each react step, residual organic content may be too low and therefore, fresh feeding is required. However, before adding the fresh feed, the clear liquid from the reactor has to be taken out. For this purpose, the settle step is required which helps in settling the biomass at the bottom of the reactor, while clear supernatant is left above. The duration of settle step may vary 10-20 min. The settling time is kept short in order to decant dispersed and slow-settling biomass.
- * **Decant** : In this step, clear supernatant is withdrawn from reactor. The amount of clear supernatant is likely to depend on the settling properties of the biomass. Rapid settling biomass may allow withdrawal of larger volume of supernatant while slowly settling biomass will allow smaller volume of supernatant. The decant step may vary in the range of 5-10 min.
- * **Wash** : To prevent progressive build-up of increasing metallic concentration (residuals from previous feedings and fresh addition in the subsequent feeding) and subsequent exposure of biomass to more than the planned metallic load, the settled mixed liquor in the reactor retained after decantation is washed once with tap water before starting the next feeding. The washing is carried out by refilling the reactors with tap water up to 1 L volume, and then gently mixing the content for 1-2 min. Thereafter, the content is allowed to settle for a period of 5 min before the supernatant is decanted. After washing, the reactor is again filled with tap water up to a 1 L volume and the clear liquid is sampled for the estimation of background or residual Cu(II) concentration left after the washing— designated as 'BG_{Cu(II)}'. The loss in the liquid volume created due to withdrawal of sample has to be made up by the new feed.

2.2.4 Reactor setup and operation

The experimental setup consists of two sets of five identical reactors with a working volume of 1 L (setup shown in Fig. 2.2). The reactors have been designated as R_{Cu0_1} and R_{Cu0_2} [control reactors with no metal in the feed], R_{Cu10_1} and R_{Cu10_2} [10 mg/L Cu(II) in the feed], R_{Cu20_1} and R_{Cu20_2} [20 mg/L Cu(II) in the feed], R_{Cu40_1} and R_{Cu40_2} [40 mg/L Cu(II) in the feed], and R_{Cu80_1} and R_{Cu80_2} [80 mg/L Cu(II) in the feed]. The reactors have been fed with copper ions along with Lactogen 3 as a source of carbon and nutrients. The composition of Lactogen 3 powder is presented in Appendix-II (Table A-II.2). The reactors have been operated at ambient conditions without pH control for two cycles per day with a fill time of 10 min, a react time of 675 min, a settling time of 10 min, a decant time of 15 min and a wash time of 10 min (as shown in Fig. 2.3). The settling time has been kept short (10 min) in order to decant dispersed and slow-settling biomass. The reactors have been initially filled with ~300 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~1500 mg/L] withdrawn from mother reactor (MR). The synthetic feed has been prepared by using Lactogen 3 (0.50 g) and lukewarm water (~100 mL), properly mixed using magnetic stirrer and then added to the reactor. The hydrated copper sulfate (CuSO₄·5H₂O) has been used as

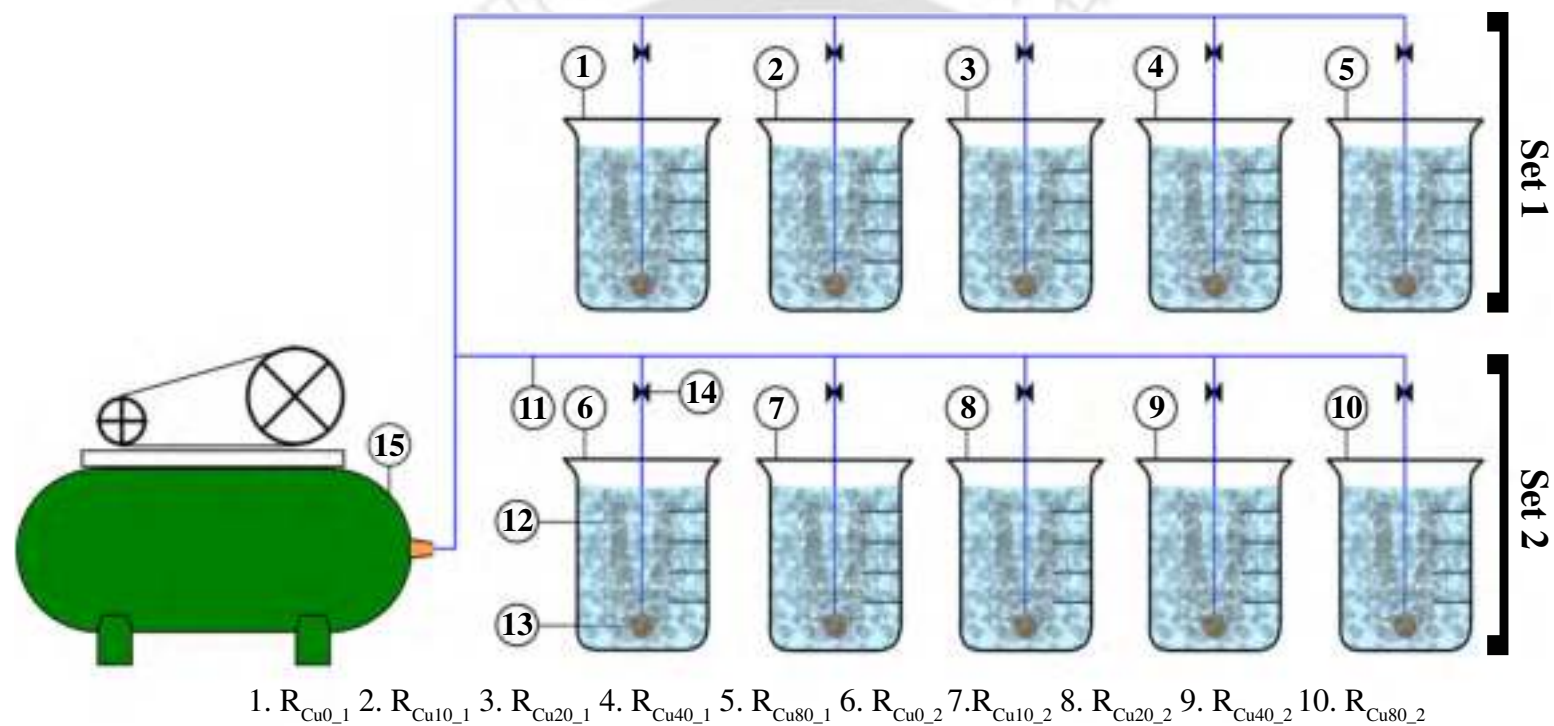


Fig. 2.2 Schematic diagram of reactor setup used for experiment.

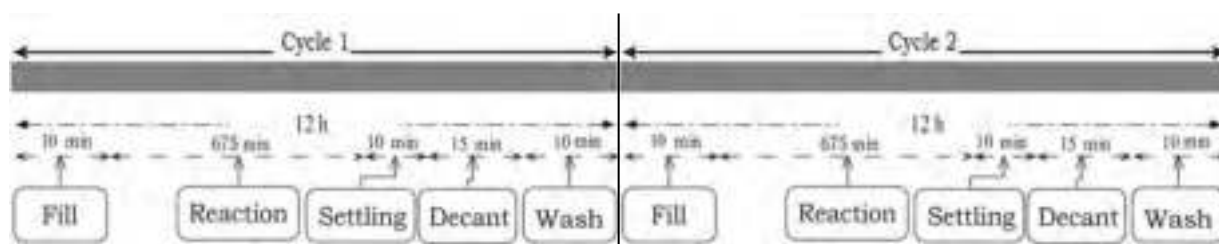


Fig. 2.3 Schematic representation of cycle time for reactor operation.

the source of Cu(II) metal in feed. A stock solution of Cu(II) metal ion has been prepared by dissolving 3.92 g of hydrated copper sulfate in distilled water, and the final volume has been made to 100 mL. After feeding 0.50 g of Lactogen 3, Cu(II) metal stock solution in volume of 0, 1, 2, 4, and 8 mL have been added, respectively, to R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} to yield the metal ion concentrations as 0, 10, 20, 40, and 80 mg/L, respectively. After addition of stock metal solution, the final volume of the reactor has been made to 1 L with tap water, which gives an initial COD concentration of 530-580 mg/L. For keeping the biomass in suspension, air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffuser.

The 1st cycle has been started around 9.30 am in the morning and room temperature has been recorded at that time, while room temperature during decanting has been recorded at 8.55 pm in the night and then 2nd cycle started around 9.30 pm. Since, the reactors have been fed with Cu(II) metal ions which causes stress on aerobic sludge biomass, hence the operation of these reactors have been termed as *stressed phase operation*. The stressed phase operation has lasted for 25 d in which the steady-state condition with respect to COD removal (%) has been achieved after 10-12 d of operation from the start and steady-state condition data obtained till 25th d of operation. Thereafter, the reactors have been operated without metal ion in the feed for the next 29 d. This phase of operation has been termed as *recovery phase operation*. The steady-state condition has been achieved after 8-10 d of operation from start of the recovery phase operation with respect to COD removal (%).

The initial liquid samples have been withdrawn after the fill stage (proper mixing the feed and metal solution ensured), and effluent samples have been collected during the decant step at the end of a completed cycle. During a settling time of 10 min of the reactors, the settled mixed liquor (sludge biomass + liquid) has occupied up to 850 mL for poorly settling biomass to 100 mL for rapidly settling biomass. It has given an overall volume exchange ratio for decanting with in the range of 10-85% throughout the study. The aerobic sludge biomass has been washed thrice at the end of the stressed phase operation but before starting the recovery phase operation.

2.2.5 Analytical methods

The frequencies of initial and effluent sample analysis are presented in [Table 2.1](#). The analytical parameters have been grouped into four different categories; (i) environmental related parameters— which helps in assessing the environmental conditions of reactor and

Table 2.1 Analysis frequency of initial and effluent sample parameters.

Category	Parameters	Frequency
Environmental related parameters	Room temperature	Daily
	Initial pH	
	Initial Cu(II) concentration	
Reactor related performance parameters	Effluent pH	Daily
	Effluent Cu(II) concentration	
	Chemical oxygen demand (COD)	
	Alkalinity	
Biomass related general parameters	Mixed liquor suspended solids (MLSS)	Every 7 th d
	Mixed liquor volatile suspended solids (MLVSS)	
	Sludge volume index (SVI)	
	Interface settling velocity (ISV)	Every 4 th d
	Aerobic sludge biomass activity (ASBA)	Towards end of stressed and recovery phase operations
Biomass related microbiological parameters	Microscopic observation after Gram's staining	Every 5 th d
	Photographic observation	
	Filament index (FI)	
	FESEM and EDX	Towards end of stressed and recovery phase operations
	Catalase activity	
	Dehydrogenase activity	
	Protease activity	
	Urease activity	
	Amplified ribosomal DNA restriction analysis (ARDRA)	

its contents, (ii) reactor performance-related parameters— which helps in assessing the performance of the reactor, (iii) biomass related general parameters— which are generally used by environmental engineers in assessing the status of reactor biomass, and (iv) biomass related microbiological parameters— are microbiological tools for assessment of biomass but being seldom used by wastewater plant operators or environmental engineers. Various analytical techniques used in this investigation are presented in the following subsections.

2.2.5.1 Temperature and pH

The room temperature has been measured using a mercury glass thermometer gifted by M/s North East Chemicals Corporation, Guwahati, India. The temperature range of the thermometer is between 0-50°C, with the least count of 2°C. pH has been measured using a digital pH meter (Thermo Scientific, Orion 3 Star, Singapore).

2.2.5.2 Determination of Cu(II)

The Cu(II) concentration in the samples has been determined using a flame emission atomic absorption spectrometer (AAS) (Varian, AA240, Netherlands) as per the methods described in Standard Methods (APHA et al., 2005). The metal stock solutions of Cu(II) of 10,000 mg/L concentration has been prepared using copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in

milli-Q water, and the stock solution has been diluted to prepare standard calibration curve. The standard curve is presented in *Appendix-I* (Fig. A-I.1).

2.2.5.3 Chemical oxygen demand

Chemical oxygen demand (COD) has been estimated using the dichromate method, as suggested in Standard Methods (APHA et al., 2005). A portion of the sample has been centrifuged at 8000 revolutions per min (rpm) to remove solids and clear liquid is used for COD estimation. In this method, 2.5 mL of a suitably diluted sample is taken in COD vials and added with 1.5 mL of standard potassium dichromate digestion solution (0.016 M) and 3.5 mL of sulfuric acid reagent, properly mixed and digested with closed reflux using a COD digester (Hach, DRB 200, USA) at 150°C for 2 h. Later, the solution mixture has been cooled to room temperature and titrated against ferrous ammonium sulfate (FAS) solution (0.1 M) using ferroin as the indicator. A sharp color change from blue-green to brick reddish has been considered as the endpoint. The FAS solution has been standardized against standard 0.1 M $K_2Cr_2O_7$ digestion solution regularly. COD in the sample has thus been calculated using the Eq. 2.1 and expressed as mg O_2 /L.

$$\text{Chemical oxygen demand (COD) (mg } O_2/\text{L)} = \frac{(A-B) \times 8000 \times M}{V} \quad (2.1)$$

where A is the volume of FAS consumed by blank (mL), B is the volume of FAS consumed by the sample (mL), V is the sample volume (mL), and M is the molarity of the FAS solution.

2.2.5.4 Alkalinity

The alkalinity of water/wastewater is its acid-neutralizing capacity determined by titration against sulphuric acid to the endpoint of the acid-base reaction, as suggested in Standard Methods (APHA et al., 2005). Alkalinity results due to presence of hydroxides, carbonates, and bicarbonates of such elements as calcium, magnesium, sodium, potassium, and radicals ammonium ion. The water samples are titrated against 0.02N sulphuric acid to pH 8.3 with 3 drops of phenolphthalein indicator and further titrated to pH 4.5 with a mixed indicator (bromocresol and methyl red). The alkalinity is calculated using Eq. 2.2 and expressed as mg/L $CaCO_3$.

$$\text{Alkalinity (mg } CaCO_3/\text{L)} = \frac{A \times N \times 50000}{V} \quad (2.2)$$

where A is the volume (mL) of titrant used, N is the normality of standard acid solution, and V is the sample volume (mL).

2.2.5.5 Solids analysis

The aerobic sludge biomass has been withdrawn during the aeration phase from reactors to measure the mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) and analysis have been performed as per Standard Methods (APHA et al., 2005). The aerobic sludge biomass samples have been filtered using 0.45 μm quantitative ashless filter paper (Axiva Sicheem Biotech, India) and kept overnight in a hot air oven (Tanco, OVEN PLT-125, India) at 105°C and further ignited at 550°C for 2 h in a muffle furnace

(Thermo Scientific, Lindberg/Blu M LGO Box furnaces, Hong Kong). The MLSS and MLVSS have been calculated using Eq. 2.3 and 2.4, respectively.

$$\text{MLSS (mg/L)} = \frac{(A_1 - B_1) \times 10^6}{V} \quad (2.3)$$

where, A_1 is the weight of dried filter + retained solids (g), B_1 is the weight of dried filter (g), and V is the sample volume (mL).

$$\text{MLVSS (mg/L)} = \frac{(A_2 - B_2) \times 10^6}{V} \quad (2.4)$$

where, A_2 is the weight of dried residue + crucible (g), B_2 is weight of dried crucible (g), and V is the sample volume (mL).

2.2.5.6 Interface settling velocity and sludge volume index

The interface settling test has been carried out in a 1 L graduated cylinder as suggested by Metcalf and Eddy (2003) and Peavy et al. (1985). The reactor content of 1 L containing aerobic sludge biomass has been poured into the graduated cylinder and mixed to make the uniform suspension; then, it is allowed to settle quietly. It forms a clear interface between sludge biomass and clarified liquid. The interface height is measured at regular time intervals. A plot is made between interface height vs time. The highest slope of the plot yields interface settling velocity (ISV).

The sludge volume index (SVI) is the volume (mL) occupied by 1 g of a suspension after 30 min settling. SVI is typically used to monitor settling characteristics of activated sludge and other biological suspensions. While carrying out ISV test, the settled sludge volume is noted down after 30 min of settling. The SVI is calculated using Eq. 2.5.

$$\text{Sludge volume index (SVI) (mL/g)} = \frac{\text{Settled sludge volume (mL/L)} \times 1000}{\text{Mixed liquor suspended solids (mg/L)}} \quad (2.5)$$

2.2.5.7 Aerobic sludge biomass activity test

The estimation of aerobic sludge biomass activity (ASBA) has been carried out by modifying methanogenic activity test procedure suggested by Jawed and Tare (1996, 1999). The aerobic sludge biomass (approximately 1000 mg/L VSS) mixture has been withdrawn from batch reactors in 1 L plastic beakers (height 14.5 cm × diameter 12.5 cm), and the total volume has been made up to 1 L using tap water. A sufficient amount of Lactogen 3 (0.50 g/L) has been added to the reaction vessel to obtain the initial feed COD concentration of approximately 500 mg/L. After that, aeration has been imparted continuously. Samples have been withdrawn from the reaction vessels at suitable time intervals, solids separated from the liquid portion through centrifuging at 8000 rpm (Remi, R-24, India) for 5 min, and residual COD has been estimated. At the end of the test, the amount of biomass present in the reactor vessels has also been estimated as volatile suspended solids (VSS). A plot has then been made between cumulative COD removed with time. The highest slope of the plot of cumulative COD removed

with time has been used to estimate the activity of aerobic sludge biomass using Eq. 2.6.

$$\text{Activity (mg COD removed/mg VSS d)} = \frac{\text{Slope (mg COD removed/h)} \times 24 \text{ h/d}}{\text{Amount of biomass (mg VSS)}} \quad (2.6)$$

2.2.5.8 Filament index

Filament index (FI) is used to measure the population of filamentous microorganisms in aerobic sludge biomass by measuring the size and length of the filament. A scale of 0 to 5 has been used ('0' for none to '5' for many filaments). The FI has been measured by the method suggested by Eikelboom (2000). Samples have been observed under a microscope at low magnification and compared with reference images provided by Eikelboom (2000).

2.2.5.9 Photographic observation

Photographic images have been captured using Asus mobile (Asus, Zenfone 2 Laser, Taiwan). The setup has been prepared using a LED light source (non-flickering LED) covered with a transparent glass sheet. The glass sheet helps to hold the glass Petri dish and allows light to pass through the Petri dish, as shown in Fig. 2.4. The biomass sample with distilled water poured into a glass Petri dish have been used for observation. The Petri dish also has a 7.0 cm long plastic scale with a least count of 1.0 mm placed over the aerobic sludge biomass to indicate the size of flocs.

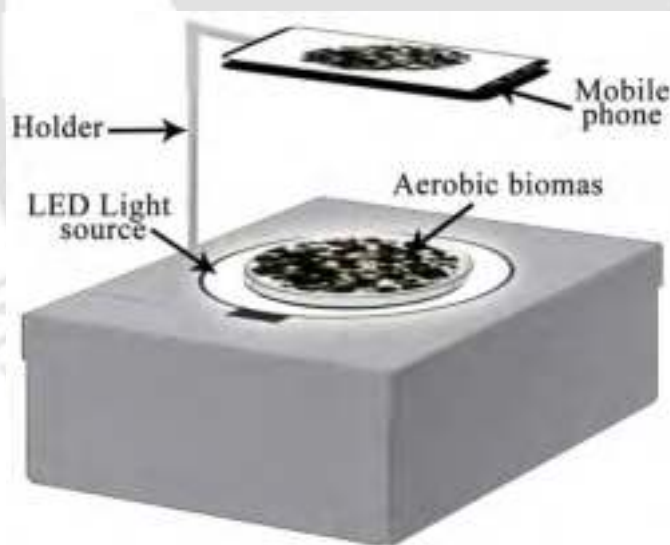


Fig. 2.4 Set-up used for photographic observation.

2.2.5.10 Gram's staining and microscopic examination

The Gram's staining of the aerobic sludge biomass has been carried out as per the method suggested by Eikelboom (2000). The Gram's staining differentiates bacteria into two large groups, Gram's-negative bacteria, and Gram's-positive bacteria. The Gram's staining kit has been purchased from HiMedia, India, which consists of crystal violet, iodine solution, a decolorizing agent, and safranin. The aerobic sludge biomass has been smeared on a glass slide, and reagents have been applied in the following order: crystal violet, Gram's iodine, decolorizing agent, and safranin. Then glass slide has been observed under light microscopy

(Olympus system microscope CX41, Japan) equipped with a digital camera (Olympus E-330, Japan). The microscopic images have been taken at 5× and 100× magnifications.

2.2.5.11 Field emission scanning electron microscope and energy-dispersive X-ray

The sludge samples have been prepared for field emission scanning electron microscope (FESEM) analysis as per the procedure suggested by Li et al. (2014). First, aerobic sludge biomass has been washed with a phosphate buffer, and 2.5% glutaraldehyde has been added and then kept for 1.5 h at 4°C. After rinsing three times with 0.1 M phosphate buffer (pH 6.8), the sludge sample has been dehydrated in a series of graded ethanol solutions (50, 70, 80, and 90%, 15 min per step) and then dehydrated in 100% ethanol three times for 15 min each time. The samples have then been placed in a desiccator and dried for 8 h. Therefore, the sample has been mounted on carbon tape over a stub and further coated with gold to make samples conductive in nature for 120 sec at 10 mbar/Pa pressure. The samples have been analyzed using field emission scanning electron microscope (Zeiss, Sigma, Germany) at 30 KX magnification. The energy-dispersive X-ray (EDX) have been performed by increasing the electron high tension (EHT) from 2.0 to 30.0 KV in the same instrument.

2.2.5.12 Enzyme activity

The activities of four enzymes, namely catalase, dehydrogenase, protease, and urease have been analyzed on the aerobic sludge biomass withdrawn from reactors towards the end of the stressed and recovery phase operations.

Catalase activity (E_{Catalase})

The mixed liquor sample of aerobic biomass (5 mL) has been mixed with distilled water (40 mL) and 0.3% (w/v) hydrogen peroxide (5 mL) and then incubated at 30°C for 30 min at 140 rpm (Remi, R-24, India). To stop the reaction, 1.5 M sulfuric acid (5 mL) has been added. The filtered sample (25 mL) has been titrated using 0.1M KMnO_4 . The catalase activity has been defined as the consumption of H_2O_2 per g biomass per h and calculated according to Eq. 2.7 as suggested by Yao et al. (2010).

$$E_{\text{Catalase}} = \frac{6.23 \times V \times C}{V_s \times \text{MLSS}} \quad (2.7)$$

where E_{Catalase} is the catalase activity (mg/g MLSS h), V is the volume of KMnO_4 used (L), C is the concentration of KMnO_4 (M), MLSS is the mixed liquor suspended solids or biomass (g/L) in sample and V_s is the volume of activated sludge sample (L).

Dehydrogenase activity ($E_{\text{Dehydrogenase}}$)

Dehydrogenase activity has been assayed as per the method suggested by Lv et al. (2008) and Yao et al. (2010). The mixed liquor of aerobic sludge biomass (5 mL) has been mixed with Tris-HCl buffer (2 mL) (0.2 M, pH 7.3), 5 mL of 2,3,5-triphenyl-tetrazolium chloride (TTC) (5 g/L), and 2 mL of glucose solution (0.1 M) and the mixture has been shaken at 140 rpm and room temperature (25°C) for 20 min and then incubated at 37°C for 12 h. The

reaction has then been stopped by the addition of 100 μL of concentrated sulfuric acid. Five mL methylbenzene has been added to extract the triphenyl formazan (TF) formed in the reaction mixture, and the sample has been shaken at 200 rpm for 30 min. The flask has been kept stable for 5 min, and then the reaction mixture has been centrifuged at 4000 rpm for 5 min. The absorbance of the supernatant has been measured at 492 nm with a UV-Vis spectrometer (Cary 100, Agilent, Singapore). The $E_{\text{Dehydrogenase}}$ has been expressed as TTC amount reduced (to TF) per g of activated sludge per hour. The dehydrogenase activity has been calculated using Eq. 2.8 suggested by Yao et al. (2010).

$$E_{\text{Dehydrogenase}} = \frac{0.08 \times M}{V_s \times \text{MLSS}} \quad (2.8)$$

where $E_{\text{Dehydrogenase}}$ is the dehydrogenase activity ($\mu\text{g/g MLSS h}$), MLSS is the mixed liquor suspended solids or biomass (g/L) in sample, M is the calculated TF quantity according to the standard line (μg) [given in Appendix-I (Fig. A-I.2)] and V_s is the volume of activated sludge sample (L).

Protease activity (E_{Protease})

Two mL of methylbenzene has been added to 5 mL mixed liquor of aerobic sludge biomass withdrawn from reactor. This mixture has then been shaken at 140 rpm at room temperature (25°C) for 20 min. Ten mL of glutin (1% w/v, dissolved in phosphate buffer, pH 7.4) has been added to the sample as the reaction substrate. Then, mixture has been incubated at 30°C for 24 h. After the incubation, the sample has been filtered (6 mL), then 0.05M H_2SO_4 (1 mL) and 20% Na_2SO_4 (w/v, 6 mL) were added to the filtrate and hand shaken gently for 1 min. Four mL of the filtrate has been mixed with 1 mL 2% ninhydrin (w/v), hand shaken for 1 min and then heated in boiling water for 10 min. Finally, the solution has then been made up to 50 mL with distilled water and the absorbance at 560 nm has been determined using a UV-Vis spectrometer. The protease activity has been defined as the amount of amino acids produced from glutin catalyzed by protease per g MLSS per hour. Protease activity has been calculated using Eq. 2.9 suggested by Yao et al. (2010).

$$E_{\text{Protease}} = \frac{0.34 \times M}{V_s \times \text{MLSS}} \quad (2.9)$$

where E_{Protease} is the protease activity (mg/g MLSS h), MLSS is the mixed liquor suspended solids or biomass (g/L) in sample, M is the calculated amino acid quantity according to the standard curve (mg) [given in Appendix-I (Fig. A-I.3)] and V_s is volume of activated sludge sample (L).

Urease activity (E_{Urease})

Urease activity has been assayed as per the method described by Lv et al. (2008) and Yao et al. (2010). Five mL of mixed liquor of aerobic sludge biomass withdrawn from reactor and has been mixed with 2 mL methylbenzene, centrifuged at 200 rpm for 20 min at room

temperature (25°C), and then 5 mL 10% urea solution (w/v) and 10 mL pH 6.7 citrate buffer have been added and incubated at 37°C for 24 h, then total volume made up to 100 mL with distilled water. One mL filtrate has been mixed with 10 mL distilled water, 4 mL 0.28 mol/L sodium phenate and 3 mL 1% sodium hypochlorite (w/v) in order. The mixture has then been gently hand shaken and kept for 20 min to develop color, finally diluted to 100 mL with distilled water and the absorbance has been measured at 578 nm with a UV-Vis spectrometer. Urease activity has been expressed as the production of ammonia (g) from urea catalyzed by urease per g MLSS per hour. Urease activity has been calculated according to Eq. 2.10 suggested by Yao et al. (2010).

$$E_{\text{Urease}} = \frac{2.08 \times M}{V_s \times \text{MLSS}} \quad (2.10)$$

where E_{Urease} is the urease activity (g/g MLSS h), MLSS is the mixed liquor suspended solids or biomass (g/L) in sample, M is the calculated NH_4^+ quantity (g/L) [standard curve shown in Appendix-I (Fig. A-I.4)] and V_s is volume of activated sludge sample (L).

The inhibition of Cu(II) on each enzyme activity has been calculated by Eq. 2.11

$$I = \left(\frac{R_0 - R}{R_0} \right) \times 100 \quad (2.11)$$

where, I is the inhibition (%); R_0 is the enzyme activity of control reactor R_{Cu0} biomass, and R is the corresponding value in the reactors biomass fed with Cu(II).

2.2.5.13 Amplified ribosomal DNA restriction analysis (ARDRA)

The aerobic sludge biomass samples have been withdrawn towards the end of the stressed and recovery phase operations from reactors for ARDRA analysis. The aerobic sludge biomass has been cultured in 5 mL Luria-Bertani media for 24 h at 37°C. DNA has been extracted from the samples with a HiPurA™ Fungal DNA Purification Kit (HiMedia, India). The extracted DNA concentration and quality have been checked by spectrophotometric analysis on BioPhotometer (Eppendorf, Germany) electrophoresis on a 1% (w/v) agarose gel. The bacterial 16S rRNA gene fragments have been PCR-amplified from genomic DNA extracted using universal primers 27f AGAGTTTGATCCTGGCTCAG and 1492r GGTTACCTTGTACGACTT (Meng et al., 2015). The primers have been synthesized by Integrated DNA Technologies (IDT), Malaysia. Polymerase chain reaction (PCR) has been carried out in 50 µL volumes containing: 5X HF Buffer, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1 unit of Phusion High-Fidelity DNA Polymerase (Thermofisher Scientific, USA), and 30 ng of genomic DNA as a template.

The PCR thermal profile has been used with an initial denaturation at 98°C for 1 min, 30 cycles of denaturation at 98°C for 30 sec, annealing at 53.8°C for 30 sec, and extension at 72°C for 30 sec. The final extension of the PCR product has been performed at 72°C for 5 min. The amplification has been conducted on a Mastercycler (EpigradientS, Eppendorf, Germany). The presence and yield of specific PCR products of approximately 1500 bp have been controlled by electrophoresis on 1.2% agarose gel containing ethidium bromide (0.5

ng/ μ L). An aliquot of each reaction has been collected and frozen at -20°C for further analysis.

Gel analysis

Three endonucleases *AluI*, *HhaI* and *MspI* (New England Biolabs, USA) have been chosen for further study of 16S rRNA amplimers. Amplified DNA has been digested for 3 h at 37°C in separate PCR tubes. The reaction mixture volume has amounted to 50 μL , which contained 15 μL of PCR product, 20 U of restriction enzyme, cut smart buffer 5 μL , and nuclease-free water to make up reaction volume. Restriction reaction has been stopped adopting to the producer's recommendation, i.e., heat treatment of sample in a water bath. Restriction fragment patterns have been analyzed by electrophoresis on 8% polyacrylamide gel stained with ethidium bromide solution (0.5 ng/ μL) for 5 h at 25 V. Gels have been analyzed under UV illumination and digitalized with a gel documentation system (Bio-rad Gel DocTM EZ, California, USA).

Dendrogram preparation

The band patterns on each sample and band size evaluation on each gel have been estimated based on the simultaneous 100 bp DNA ladder (Fermentas, Lithuania) - DNA molecular weight standard gel separation. The computation analysis and dendrogram preparation have been performed using the GelJ application tool (Heras et al., 2015). In the cluster analysis, a dendrogram has been constructed from Dice's coefficients (SD) using the unweighted pair group method with arithmetic averages (UPGMA). Pairwise community similarities have been quantified using the Dice index (C) as $C = 2j/(a + b)$, where j is the number of bands common to samples A and B, and a and b are the number of bands in samples A and B, respectively.

2.3 Results and Discussion

The reactors have been operated during 0-25 d under stressed phase with Cu(II) in the feed and then during 26-54 d under recovery phase without Cu(II) in the feed. The results obtained are discussed below in subsequent subsections. The average value of duplicate reactors (the average of R_{Cu0_1} and R_{Cu0_2} represented as R_{Cu0} and those for other reactors as R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80}) with the standard deviation in the respective tables.

2.3.1 Stressed phase operation

2.3.1.1 Environmental and performance parameters

The variation in environmental and performance-related parameters have been shown in Fig. 2.5 to 2.9 and data has been summarized and presented in Table 2.2. The reactors have been operated under ambient conditions throughout the operation; hence pH and room temperature have not been adjusted/controlled. The room temperature variation has been mainly observed due to weather changes. Since, all reactors have been operated at room temperature, variation in room temperature recorded at 9.30 am and 8.55 pm have been shown in Fig. 2.5. The initial and effluent pH for reactor R_{Cu0} has ranged from 7.12-7.61 under stressed phase operation and it has not shown much variation during operation (Fig. 2.5).

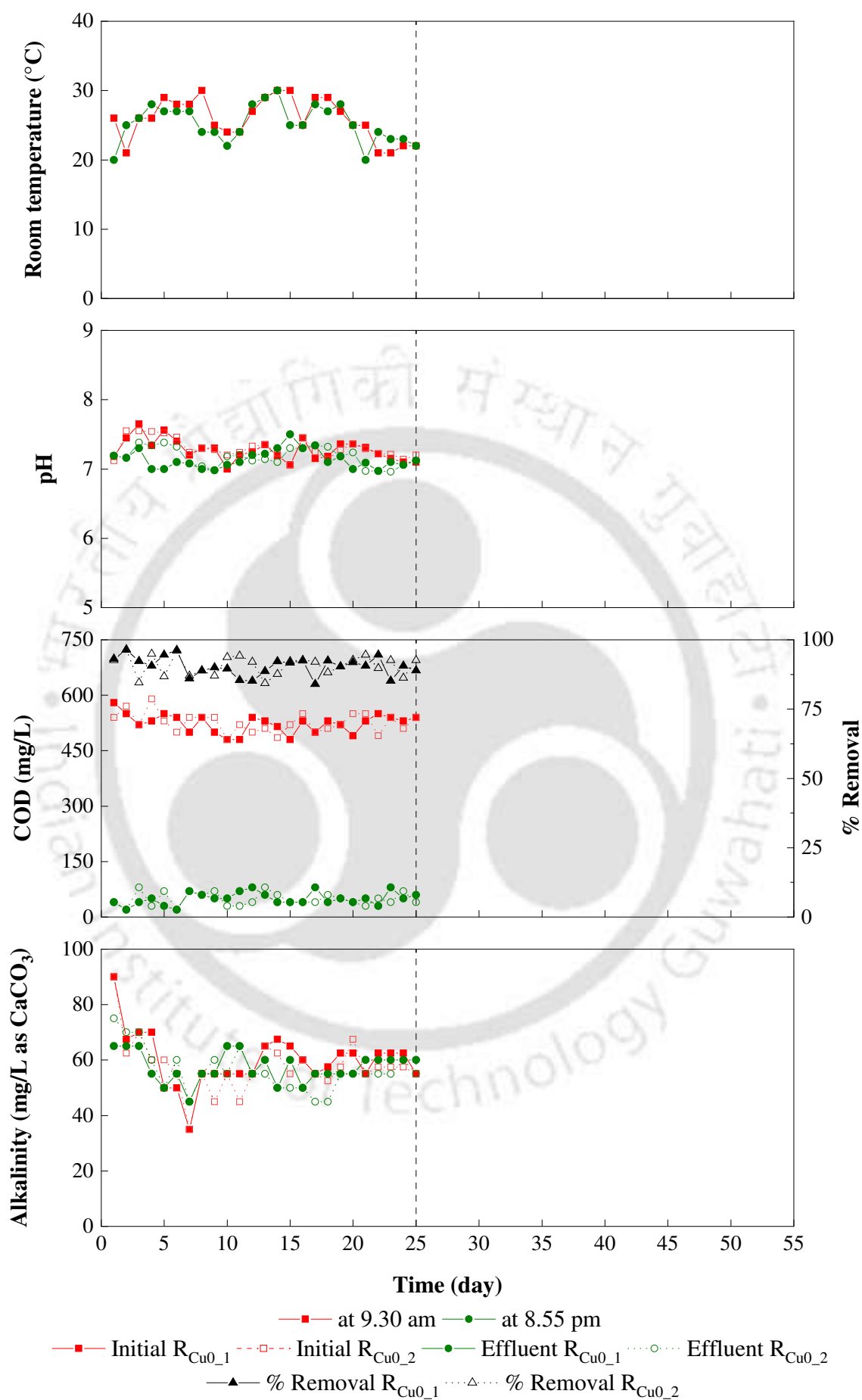


Fig. 2.5 Variation in environmental and performance related parameters for reactor R_{Cu0} during 0-25 d of operation.

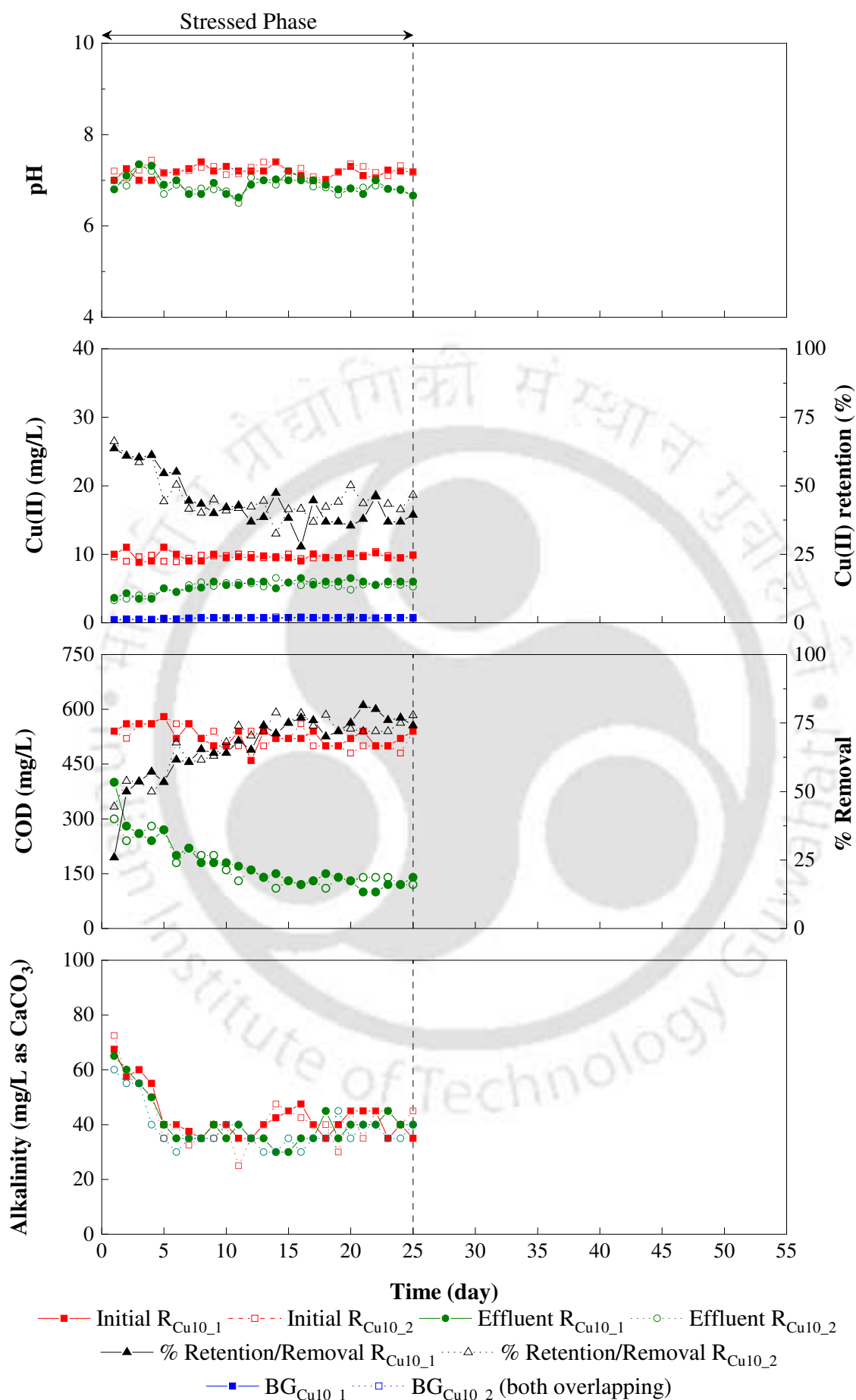


Fig. 2.6 Variation in environmental and performance related parameters for reactor R_{Cu10} during 0-25 d of stressed phase operation.

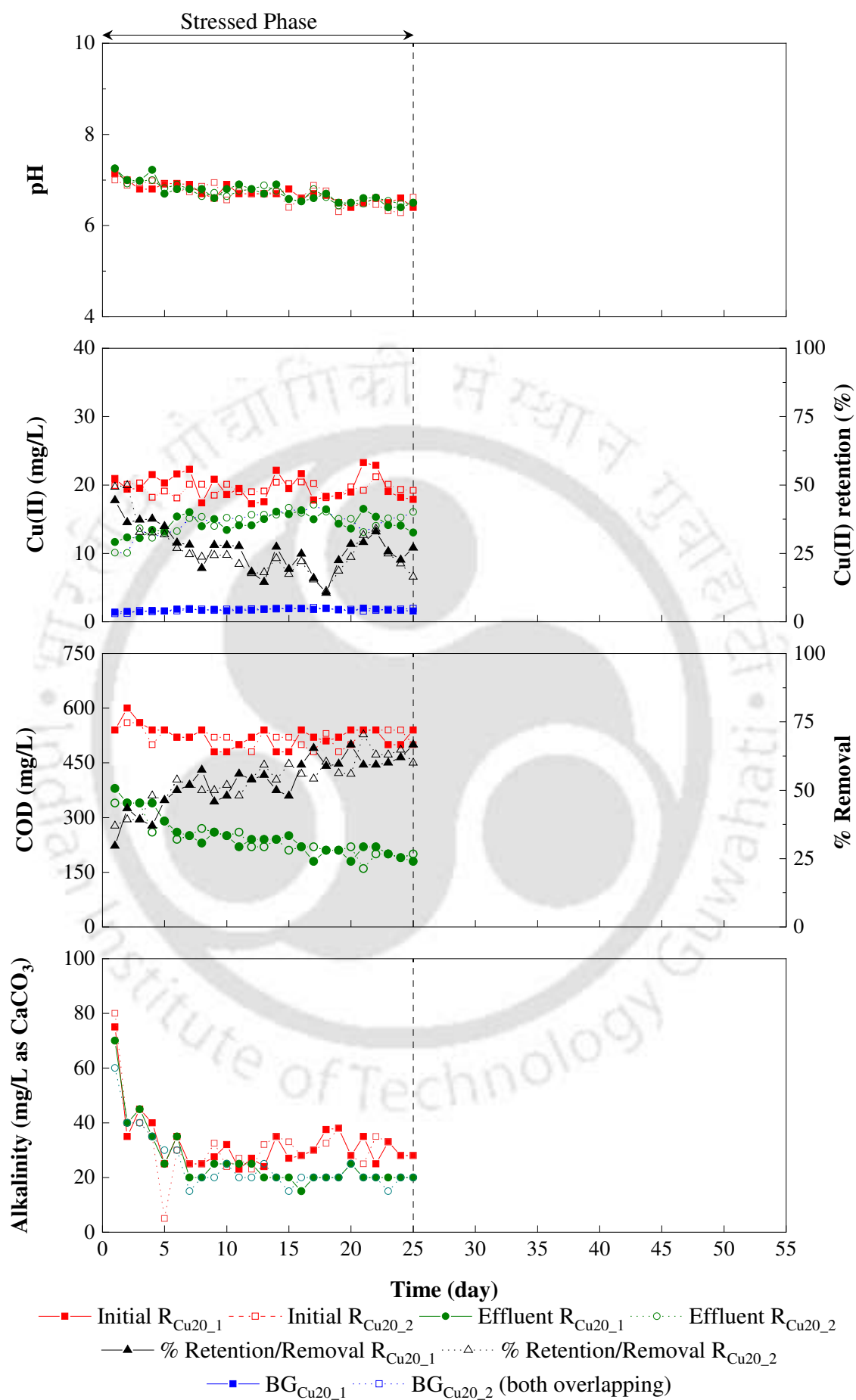


Fig. 2.7 Variation in environmental and performance related parameters for reactor R_{Cu20} during 0-25 d of stressed phase operation.

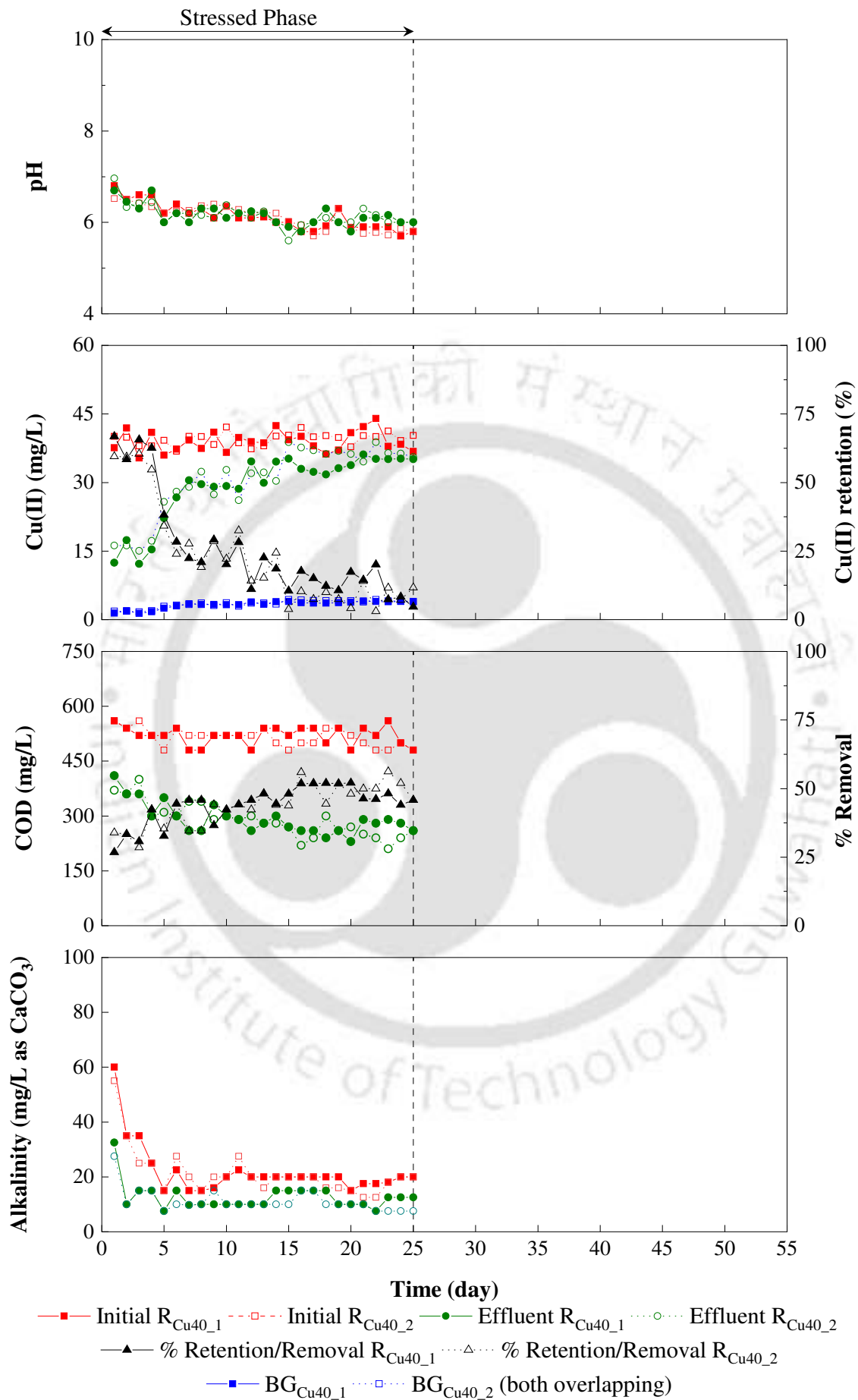


Fig. 2.8 Variation in environmental and performance related parameters for reactor R_{Cu40} during 0-25 d of stressed phase operation.

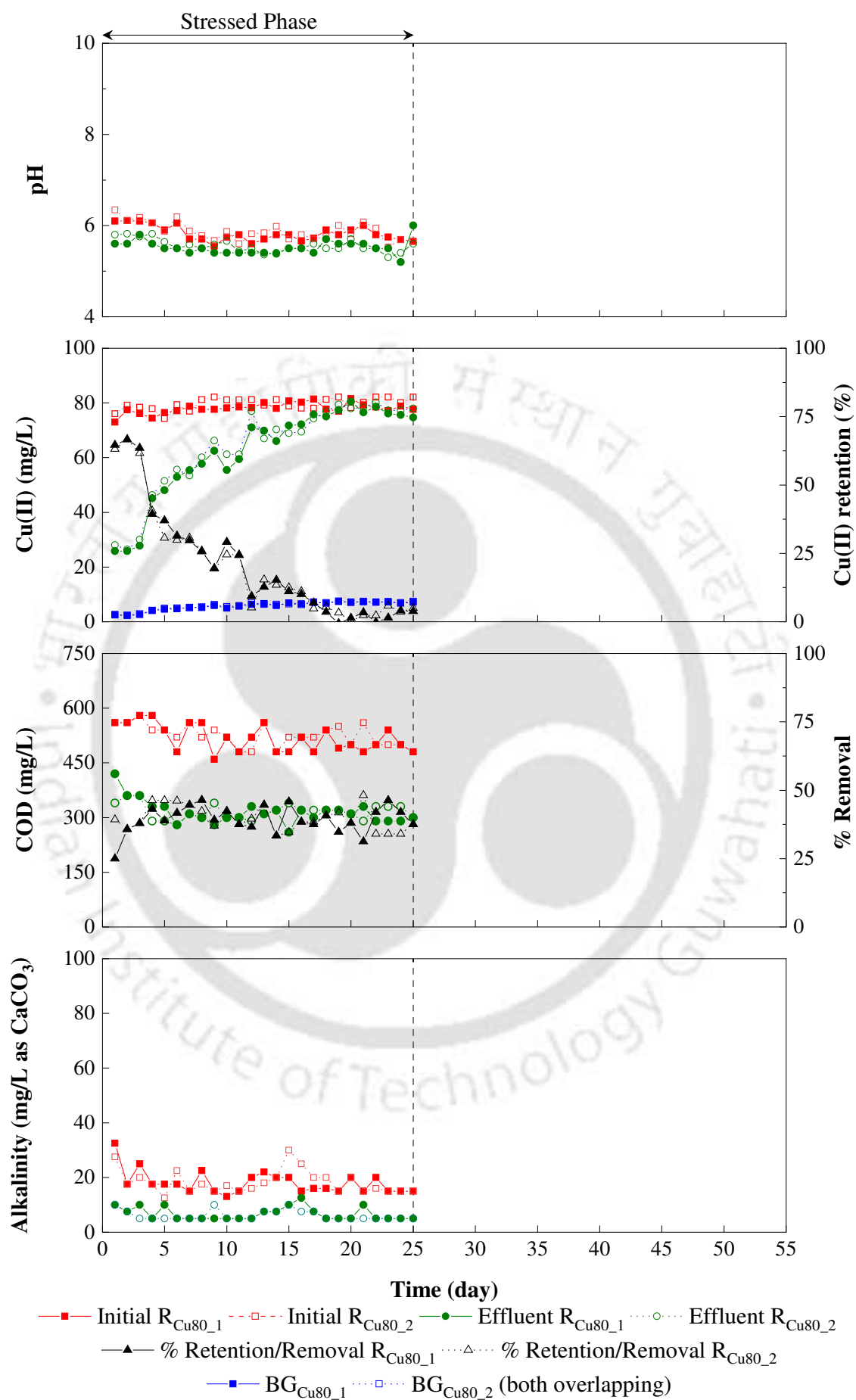


Fig. 2.9 Variation in environmental and performance related parameters for reactor R_{Cu80} during 0-25 d of stressed phase operation.

Table 2.2 Summary of environmental and performance related parameters for reactors operated during 0-25 d of stressed phase operation.

Parameters	Day-wise	0-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	10	20	40	80
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	26.0±3.0 (25)	26.0±3.0 (25)	26.0±3.0 (25)	26.0±3.0 (25)
		Room temperature (°C) at 8.55 pm	25.2±2.7 (25)	25.2±2.7 (25)	25.2±2.7 (25)	25.2±2.7 (25)
		Initial pH	7.28±0.15 (50)	7.19±0.12 (50)	6.60±0.20 (50)	6.12±0.28 (50)
		Initial Cu(II) (mg/L)	0	9.64±0.45 (50)	19.65±1.33 (50)	39.23±1.82 (50)
	Performance	Effluent pH	7.15±0.13 (50)	6.89±0.19 (50)	6.72±0.21 (50)	6.15±0.24 (50)
		Effluent Cu(II) (mg/L)	0	5.29±0.85 (50)	14.47±1.58(50)	28.99±7.44 (50)
		Cu(II) retention (%)	0	45.12±8.80 (50)	26.12±8.64 (50)	23.97±18.46 (50)
		Background Cu(II) (mg/L)	0	0.67±0.11 (50)	1.73±0.19 (50)	3.36 ±0.84 (50)
		Initial soluble COD (mg/L)	524.80±27.79 (50)	524.80±23.29 (50)	522.40±25.96 (50)	517.60±24.93 (50)
		Effluent soluble COD (mg/L)	48.80±17.61 (50)	173.60±64.08 (50)	243.60±50.70 (50)	290.00±44.51 (50)
		COD removal (%)	90.66±2.91 (50)	67.19±10.99 (50)	53.47±8.78 (50)	44.03±7.17 (50)
		Initial alkalinity (mg CaCO ₃ /L)	58.90±9.85 (50)	42.6±9.15 (50)	32.46±11.40 (50)	21.60±8.55 (50)
		Effluent alkalinity (mg CaCO ₃ /L)	60.29±6.55 (50)	37.79±8.28 (50)	28.02±10.87 (50)	11.99±4.55 (50)
						6.40± 2.07 (50)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

However, pH for Cu(II) fed reactors have decreased under stressed phase operation (as shown in Fig. 2.6 to 2.9). The average pH for Cu(II) fed reactors has decreased with increased Cu(II) concentration in the feed. The average influent and effluent pH values for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been obtained in the range of 6.40-7.34, 6.11-7.70, 5.60-6.75, and 5.65-6.22, respectively. The changes in pH for Cu(II) fed reactors is due to the addition of $CuSO_4$. The copper ions are the dominant species at low pH values up to pH 7.5. At higher pH values, copper hydroxide, $Cu(OH)_2$, is the dominant species up to pH 12.3 (Albrecht et al., 2011). The lower concentration of Cu(II) (10 mg/L) has not affected pH as compared to the higher Cu(II) concentration (80 mg/L).

The reactor R_{Cu0} has shown uniform COD removal throughout the operation while Cu(II) fed reactors have clearly indicated hindrance in COD removal under stressed phase operation (Fig. 2.5-2.9). Initially, COD removal (till 7th d) has been affected with decreasing trend for all Cu(II) fed reactors. The possible reason might be adaptation of microbes to the new environment [i.e., presence of Cu(II)]. When microbes have adapted the new situation, the COD removals for reactors R_{Cu10} and R_{Cu20} slowly improved, while reactors R_{Cu40} and R_{Cu80} have shown stabilization in COD removal as shown in Fig. 2.6-2.9. The average COD removal during 1-12 d for reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 91.1%, 57.9%, 46.3%, 40.3%, and 38.2%, respectively. The effluents of the reactors which have been fed with 10, 20, 40, and 80 mg/L Cu(II) have shown predominantly lower quality than those of reactor R_{Cu0} (Fig. 2.5-2.9). The average COD removal under stressed phase operation for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been 67.2%, 53.5%, 44.0%, and 39.5%, respectively, while reactor R_{Cu0} resulted in 90.7% COD removal (Fig. 2.5-2.9). It has been observed that an increase in Cu(II) concentration in the reactors has decreased the COD removal efficiency.

On the 1st d of stressed phase, Cu(II) retention has been observed to be higher for all Cu(II) fed reactors [64.9%, 46.7%, 3.0%, and 3.8% for reactors fed with 10, 20, 40, and 80 mg/L of Cu(II), respectively]. However, later on 12th d and 25th d of operation 39.67%, 17.94%, 12.64%, 7.19% and 43.0%, 21.5%, 8.3%, and 4.4% of Cu(II) retention have been observed, respectively, for 10, 20, 40, and 80 mg/L of Cu(II) fed reactors (Fig. 2.6-2.9). The available literature suggests that sorption and accumulation of Cu(II) are common processes for Cu(II) retention by aerobic sludge biomass. Initially, the aerobic sludge biomass has a higher capacity to sorb Cu(II) on its surface, and later on, all sorption sites have been occupied by Cu(II) ion; hence the Cu(II) retention has decreased. The reactors which have been fed with a high dose of Cu(II) got saturated faster than the reactors fed with lower doses of Cu(II). The average Cu(II) retention of reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 45.1%, 26.1%, 24.0%, and 20.4%, respectively, as shown in Fig. 2.6-2.9. This has severely affected COD removal. Similar results have also been reported by Nicolau et al. (2005) and Oviedo et al. (2002) that high concentrations of Cu(II) (20 and 50 mg/L) have negatively affected the COD removal efficiency. The stress caused by the higher concentration of Cu(II) might have inhibited the microbial activity resulting in lower COD removal. Zhang

et al. (2017) have reported that the presence of 20 and 100 mg/L of copper sulphate led to a reduction in COD removals to 44.8% and 7.3%, respectively. The background Cu(II) concentrations have also been estimated during stressed phase operation for Cu(II) fed reactors. The results have been shown in Fig. 2.6-2.9 (data points have overlapped) and summary of data is presented in Table 2.2. Very less amount of Cu(II) (in the range of 1-7 mg/L of) has remained in the reactors after washing. The average background Cu(II) for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 0.67, 1.73, 3.36, and 5.83 mg/L, respectively, under stressed phase operation.

The steady-state condition under stressed phase operation have been observed from 19th d of operation in terms of COD removal (%), and hence summary of steady-state data has been presented in Table 2.3. The average COD removal (%) for reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been obtained as 90.8%, 74.9%, 61.9%, 49.1%, and 38.5%, respectively, with minimum variation as shown in Table 2.3. The minimum variation in all other performance related parameters have been observed. The Cu(II) retention at steady-state condition has been observed as 41.8%, 25.6%, 10.2%, and 2.6% for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} , respectively. The addition of Cu(II) has also changed the alkalinity of Cu(II) fed reactors, as shown in Fig. 2.5-2.9. The initial and effluent alkalinity for all reactors have ranged from 5.36 ± 0.94 to 59.29 ± 3.96 mg $CaCO_3$ /L under stressed phase operation, as presented in Table 2.2. It can be observed that a lower concentrations of Cu(II) (10 and 20 mg/L) have not shown much effect on reactor performance. In contrast, higher doses of Cu(II) (40 and 80 mg/L) have caused higher stress on reactors R_{Cu40} and R_{Cu80} leading to deterioration in its performance as compare to reactor R_{Cu0} . It has also been observed that an increase in initial Cu(II) concentration has resulted in a higher concentration of Cu(II) in the effluent. Wang et al. (2010) have reported that the presence of Cu(II) is toxic at relatively high concentrations, and therefore Cu(II) concentration of 40 and 80 mg/L may be considered as the toxic range for biomass.

2.3.1.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The impact of Cu(II) on characteristics of aerobic sludge biomass have been assessed grossly through biomass solids analysis in terms of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS), interface settling velocity (ISV), sludge volume index (SVI) and aerobic sludge biomass activity (ASBA) test. The MLSS and MLVSS are useful to assess the growth of aerobic sludge biomass in reactors. The SVI and ISV contain information about sludge compactness and settling behavior of aerobic sludge biomass, while activity test indicates organic degradation capability of biomass.

The MLSS, MLVSS, and SVI have been estimated on every 7th d of the operation throughout for all the reactors and are shown in Fig. 2.10, while a summary of data is presented in Table 2.4. The MLSS, MLVSS, and SVI for reactor R_{Cu0} on 1st and 24th d of operation have been observed 1512 mg/L, 1482 mg/L, and 89 mL/g; and 4152 mg/L, 3861

Table 2.3 Summary of environmental and performance related parameters for reactors operated during 19-25 d of steady-state of stressed phase operation.

Parameters	Day-wise	19-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	10	20	40	80
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	23.29±2.36 (7)	23.29±2.36 (7)	23.29±2.36 (7)	23.29±2.36 (7)
		Room temperature (°C) at 8.55 pm	23.57±2.51 (7)	23.57±2.51 (7)	23.57±2.51 (7)	23.57±2.51 (7)
		Initial pH	7.24±0.09 (14)	7.20±0.09 (14)	6.46±0.10 (14)	5.89±0.19 (14)
		Initial Cu(II) (mg/L)	0	9.77±0.30 (14)	19.70±1.56 (14)	39.70±1.93 (14)
		Effluent pH	7.07±0.09 (14)	6.79±0.10 (14)	6.50±0.07 (14)	6.04±0.12 (14)
		Effluent Cu(II) (mg/L)	0	5.68±0.28 (14)	14.61±1.05 (14)	35.60±1.15 (14)
		Cu(II) retention (%)	0	41.79±3.22 (14)	25.60±5.10 (14)	10.16±4.90 (14)
		Background Cu(II) (mg/L)	0	0.71±0.04 (14)	1.75±0.13 (14)	4.00±0.14 (14)
	Performance	Initial soluble COD (mg/L)	528.57±21.10 (14)	508.57±19.00 (14)	522.86±22.42 (14)	508.57±27.28 (14)
		Effluent soluble COD (mg/L)	48.57±14.23 (14)	127.14±13.14 (14)	198.57±18.11 (14)	258.57±20.68 (14)
		COD removal (%)	90.78±2.78 (14)	74.94±2.77 (14)	61.92±4.21 (14)	49.17±3.21 (14)
		Initial alkalinity (mg CaCO ₃ /L)	59.29±3.96 (14)	40.71±5.29 (14)	30.71±4.68 (14)	17.57±2.67 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	57.14±2.16 (14)	39.29±3.33 (14)	20.36±2.39 (14)	9.64±1.59 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

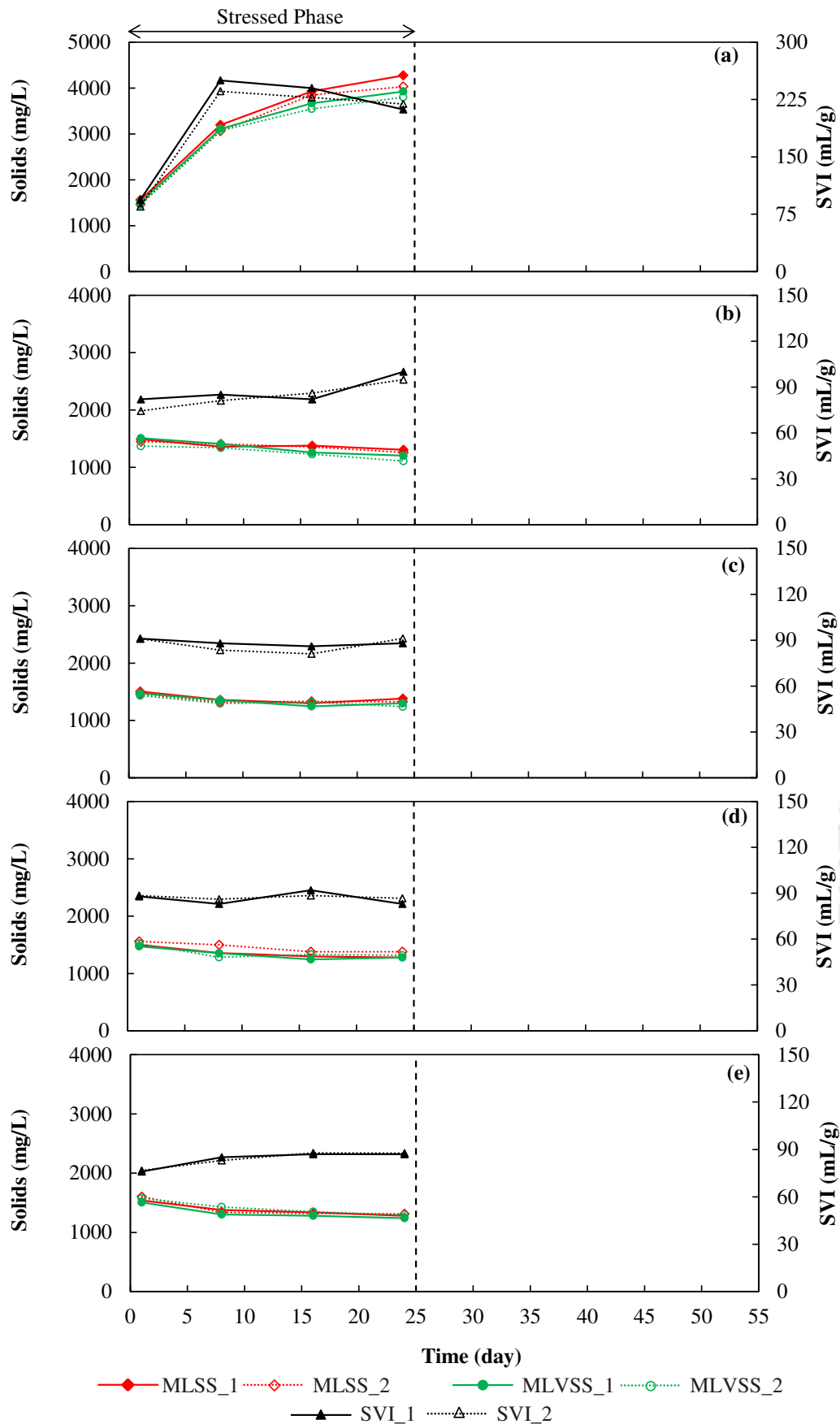


Fig. 2.10 Variation in MLSS, MLVSS, and SVI values for reactor biomass from (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} during 0-25 d of stressed phase operation.

Table 2.4 Summary of solids characteristics estimated during stressed phase operation.

Gross parameters	Stressed phase operation					
	Day-wise	0-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
MLSS (mg/L)		3171.06±1188.55 (8)	1376.50±79.55 (8)	1373.50±77.28 (8)	1407.50±94.67 (8)	1389.50±125.85 (8)
MLVSS (mg/L)		3010.47±1068.55 (8)	1304.41±129.42 (8)	1333.28±89.74 (8)	1351.85±104.89 (8)	1372.64 ±120.03 (8)
MLVSS/MLSS ratio		0.96±0.03 (8)	0.95±0.05 (8)	0.97±0.03 (8)	0.95±0.04 (8)	0.93±0.07 (8)
SVI (mL/g)		195.40±71.71 (8)	85.68±8.60 (8)	87.45±3.61 (8)	98.18±2.78 (8)	83.76±5.23 (8)
ISV (m/h)		2.87±5.80 (14)	17.22±0.55 (14)	17.89±0.85 (14)	17.64±0.85 (14)	18.63±0.93 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

mg/L, and 215 mL/g, respectively (Fig. 2.10). The reactor R_{Cu0} has shown continuous growth of aerobic sludge biomass, and an increase in SVI has been observed because of abundance of filamentous biomass. The average MLSS, MLVSS, and SVI for reactor R_{Cu0} has been 3171.06 ± 1188.55 mg/L, 3010.47 ± 1068.55 mg/L, and 195.40 ± 71.71 mL/g, respectively, from 0-25 d operation. The addition of Cu(II) to the reactors has affected the growth of aerobic sludge biomass. It can be seen from Fig. 2.10(b)-(e) that the MLSS and MLVSS concentration for all Cu(II) fed reactors have stabilized, and no growth have been observed under stressed phase operation. The lower SVI has been observed for Cu(II) fed reactors under stressed phase operation. However, low SVI with no increase of MLSS concentration along with poor COD removal indicates that the solids settling characteristics are changing, and a plant upset can likely to occur (Liu and Liptak, 1999). The low SVI with a good COD removal profile is desirable for reactor operation. In this study, lower SVI have been obtained with lower COD removal during the stressed phase operation, which is undesirable. The decrease in SVI for stressed reactors has been due to the addition of Cu(II) in the feed, which has reduced the growth of filamentous organisms but led to the formation of granular biomass. Wong (1999) reported that 40 mg/L of Cu(II) has slightly decreased the SVI, whereas 80 mg/L of Cu(II) has significantly decreased the SVI of aerobic sludge biomass.

The results obtained during the stressed phase of reactor operation also depict a reduction in MLSS and MLVSS concentration in all the reactors except the reactor R_{Cu0} which showed a continuous increase in MLSS and MLVSS. The MLVSS/MLSS ratio of reactor R_{Cu0} has remained almost the same throughout the stressed phase operation. The average variation in MLVSS/MLSS ratio has been presented in Table 2.4. However, no variations in average MLVSS/MLSS ratio has been observed with increase in Cu(II) concentration in the feed. On the 1st d of stressed phase, the MLVSS/MLSS ratio for all reactors appear to be same, i.e., 0.98. However, on the 24th d, MLVSS/MLSS ratio have decreased to 0.90, 0.92, 0.91, and 0.85 for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} , respectively. Zhang et al. (2017) reported that Cu(II) ions exhibit severe toxicity towards microbes, leading to disruption and cell lysis. The addition of Cu(II) to the reactors inhibits aerobic sludge biomass growth and causes disruption of flocs, which has resulted in flocculent (pinpoint) aerobic sludge biomass.

The information about the settling behavior of aerobic sludge biomass is an important input for solid-liquid separation during the reactor operation. The settling tests for all reactor biomasses have been carried out on every 4th d throughout the operation, as shown in Fig. 2.11 and ISV for all reactors have been estimated on every 4th d, as shown in Fig. 2.12 and summarized data is presented in Table 2.4. The results suggest that settling velocity for reactor R_{Cu0} has decreased on 8th d of operation from 15.72 m/h on 1st d to 0.22 m/h on 8th d and has remained similar for further operation. Initially, growth of filamentous microorganisms has been slow, and aerobic sludge biomass appears to be flocculant in nature in reactor R_{Cu0} .

After 8th d of operation, reactor R_{Cu0} has filled with filamentous microorganisms, causing bulking of sludge with decreasing settling velocity. Whereas Cu(II) fed reactors have shown faster and uniform settling velocity throughout the operation (Table 2.4), as compared to the

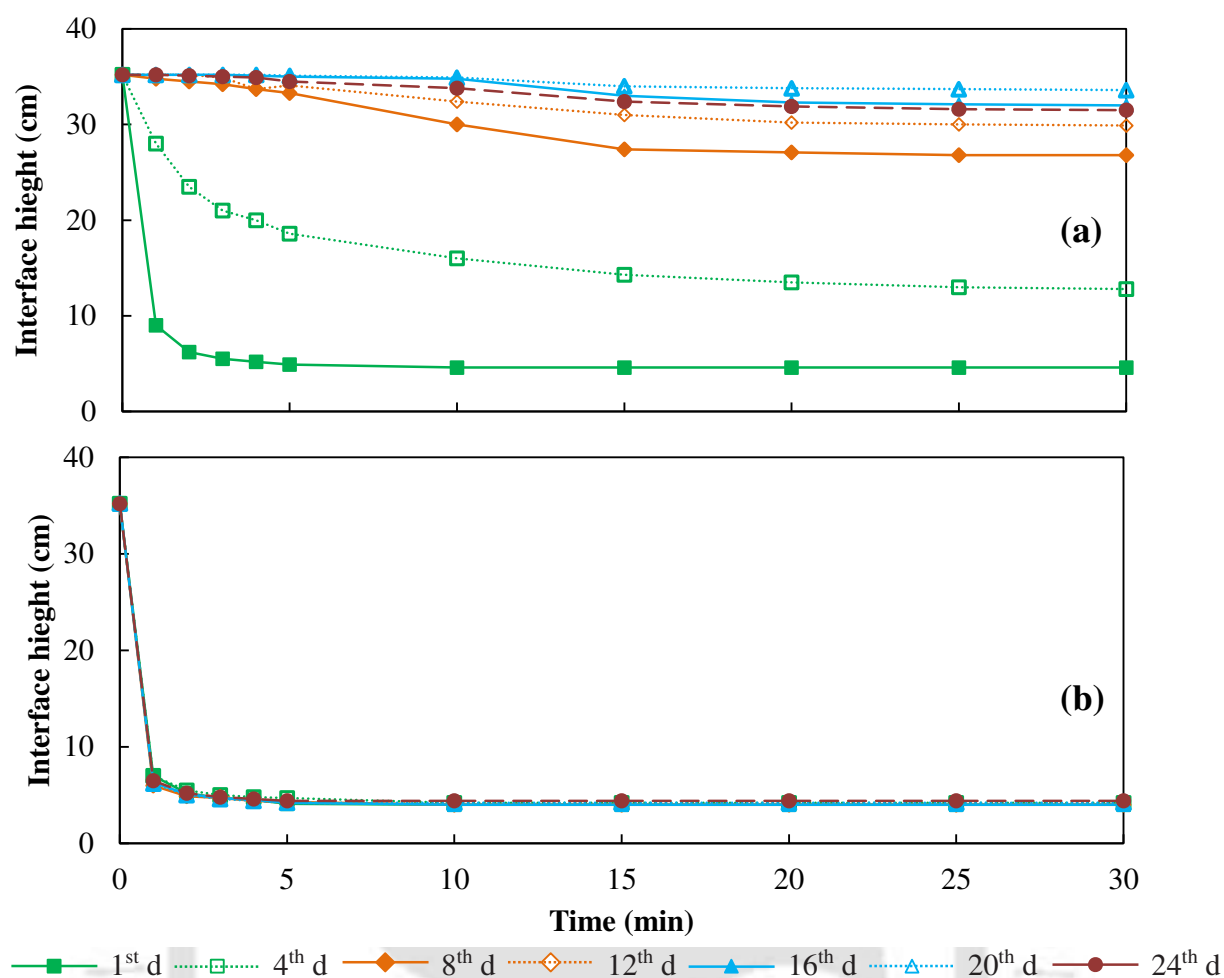


Fig. 2.11 Typical settling test results for reactor (a) R_{Cu0} and (b) R_{Cu10} biomasses during 0-25 d of stressed phase operation.

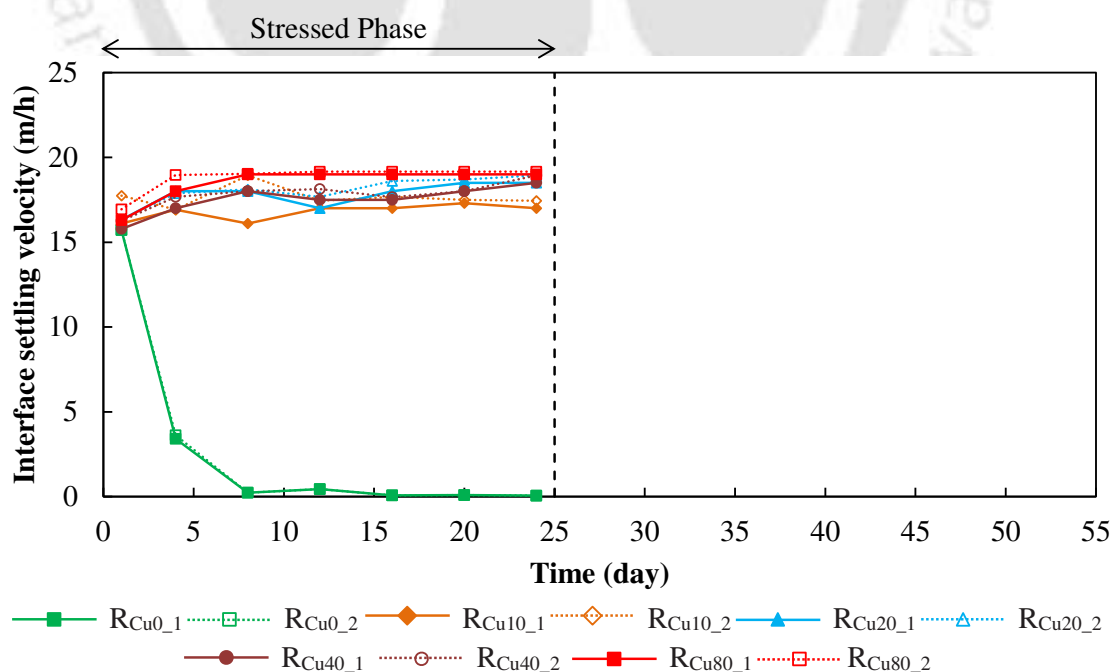


Fig. 2.12 Variation in interface settling velocities of reactor biomasses during 0-25 d of stressed phase operation.

reactor R_{Cu0} . Low SVI and high ISV both are desirable for a better reactor operation; however, without a good COD removal profile, these parameters are not adequate for defining the treatment process either as good or bad.

Aerobic sludge biomass activity test

The aerobic sludge biomass activity (ASBA) test provides information about the food utilization rate by aerobic sludge biomass microorganisms. The aerobic sludge biomass has been withdrawn from all reactors at steady-state conditions under the stressed phase operation on 20th d, and ASBA tests have been carried out as shown in Fig. 2.13, while

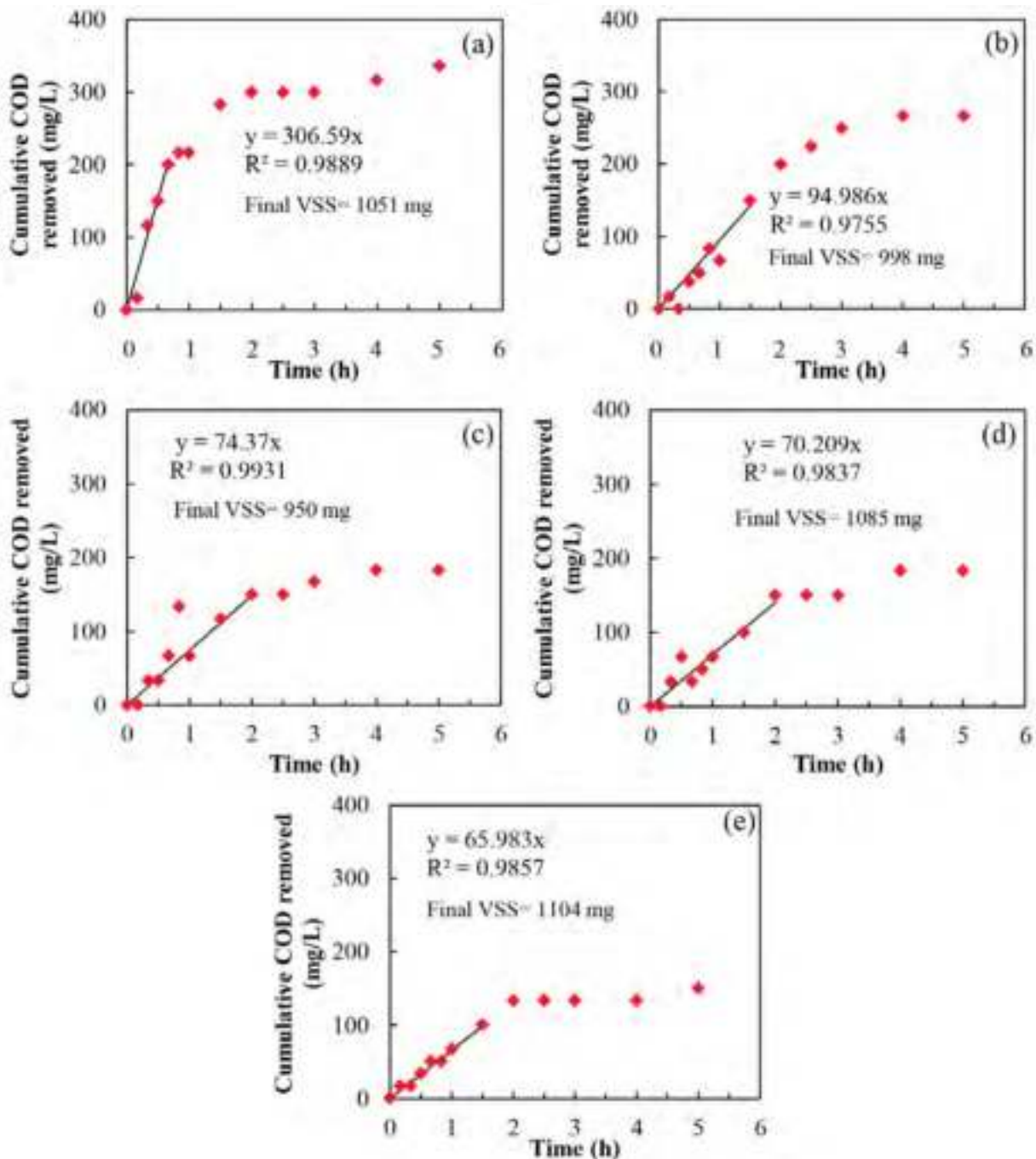


Fig. 2.13 Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactor (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} on 20th d during steady-state condition of stressed phase operation.

Table 2.5 Aerobic sludge biomass activity test results carried out towards the end of stressed phase operation.

Stressed phase operation					
Biomass withdrawn on	20 th d				
Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
ASBA (mg COD removed/mg VSS d)	7.00	2.28	1.89	1.55	1.43

the results are presented in Table 2.5. The ASBA values for reactor R_{Cu0}, R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses have been observed as 7.00, 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d, respectively. The activity test results for Cu(II) fed reactors have shown a decrease in activity values with an increase in Cu(II) concentration in the feed, indicating an increased inhibitory effect of Cu(II) on aerobic sludge biomass activity (Fig. 2.13). Even Cu(II) concentration of 10 mg/L in the feed of reactor R_{Cu10} has shown a drastic reduction in biomass activity compared to reactor R_{Cu0} biomass.

The increase of Cu(II) concentration has enhanced the inhibitory effects, and hampered the bio-oxidation process carried out by aerobic sludge biomass. This result can be correlated with the COD removal efficiency of aerobic sludge biomass, which has also been affected due to the presence of Cu(II) in the feed. Madoni et al. (1999) and Oviedo et al. (2002) have reported that an increase in the concentration of Cu(II) has significantly affected the soluble oxygen uptake rate (sOUR). Wang et al. (2010) reported that 5 mg/L of Cu(II) has caused a drastic effect on the bio-activity of aerobic sludge biomass.

Cu(II) stress (between 10 to 80 mg/L) to the biomass has resulted in 39-75% COD removal during the steady-state condition of stressed phase operation. Considering biomass related general parameters, it has been revealed that aerobic sludge biomass has changed its behavior and characteristics when stressed to Cu(II), which has varied with Cu(II) doses in feed. The biomass growth in the control reactor (R_{Cu0}) has been continuously increased and SVI has also risen from 89 mL/g to 215 mL/g from the 1st d to the 24th d. The filaments have been present in the control reactor throughout the operation; hence, the average SVI for reactor R_{Cu0} has been observed as 195 mL/g. However, Cu(II) stress has significantly inhibited the aerobic sludge biomass growth in reactors R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80}. The biomass growth has been reduced, and SVI has been stabilized under the stressed phase operation. The average SVI for reactors R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} have ranged between 83-93 mL/g. It is reported that a low SVI with no increase in MLSS concentration indicates that the solids settling characteristics are changing, and a plant upset can likely to occur (Liu and Liptak, 1999). The addition of Cu(II) has increased ISV for Cu(II) fed reactor. The average ISV for Cu(II) fed reactor biomasses have ranged between 17.22-18.63 m/h, which is much higher than the ISV of reactor R_{Cu0} biomass (2.87 m/h) during stressed phase operation. The settling test results have indicated that the majority of the aerobic sludge biomass fraction have settled in the measuring cylinder within 5 minutes for reactor R_{Cu10}. The heavy metal reduces the filament population (Mcdermott et al., 1963), which has resulted in lower SVI

with increased ISV. Pinpoint flocs and overgrowth of filaments result in high SVI (above 200 mL/g) and decreased ISV, whereas strong and dense flocs give optimum SVI (below 150 mL/g) with increased SVI (Neufeld, 1976). An activated sludge that settles very rapidly (SVI of less than approximately 70 mL/g) can leave behind a turbid supernatant (Palm et al., 1980), which is undesirable for the treatment process. The ASBA for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have also been declined due to the addition of Cu(II) in feed, which has been observed as 7.00, 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d, respectively. The reduction in ASBA might have led to a drop in COD removal. Similarly, COD removal for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} are 75%, 62%, 49%, and 39%, respectively, whereas reactor R_{Cu0} has shown 91% of COD removal during the steady-state condition of stressed phase operation. Therefore, mainly SVI, ISV, and ASBA parameters may indirectly be used as an indicator for deterioration of biomasses under stressed phase operation.

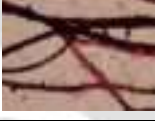
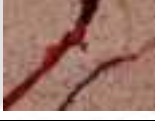
2.3.1.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology

The morphology of aerobic sludge biomass has been observed through microscopic examination of Gram's stained samples (at $5\times$ and $100\times$ magnification) and photographic images captured at regular intervals, as presented in Table 2.6 and 2.7, respectively. Microscopic examinations have indicated significant changes in biomass morphology of reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses due to presence of Cu(II) in the feed, as presented in Table 2.6 compared to that of reactor R_{Cu0} biomass. On the 1st d of operation, all reactors have similar flocs structure, and there has been an abundance of filamentous microorganisms. The images taken at $5\times$ magnification have shown the complete flocs structure; however, filamentous microorganisms can be seen at $100\times$ magnification. The filaments abundance has decreased from 5th d to 25th d gradually for reactor R_{Cu10} biomass (clearly visible at $100\times$). The gradual decrease in filaments have also been observed for reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses due to presence of Cu(II) from 5th d to 25th d (Table 2.6). The lower doses of Cu(II) (10 and 20 mg/L) have slightly affected the filamentous growth, whereas higher doses (40 and 80 mg/L) have completely inhibited the filaments growth and has resulted into disintegration of flocs.

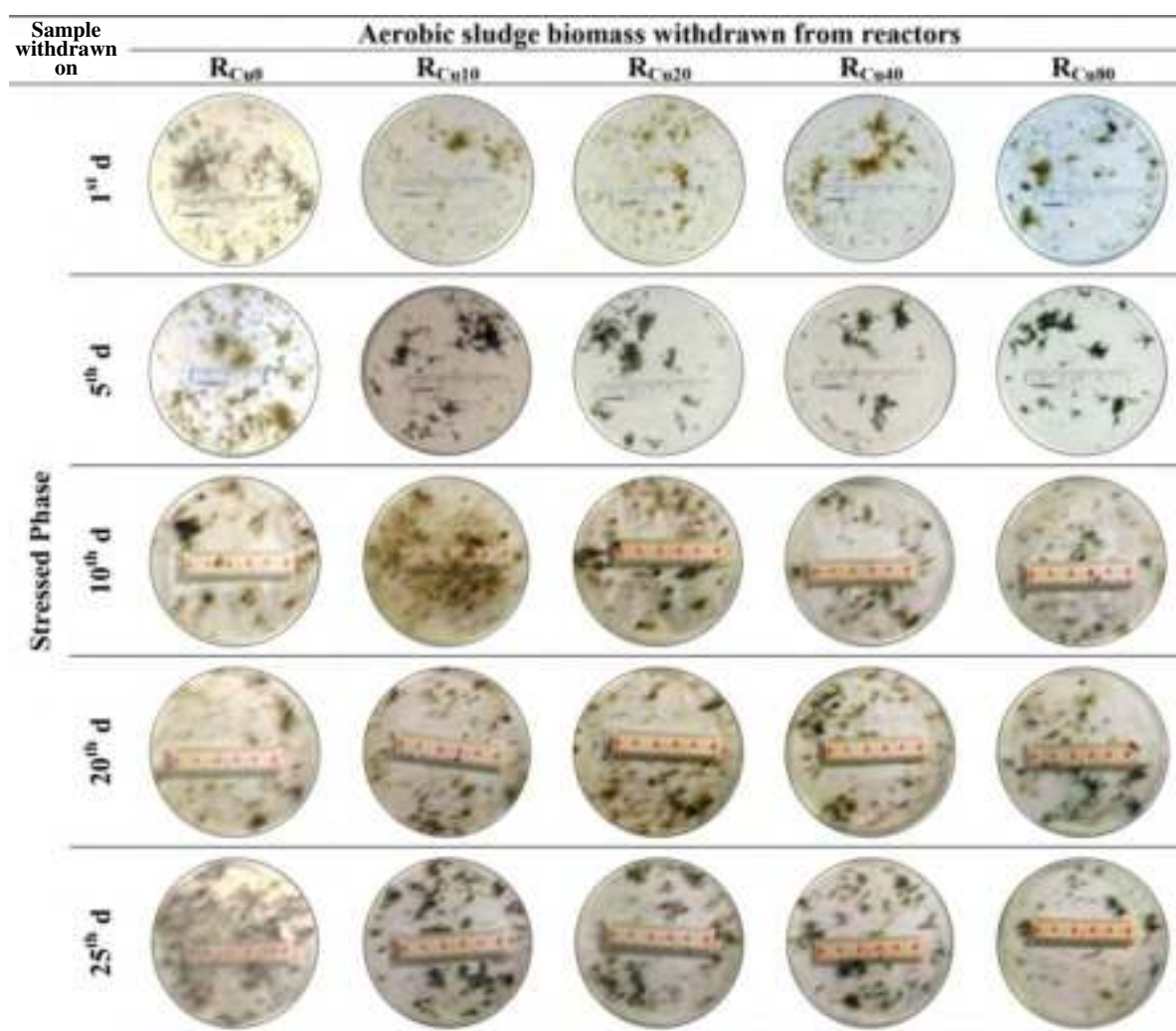
Photographic observations indicate that aerobic sludge biomass has disintegrated with an increase in Cu(II) concentration from 10 to 80 mg/L as significant changes have been observed in the physical characteristics of biomass such as flocs size and color as presented in Table 2.7. On the 1st d, the size of the flocs has ranged from 2-16 mm for all reactors, while on 25th d, the size of the flocs for Cu(II) fed reactors has reduced in the range of 1-4 mm. An increasing Cu(II) concentration has negatively influenced the relatively large flocs of aerobic sludge biomass and has resulted in flocs disintegration. The high concentration of Cu(II) has resulted in biomass color change from dark green to blue (visible with naked eyes). The floc disintegration has resulted from Cu(II) inhibition on bacterial activity and their extra-polymeric substance (EPS) production (Cui et al., 2015), which are key factors

Table 2.6 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} during stressed phase operation.

Reactor	Mag.	Aerobic sludge biomass samples withdrawn on			
		5 th d	10 th d	20 th d	25 th d
R_{Cu0}	5×				
	100×				
R_{Cu10}	5×				
	100×				
R_{Cu20}	5×				
	100×				
R_{Cu40}	5×				
	100×				
R_{Cu80}	5×				
	100×				

affecting floc flocculation. The presence of Cu(II) has affected the abundance of filamentous biomass during the initial period. [Shuttleworth and Unz \(1988\)](#) have reported that Cu(II) has the most inhibitory effect on filamentous bacteria.

The filament index (FI) of aerobic sludge biomass for all the reactors are presented in [Table 2.8](#). The FI for reactor R_{Cu0} biomass has been recorded 5 on the scale of 0-5 from the start to end of the study. When the biomasses have been stressed to Cu(II), the FI values have started to decrease. The FI for reactor R_{Cu10} biomass has been 5 on the 1st d; however, FI then has been reduced to 2 when the stressed phase achieves a steady-state on 20th d of

Table 2.7 Photographic images of aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} during stressed phase operation.

operation. The FI values for reactor R_{Cu40} and R_{Cu80} biomasses have drastically decreased to 1 towards the end of the stressed phase operation. Filamentous organisms are susceptible to heavy metals exposure. Even a trace concentration might be lethal for them. The Cu(II) stress has severely affected the filamentous microorganism and has resulted in a decreased FI values.

The samples have been withdrawn on 25th d of operation for observation under FESEM as shown in Fig. 2.14. It could be clearly seen that bacterial cells appear to adhere to EPS

Table 2.8 Filament index of aerobic sludge biomasses estimated during stressed phase operation.

Biomass withdrawn on	Reactors				
Day	R_{Cu0}	R_{Cu10}	R_{Cu20}	R_{Cu40}	R_{Cu80}
1 st d	5	5	5	5	5
5 th d	5	5	2	5	1
10 th d	5	5	3	1	1
20 th d	5	2	1	1	1

to form microbial clusters in reactor R_{Cu0} biomass. Few filaments have only been observed in reactor R_{Cu10} biomass with a thick EPS structure. Only cocci shaped bacteria have been observed in reactor R_{Cu20} biomass, with some of it is affected by Cu(II). The image clearly shows the affected biomass withdrawn from reactor R_{Cu40} . The EDX analysis has also been performed to examine the distribution of Cu(II) on the surface of aerobic sludge biomass, as shown in Fig. 2.15. The distribution of green color over the sample represent the existence of Cu(II) on aerobic sludge biomass, visible in Fig. 2.15(a) and the EDX spectrum shows the peaks and quantity of Cu(II) on the surface of aerobic sludge biomass as shown in Fig. 2.15(b).

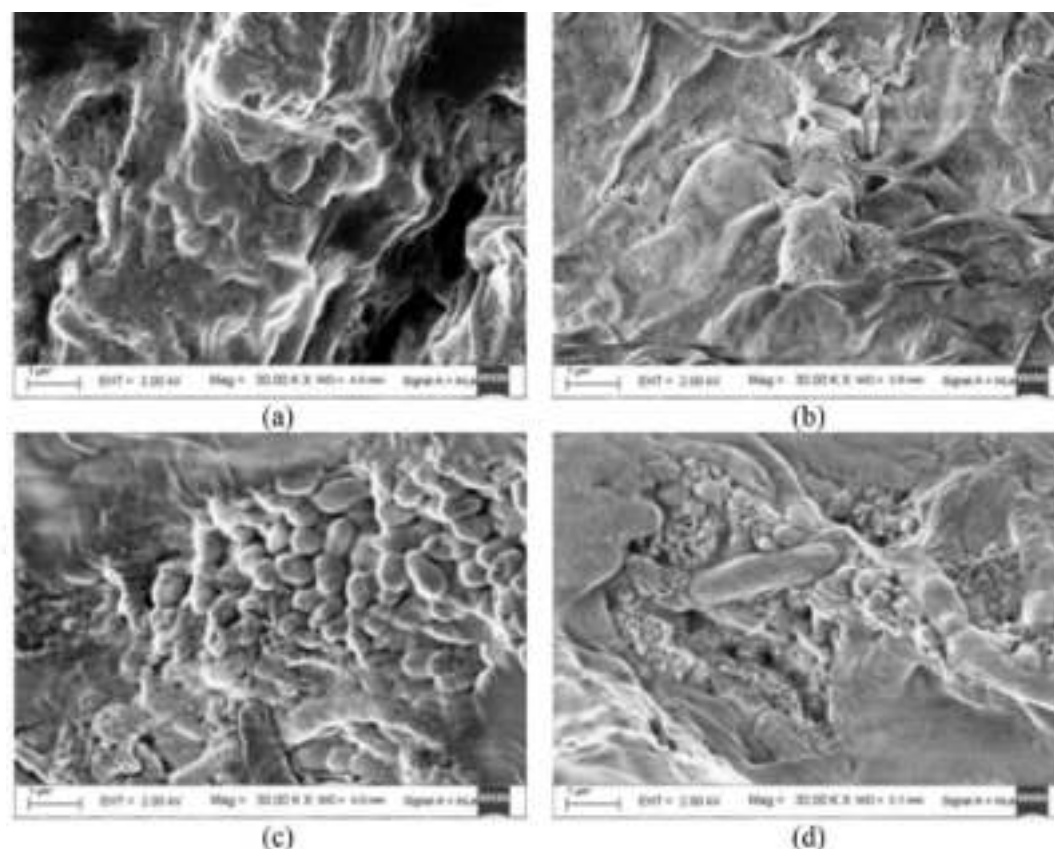


Fig. 2.14 FESEM micro-images of aerobic sludge biomass withdrawn on 25th d of stressed phase operation from reactors (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , and (d) R_{Cu40} .

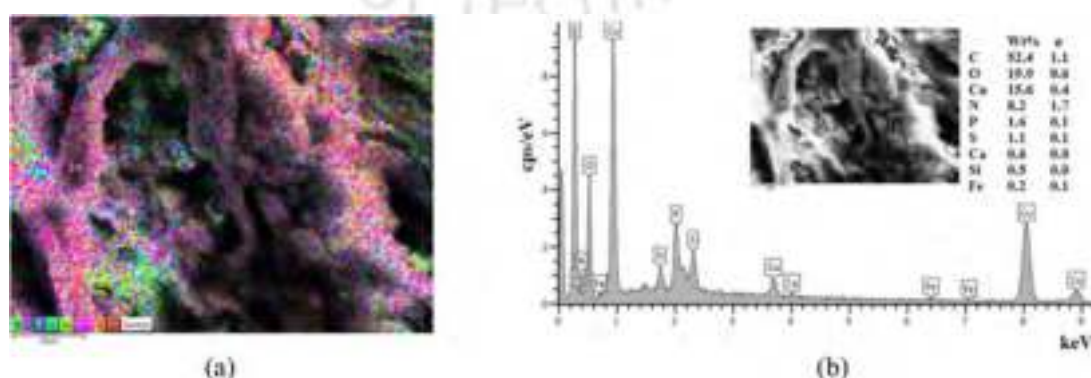


Fig. 2.15 EDX analysis of aerobic sludge biomass withdrawn on 25th d of stressed phase operation from reactor R_{Cu40} (a) EDX mapping and (b) EDX spectrum.

Enzyme activity

Catalase, a terminal respiratory enzyme, is present practically in all aerobic living cells and breaks down hydrogen peroxide (H_2O_2), a toxic product of oxidative metabolism, into water and oxygen (Gabbita and Huang, 1984). $E_{Catalase}$ for all the reactors appear to be approximately similar on the 1st d of operation as shown in Fig. 2.16(a), which has ranged between 0.259-0.286 mg/g MLSS h. $E_{Catalase}$ values have decreased with the increase of Cu(II) concentration during the stressed phase operation. The summary of $E_{Catalase}$ values for all reactors during stressed phase operation are given in Table 2.9. The percent inhibition of $E_{Catalase}$ for Cu(II) fed reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been 71.9%, 84.0%, 91.5% and 95.9%, respectively, at the end of the stressed phase operation. It is clear from Fig. 2.16(a) that presence of high concentration of Cu(II) in feed has inhibited $E_{Catalase}$ of the aerobic sludge biomass due to the formation of the metal complexes and also change in the pH levels. Achuba and Okoh (2008) have reported that a slight change in pH have

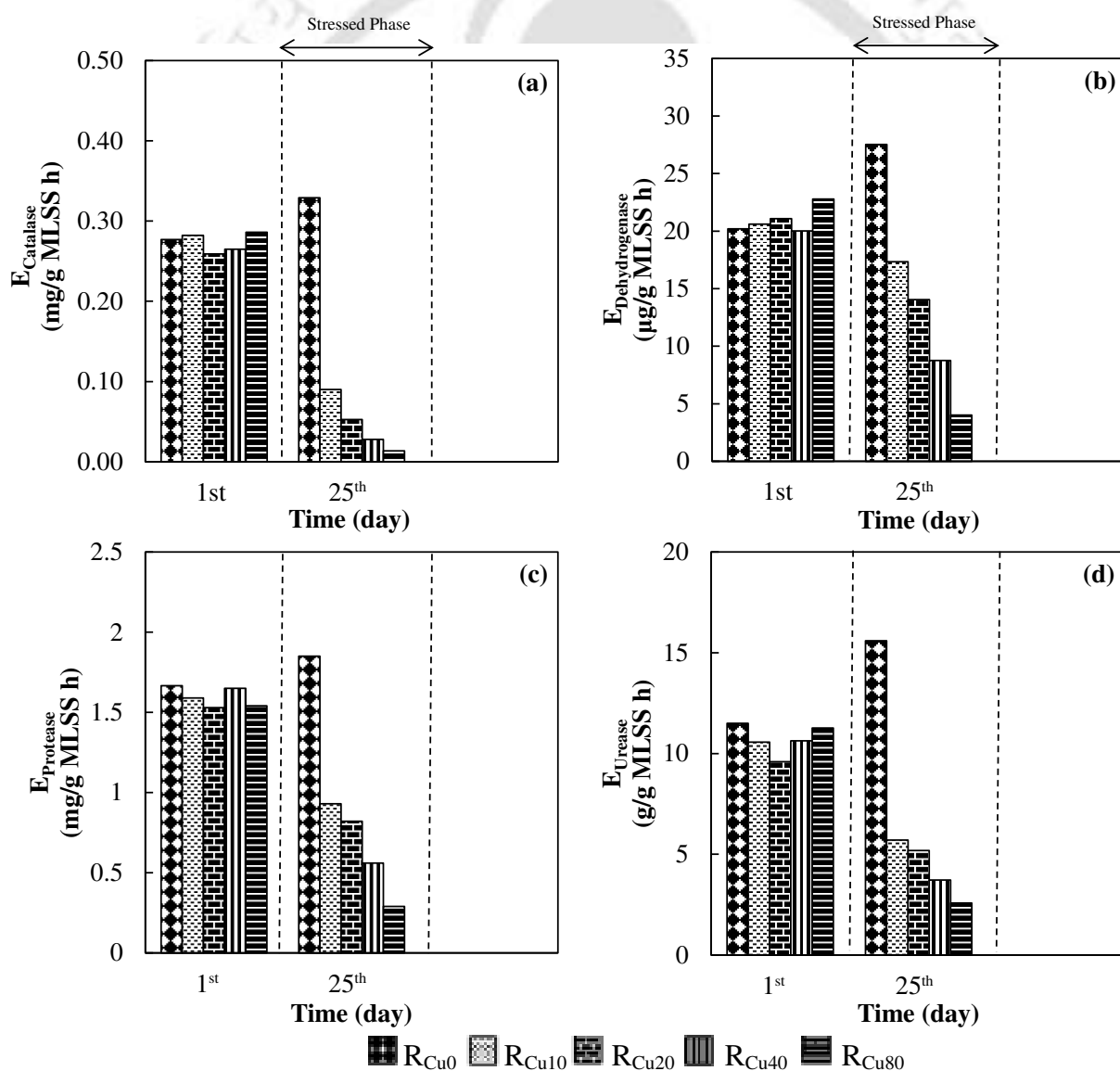


Fig. 2.16 Enzyme activities of aerobic sludge biomass (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} estimated during stressed phase operation.

Table 2.9 Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase operation.

Biomass withdrawn on	Reactor biomass	E _{Catalase} (mg/g MLSS h)	E _{Dehydrogenase} (μg/g MLSS h)	E _{Protease} (mg/g MLSS h)	E _{Urease} (g/g MLSS h)
Stressed phase operation					
1 st d	R _{Cu0}	0.28±0.03	20.19±1.76	1.67±0.07	11.50±1.13
	R _{Cu10}	0.28±0.03	20.61±1.76	1.59±0.04	10.57±2.12
	R _{Cu20}	0.26±0.04	21.08±1.82	1.53±0.07	9.59±0.95
	R _{Cu40}	0.27±0.02	20.02±1.92	1.65±0.09	10.63±0.95
	R _{Cu80}	0.29±0.04	22.77±2.15	1.54±0.05	11.26±1.32
25 th d	R _{Cu0}	0.33±0.12	27.52±1.85	1.85±0.05	15.59±2.15
	R _{Cu10}	0.09±0.01	17.34±2.54	0.93±0.07	5.71±0.83
	R _{Cu20}	0.05±0.01	14.05±1.41	0.82±0.04	5.19±0.97
	R _{Cu40}	0.03±0.01	8.75±1.28	0.56±0.07	3.72±0.68
	R _{Cu80}	0.01±0.01	4.02±0.87	0.29±0.05	2.58±0.74

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation.]

significantly affected E_{Catalase}. The pH is also an important parameter for the growth and activity of microorganisms. The decreased pH has also resulted in a decreased COD removal as it affects the microbial activity of aerobic sludge biomass.

Dehydrogenases are the enzymes mostly used in toxicity testing in wastewater treatment plants (Bitton, 2005). E_{Dehydrogenase} for all the reactors appears to be similar on the 1st d of operation as shown in Fig. 2.16(b), which has ranged between 20.02–22.77 μg/g MLSS h. It can be clearly observed that the E_{Dehydrogenase} has significantly dropped with the increase of Cu(II) concentrations [Fig. 2.16(b)]. The lowest E_{Dehydrogenase} value has been observed from the reactor fed with 80 mg/L of Cu(II) concentration (4.02 μg/g MLSS h). Wang et al. (2010) and Liu et al. (2017) have reported that the presence of 5 mg/L of Cu(II) in influent has caused a significant inhibitory effect on E_{Dehydrogenase}. It can be observed that at higher concentrations of Cu(II), not only Cu(II) ions but pH also play an important role, which imparts an inhibitory effect on E_{Dehydrogenase} (Trevors, 1984). E_{Dehydrogenase} for reactor R_{Cu0}, R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses have been 27.52, 17.34, 14.05, 8.75, and 4.02 μg/g MLSS h, respectively, towards the end of the stressed phase operation. The summary of E_{Dehydrogenase} for all reactors during stressed phase operation is given in Table 2.9. The percent inhibition of E_{Dehydrogenase} for Cu(II) fed reactor R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses have been 36.9%, 48.9%, 68.2%, and 85.4%, respectively, at the end of the stressed phase operation. The maximum inhibition has been obtained for a high dose of Cu(II) concentration (80 mg/L). Similar results have been reported by Feng et al. (2016) and Pamukoglu and Kargi (2007b).

Protease is a hydrolytic enzyme, plays an important role in the degradation of protein in wastewater and affects the removal of organic matter and microbial metabolism (Lv et al., 2008; Liu et al., 2017). Fig. 2.16(c) shows that E_{Protease} of aerobic sludge biomasses which have been inhibited by Cu(II) at all the selected concentrations [10, 20, 40, and 80 mg/L of Cu(II)] during the stressed phase operation. E_{Protease} for all the reactors on the 1st d of

operation as shown in Fig. 2.16(c) have ranged between 1.523-1.666 mg/g MLSS h. E_{Protease} has been 1.85, 0.93, 0.82, 0.56, and 0.29 mg/g MLSS h for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively, towards the end of the stressed phase operation. The summary of E_{Protease} for all the reactors during stressed phase operation is given in Table 2.9. Liu et al. (2017) have reported that Cu(II) has no significant effect on E_{Protease} . The protease enzyme is unstable at lower pH values, and its activity decreases due to low pH (Racheal et al., 2015). The percent inhibition of E_{Protease} for Cu(II) fed reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been 49.9%, 55.7%, 69.9%, and 84.2%, respectively towards the end of the stressed phase operation.

Urease can catalyze urea to break it down into ammonia and carbon dioxide (CO_2) and serve as an important indicator as there is plenty of urea in activated sludge biomass. E_{Urease} has decreased with increasing concentration of Cu(II), which is shown in Fig. 2.16(d). It has been suggested that the presence of Cu(II) could significantly inhibit E_{Urease} , and there exist a close relationship between Cu(II) dose and its inhibition of urease. E_{Urease} for all the reactors on the 1st d of operation ranged between 9.587-11.497 g/g MLSS h. E_{Urease} has been 15.59, 5.71, 5.19, 3.72, and 2.58 g/g MLSS h for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively, towards the end of the stressed phase operation. The summary of E_{Urease} for all the reactors during stressed phase operation is given in Table 2.9. The percent inhibition of E_{Urease} for Cu(II) fed reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been 63.4%, 66.6%, 76.1% and 83.4%, respectively, towards the end of the stressed phase operation. The obtained results suggest that E_{Urease} has got significantly influenced due to the addition of Cu(II) in the feed. As the Cu(II) doses increase, E_{Urease} decrease for reactor R_{Cu40} and R_{Cu80} biomasses.

It can be observed from the results that the presence of Cu(II) inhibits enzyme activity, which may be directly responsible for the decrease in organic matter removal and biomass activity as well. The possible mechanism of inhibition of enzyme activities by Cu(II) may be binding of the metal ions to functional groups involved in the catalytic action of enzymes. Metal accumulates in the cellular compartment of the enzyme and occupies the active site of the enzyme. The most widely known mechanisms for heavy metal toxicity are the irreversible inhibition of extracellular or intracellular enzymes upon binding with metal ions (Assche and Clijsters, 1990; Pamukoglu and Kargi, 2007b).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is one of the most useful tool for studying bacterial phylogeny as it is highly cost-effective and less time-consuming. This technique can be performed in any standard laboratory conditions with regular molecular biology facilities (Das and Dash, 2015). Based upon the banding pattern of the restriction products, the phylogenetic relationship among the strains can be derived. Related organisms are supposed to provide the same restriction pattern after digestion with the restriction enzyme. A tetra-cutter restriction enzyme digests the 16S rRNA gene at particular sites to provide a unique restriction pattern, which is considered the

signature of that organism. Thus, the restriction pattern of one organism can be compared with the restriction pattern of another to deduce the phylogenetic relationship between them. ARDRA gives only a little information about the type of microorganisms present in the sample, but allows to search for changes in the community over time, or even to compare those changes to current environmental conditions (Błaszczuk et al., 2011).

The restriction enzymes *AluI* and *MspI* have produced a differential pattern of fragments for all the aerobic sludge biomass samples (withdrawn towards the end of the stressed phase operation) which are shown in Fig. 2.17. The similarity matrix for all the stressed phase aerobic sludge biomass samples is summarized in Table 2.10 (data generated using GelJ software). The dendrogram generated by digestion of aerobic sludge biomass samples with *AluI* as shown in Fig. 2.17(a), suggest that reactor R_{Cu10} biomass is similar to reactor R_{Cu0} biomass and the similarity has decreased between reactor R_{Cu0} biomass and other reactor biomasses (R_{Cu20} , R_{Cu40} , and R_{Cu80}) with increase in Cu(II) doses. The reactor R_{Cu10} and R_{Cu20} biomasses have shown 71.4% and 64.7% similarity, respectively, under stressed phase operation, as compared to the reactor R_{Cu0} biomass. The reactor biomasses fed with Cu(II) at 40 mg/L and 80 mg/L have shown 56.8% and 48.1% similarity as compare to reactor R_{Cu0} biomass, which suggest that 10 mg/L Cu(II) concentration is sufficient to change the microbial community structures of the reactor biomasses. The similarity matrix for all stressed phase reactor biomasses digested with *MspI* are presented in Table 2.10. The dendrogram generated by the digestion of aerobic sludge biomass samples with *MspI* has been shown in

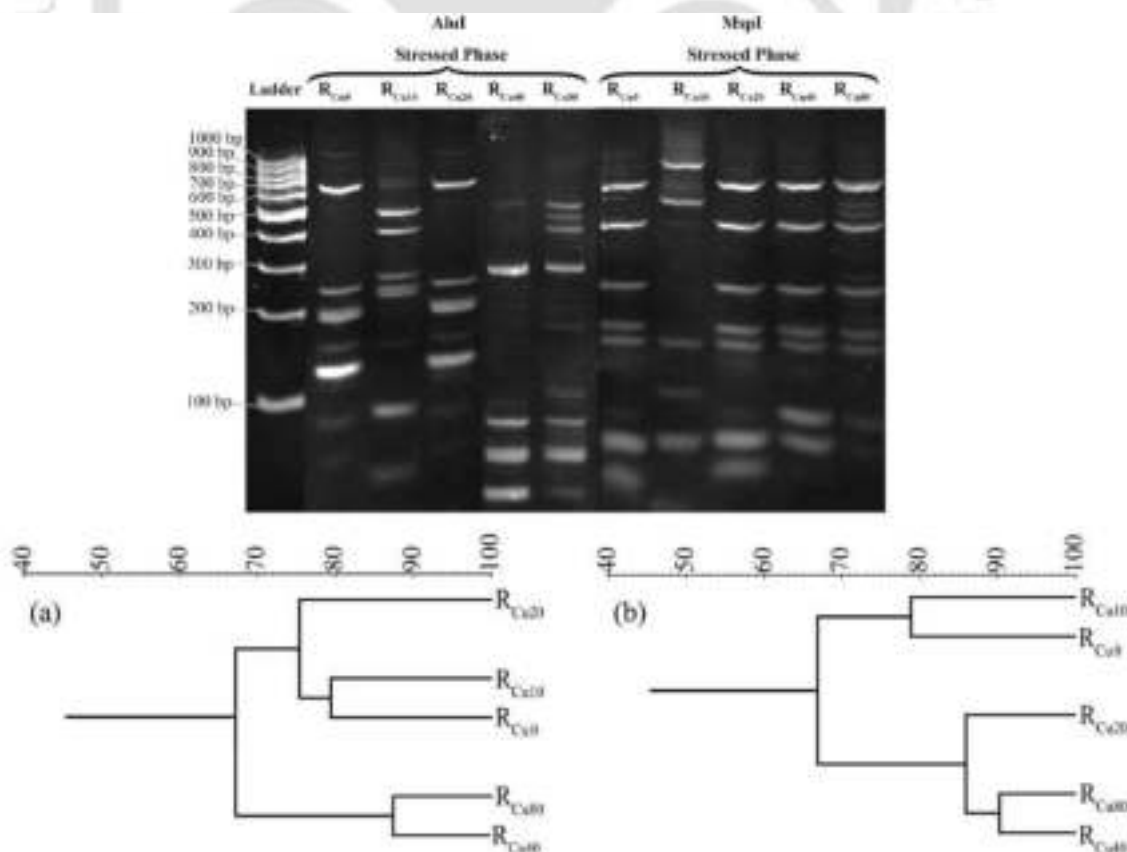


Fig. 2.17 Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase treated with (a) *AluI* and (b) *MspI* restriction enzymes.

Table 2.10 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase operation.

Reactor	Stressed phase operation									
	<i>AluI</i>					<i>MspI</i>				
	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
R _{Cu0}	1	0.7142	0.6471	0.5682	0.4818	1	0.6584	0.5156	0.4287	0.3797
R _{Cu10}	0.7142	1	0.6715	0.5449	0.4987	0.6585	1	0.5783	0.4972	0.4142
R _{Cu20}	0.6471	0.6715	1	0.6486	0.5544	0.5157	0.5781	1	0.8141	0.7264
R _{Cu40}	0.5682	0.5449	0.6486	1	0.8210	0.4284	0.4974	0.8141	1	0.8378
R _{Cu80}	0.4818	0.4987	0.5544	0.8210	1	0.3797	0.4142	0.7264	0.8378	1

Fig. 2.17(b), which has indicated decrease in similarities between Cu(II) fed reactor (R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80}) biomasses and reactor R_{Cu0} biomass. The reactors biomass fed with Cu(II) concentration of 10, 20, 40, and 80 mg/L have shown similarity of 65.8%, 51.5%, 42.8% and 37.9%, respectively, under stressed phase as compared to the reactor R_{Cu0} biomass. *MspI* digestion pattern reveals that 10 mg/L Cu(II) concentration could change the microbial community. The presence of Cu(II) up to 80 mg/L might have changed the microbial community up to 37.9%. The results suggest that similarities between aerobic sludge biomass, stressed with different concentrations of Cu(II) have decreased with increase in Cu(II) doses. ARDRA has proved to be a suitable and rapid method for taxonomic studies. The microbial communities in the reactor biomasses have showed distinct variation with increasing Cu(II) concentration. The differences between the patterns do not ensure that the composition of the communities is exactly the same. However, significant compositional changes in the community should be detected with the restriction enzymes used (Moyer et al., 1996; Smit et al., 1997; Shah, 2014).

Under microbiological parameters, visual and microscopic techniques have been used to assess the morphology of aerobic sludge biomass. The microscopic and photographic analysis have revealed that Cu(II) stress has inhibited the growth of filamentous biomass in reactors. Flocs have been transformed from dense to pinpoint, resulting in lower SVI and higher ISV for Cu(II) fed reactors. The filaments abundance has decreased from 5th d to 25th d gradually for reactor R_{Cu10} biomass. The disintegration of flocs has also been observed for reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses due to Cu(II) presence from 5th d to 25th d. At 80 mg/L of Cu(II), aerobic sludge biomass has disintegrated completely. The photographic observation has revealed that reactor R_{Cu0} biomass flocs size range 2.0-14.0 mm, while on 25th d, the flocs size for Cu(II) fed reactors has reduced to the range of 1.0-4.0 mm as well as has transformed into pinpoint flocs. The FI value indicates the existence of filamentous biomass, which has been drastically reduced due to the addition of Cu(II). The FI has also hindered due to Cu(II) stress. The FI value for reactor R_{Cu10} biomass has declined from 5 to 2 around 20th d, while FI value for reactor R_{Cu80} biomass has declined from 5 to 1 within 5th d of operation. FESEM has revealed the morphology of aerobic sludge biomass, and EDX has confirmed that Cu(II) has been retained on the surface of aerobic sludge biomass. $E_{Catalase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have ranged 0.01-0.09 mg/g VSS, while 0.33 mg/g VSS for reactor R_{Cu0} biomass. $E_{Dehydrogenase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have ranged 4.02-17.34 μ g/g VSS, while 27.52 μ g/g VSS for reactor R_{Cu0} biomass. $E_{Protease}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have ranged 0.29-0.93 mg/g VSS, while 1.85 mg/g VSS for reactor R_{Cu0} biomass. Similarly, E_{Urease} for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have ranged from 2.58-5.71 g/g VSS, while 15.59 g/g VSS for reactor R_{Cu0} biomass. The enzyme activities have been drastically affected due to increased Cu(II) doses. The percent inhibition of $E_{Catalase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been 71.9%, 84.0%, 91.5%, and 95.9%, respectively. Similarly, percent inhibition of $E_{Dehydrogenase}$ of 36.9%, 48.9%, 68.2%, and 85.4%; $E_{Protease}$ of 49.9%, 55.7%,

69.9%, and 84.2%; and E_{Urease} of 63.4%, 66.6%, 76.1%, and 83.4% have been obtained for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. The enzyme activity test has indicated that all four enzymes had been severely inhibited by Cu(II) stress. ARDRA has revealed the changes in microbial community structure. *AluI* has shown 71.4%, 64.7%, 56.8%, and 48.1% of similarity, while *MspI* has shown 65.8%, 51.5%, 42.8%, and 37.9%, of similarity for reactor biomasses fed with Cu(II) concentration of 10, 20, 40, and 80 mg/L, respectively, as compared to reactor R_{Cu0} biomass. Cu(II) stress may be responsible for the alterations in microbial populations in reactors that may be unable to survive. A decrease in the breakdown of organics may also be related to an insufficient variety of bacteria.

2.3.2 Recovery phase operation

The addition of Cu(II) has been discontinued in the feed when reactor operation have achieved steady-state condition during stressed phase and thereafter further operated for 26-54 d under recovery phase operation without Cu(II) in the feed. Literature have mostly filled with stressed phase studies and a few literature are available on recovery phase study which have been used to compare with the results obtained in this study. For the sake of highlighting the differences between observations and results of recovery phase operation with the stressed phase operation, most of the figures and tables have been reproduced with the results of stressed phase operation along with recovery phase operation.

2.3.2.1 Environmental and performance parameters

The reactors have been operated for 29 d (26-54 d) during recovery phase operation. The results obtained during recovery phase operation have been shown in Fig. 2.18-2.22 and summary of data has been presented in Table 2.11. The variation in room temperature has been shown in Fig. 2.18. The change in weather conditions have resulted in variations in room temperature. The initial and effluent pH for reactor R_{Cu0} has ranged uniformly between 6.57-7.45. Moreover, discontinuation of Cu(II) has resulted in neutral pH conditions of reactors towards the end of the recovery phase. The pH values have started to increase after Cu(II) discontinuation. The pH values for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have ranged 6.86-7.33, 6.19-7.18, 6.10-7.15, and 6.12-7.26, respectively, under recovery phase operation, as shown in Fig. 2.19-2.22. During stress phase operation, average initial pH values for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} and R_{Cu80} have been observed as 7.19, 6.60, 6.12, and 5.85, respectively, while effluent pH have been observed as 6.89, 6.72, 6.15, and 5.53, respectively. Whereas, pH values in all reactors have reached to neutral at the end of recovery phase operation. The average initial pH values for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 7.14, 6.88, 6.93, and 6.85 respectively, while effluent pH have been observed as 7.09, 7.04, 7.01, and 6.96, respectively, during recovery phase (Table 2.11).

Initially, reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have not shown significant COD removal after discontinuation of Cu(II) in the feed. This suggests that Cu(II) in feed has damaged the biomass under stressed phase operation and post-effects of Cu(II) stress on aerobic sludge biomass have lasted for 13th d after the start of recovery phase. On the 14th d from the start

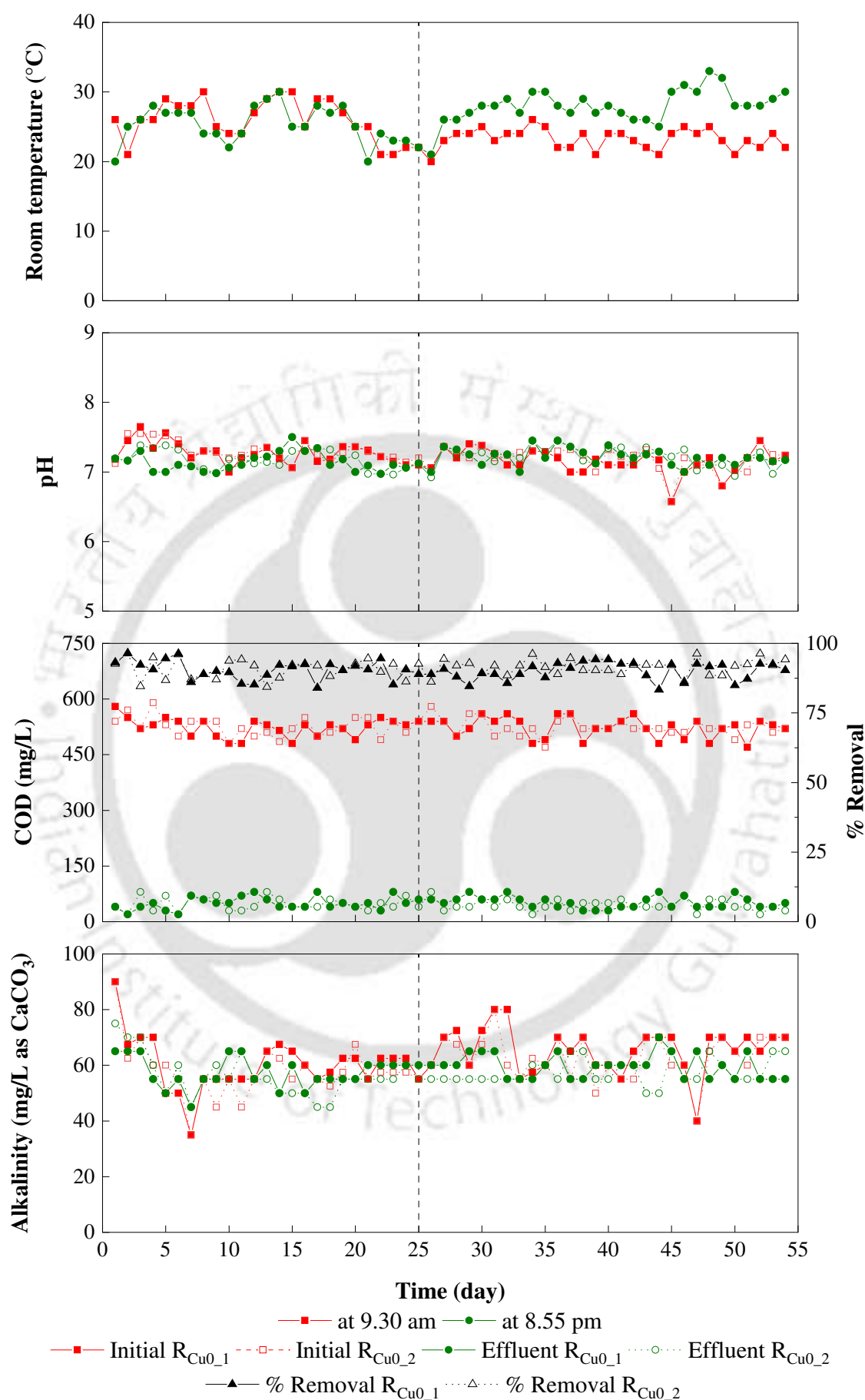


Fig. 2.18 Variation in environmental and performance related parameters for reactor R_{Cu0} during 26-54 d of operation along with 0-25 d of operation [without Cu(II) in the feed].

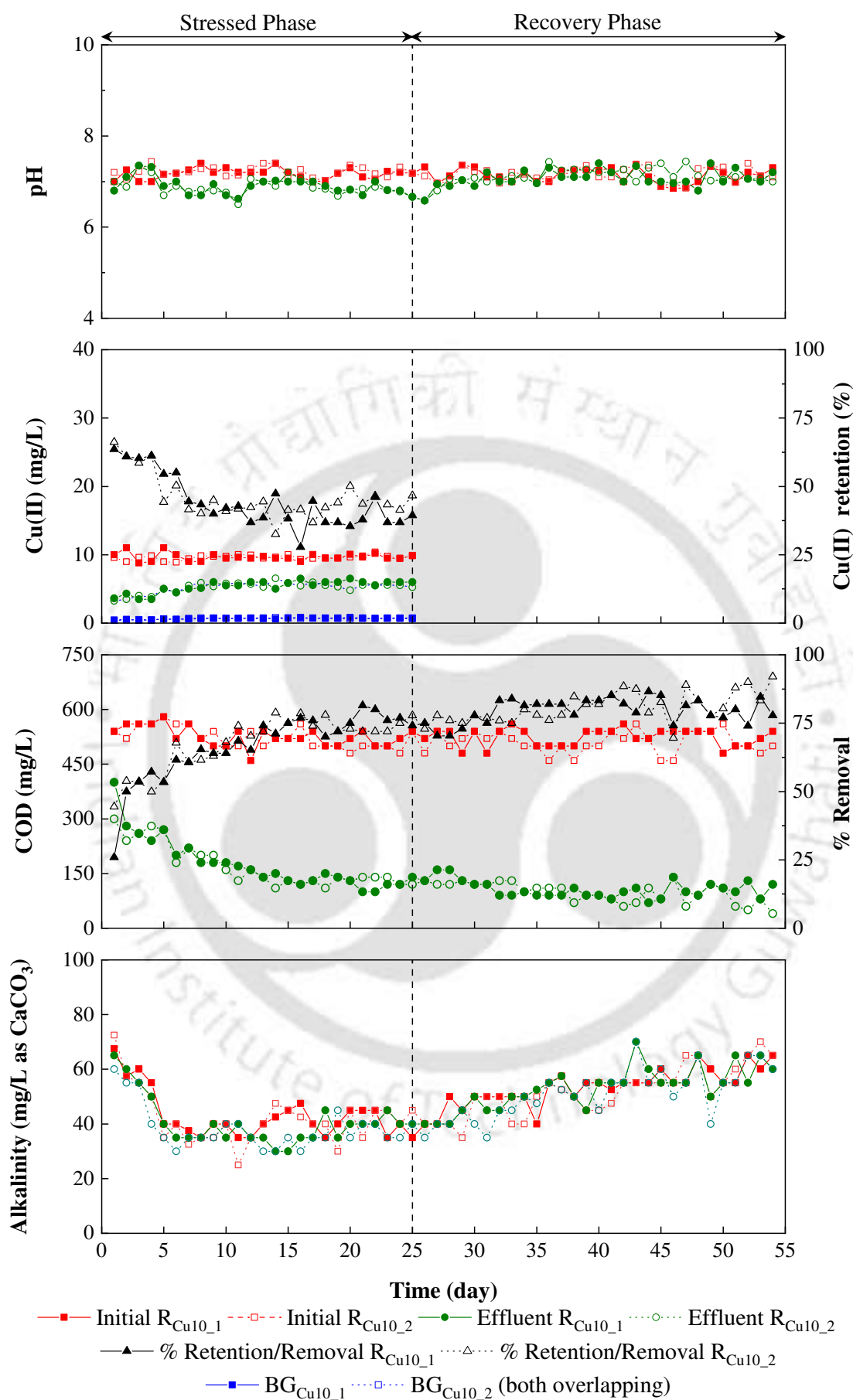


Fig. 2.19 Variation in environmental and performance related parameters for reactor R_{Cu10} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.

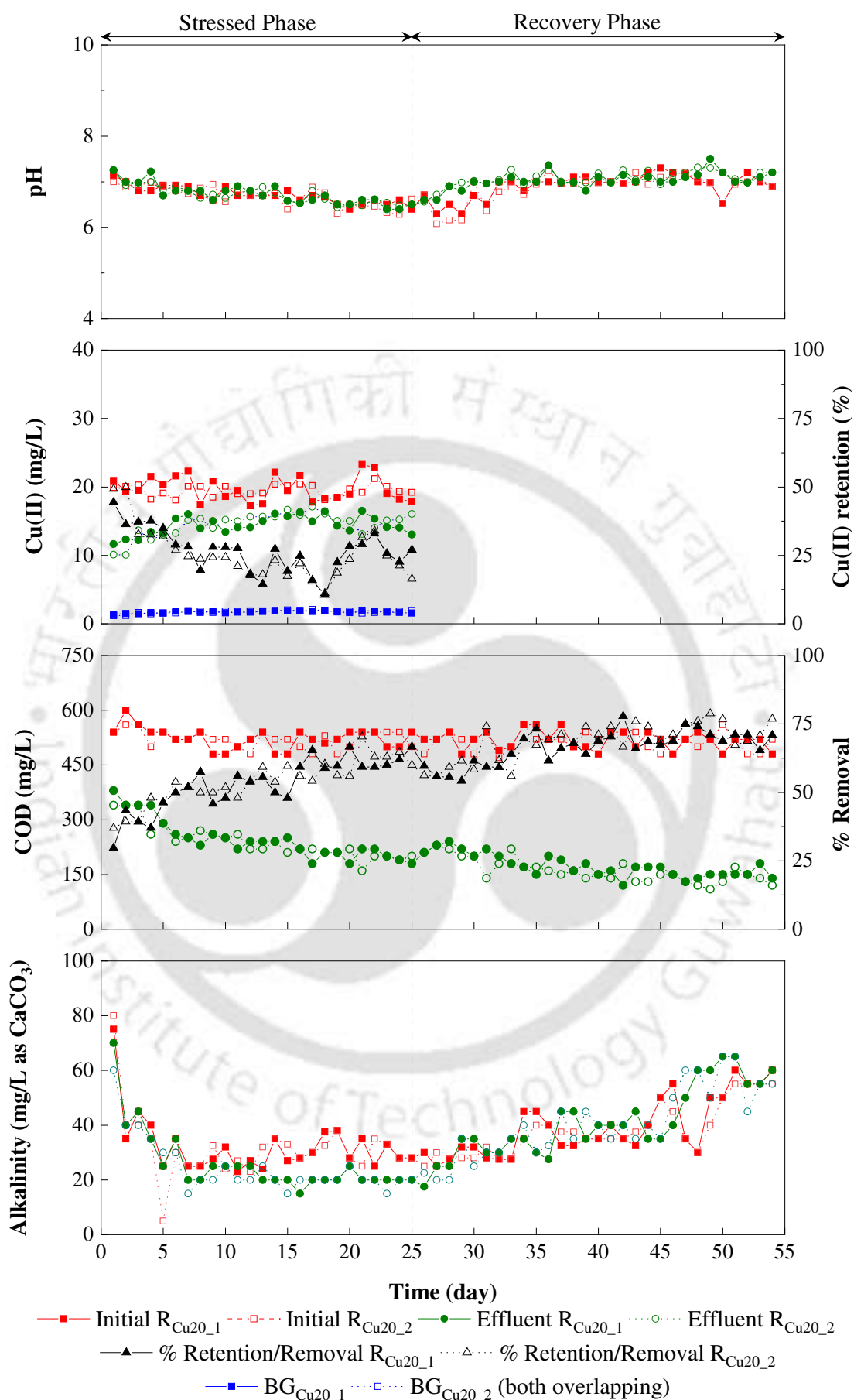


Fig. 2.20 Variation in environmental and performance related parameters for reactor R_{Cu20} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.

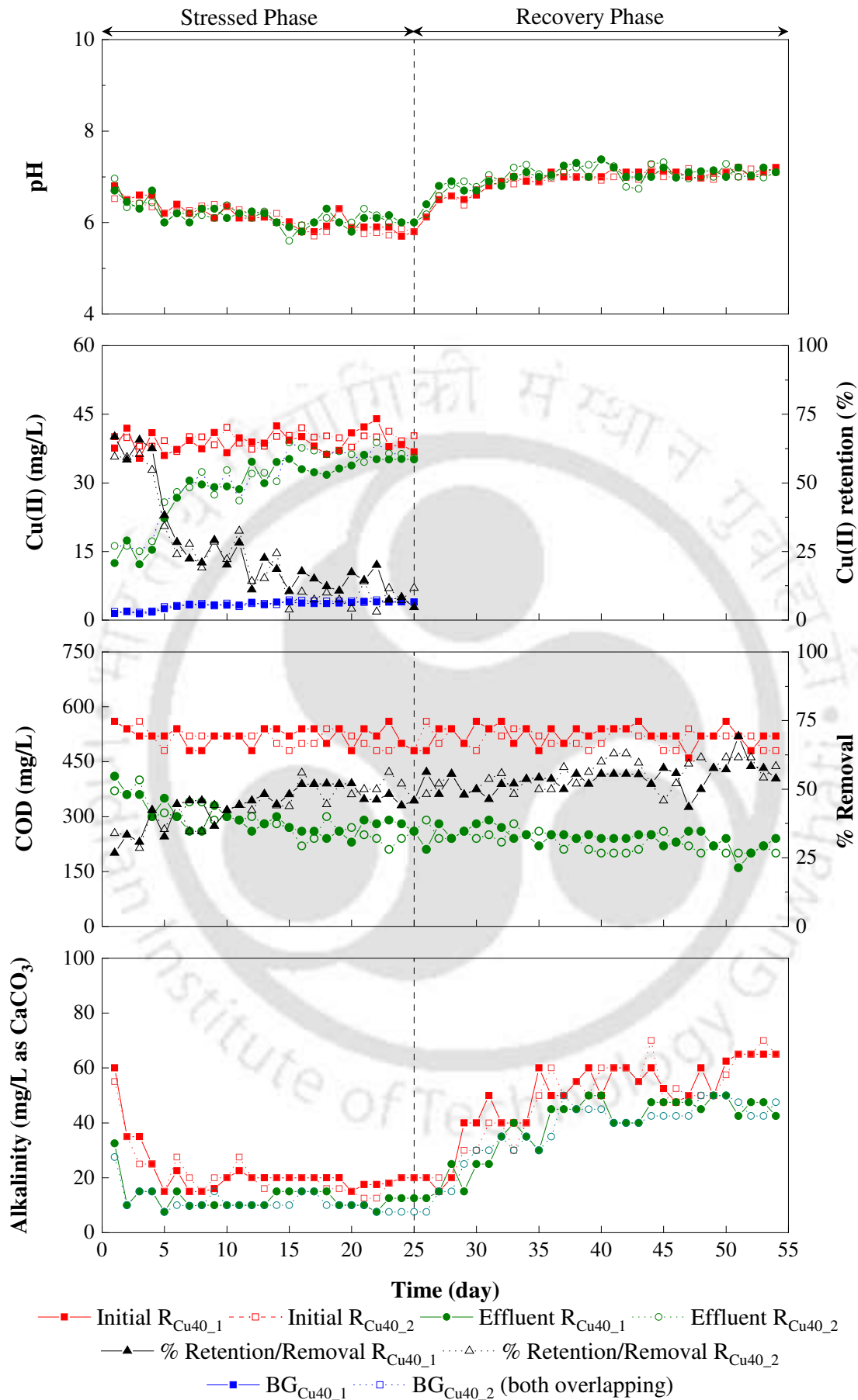


Fig. 2.21 Variation in environmental and performance related parameters for reactor R_{Cu40} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.

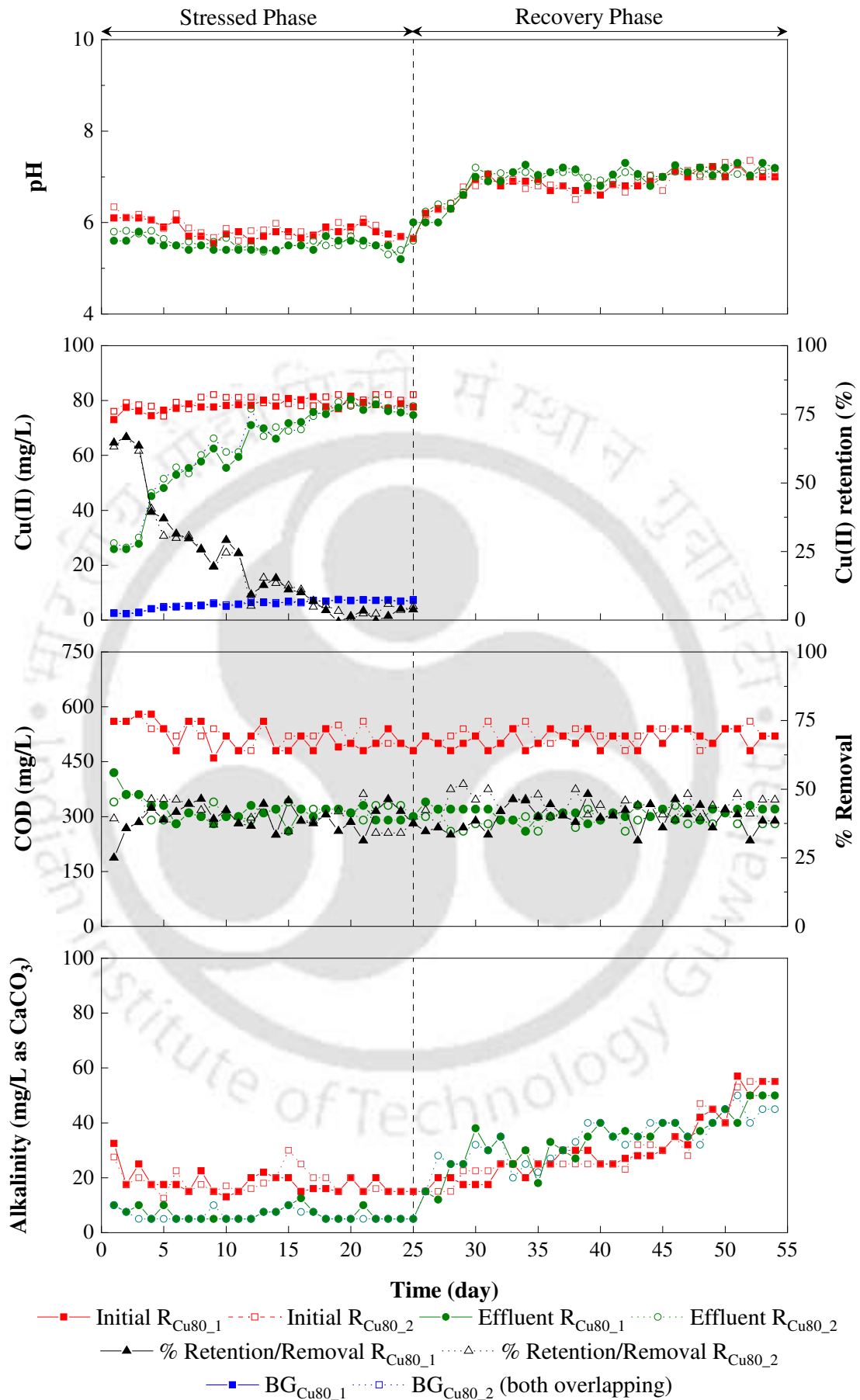


Fig. 2.22 Variation in environmental and performance related parameters for reactor R_{Cu80} operated during 26-54 d under recovery phase operation along with data for stressed phase operation.

Table 2.11 Summary of environmental and performance related parameters for reactors operated during 26-54 d under recovery phase operation along with data for stressed phase operation (0-25 d).

Parameters	Day-wise	0-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	10	20	40	80
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	26.0±3.0 (25)	26.0±3.0 (25)	26.0±3.0 (25)	26.0±3.0 (25)
		Room temperature (°C) at 8.55 pm	25.2±2.7 (25)	25.2±2.7 (25)	25.2±2.7 (25)	25.2±2.7 (25)
		Initial pH	7.28±0.15 (50)	7.19±0.12 (50)	6.60±0.20 (50)	6.12±0.28 (50)
		Initial Cu(II) (mg/L)	0	9.64±0.45 (50)	19.65±1.33 (50)	39.23±1.82 (50)
	Performance	Effluent pH	7.15±0.13 (50)	6.89±0.19 (50)	6.72±0.21 (50)	6.15±0.24 (50)
		Effluent Cu(II) (mg/L)	0	5.29±0.85 (50)	14.47±1.58(50)	28.99±7.44 (50)
		Cu(II) retention (%)	0	45.12±8.80 (50)	26.12±8.64 (50)	23.97±18.46 (50)
		Background Cu(II) (mg/L)	0	0.67±0.11 (50)	1.73±0.19 (50)	3.36 ±0.84 (50)
		Initial soluble COD (mg/L)	524.80±27.79 (50)	524.80±23.29 (50)	522.40±25.96 (50)	517.60±24.93 (50)
		Effluent soluble COD (mg/L)	48.80±17.61 (50)	173.60±64.08 (50)	243.60±50.70 (50)	290.00±44.51 (50)
		COD removal (%)	90.66±2.91 (50)	67.19±10.99 (50)	53.47±8.78 (50)	44.03±7.17 (50)
		Initial alkalinity (mg CaCO ₃ /L)	58.90±9.85 (50)	42.6±9.15 (50)	32.46±11.40 (50)	21.60±8.55 (50)
		Effluent alkalinity (mg CaCO ₃ /L)	60.29±6.55 (50)	37.79±8.28 (50)	28.02±10.87 (50)	11.99±4.55 (50)
Parameters	Day-wise	26-54 d				
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	0	0	0	0
Recovery phase	Environmental	Room temperature (°C) at 9.30 am	23.2±1.5 (29)	23.2±1.5 (29)	23.2±1.5 (29)	23.2±1.5 (29)
		Room temperature (°C) at 8.55 pm	28.1±2.3 (29)	28.1±2.3 (29)	28.1±2.3 (29)	28.1±2.3 (29)
		Initial pH	7.16±0.18 (58)	7.14±0.16 (58)	6.88±0.29(58)	6.93±0.25 (58)
		Effluent pH	7.21±0.13 (58)	7.09±0.18 (58)	7.04±0.18 (58)	7.01±0.23 (58)
	Performance	Initial soluble COD (mg/L)	522.06±25.54 (58)	517.24±26.55 (58)	516.55±18.56 (58)	518.62±24.79 (58)
		Effluent soluble COD (mg/L)	48.96±15.23 (58)	102.06±25.52 (58)	166.89±31.99 (58)	235.51±26.20 (58)
		COD removal (%)	90.59±2.91 (58)	80.23±5.07 (58)	67.63±6.46 (58)	54.56±4.93 (58)
		Initial alkalinity (mg CaCO ₃ /L)	64.31±8.09 (58)	52.58±7.87 (58)	38.79±10.04 (58)	49.31±14.25 (58)
		Effluent alkalinity (mg CaCO ₃ /L)	59.74±4.49 (58)	44.81±8.50 (58)	43.18±12.83 (58)	38.36±11.27 (58)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

of recovery phase, the COD removal for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 82.7%, 69.2%, 54.0%, and 44.4%, respectively. However, average COD removal for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 80.2%, 67.6%, 54.6%, and 42.0%, respectively, while reactor R_{Cu0} has showed 90.6% COD removal during recovery phase operation (Fig. 2.18-2.22). During steady-state of stressed phase operation, the average COD removal for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been obtained as 74.9%, 61.9%, 49.1%, and 38.5%, respectively (Table 2.12). The results suggest that reactor R_{Cu10} and R_{Cu20} biomasses have shown improvement in COD removal; however, reactor R_{Cu40} and R_{Cu80} biomasses have shown slight improvement in COD removal towards the end of the recovery phase operation. The discontinuation of Cu(II) in the feed has shown improved COD removal in the recovery phase for reactors R_{Cu10} , R_{Cu20} , and R_{Cu40} while very slow recovery has been observed for reactor R_{Cu80} . The improvement in COD removal efficiency under the recovery phase operation suggests that microbes present in aerobic sludge biomass probably trying to repair their cellular machinery and exhibit improved bio-activity.

The discontinuation of Cu(II) has resulted in an increase in alkalinity values that has ranged between 31.03 ± 12.09 to 64.31 ± 8.09 mg $CaCO_3$ /L for initial and effluent, respectively, under recovery phase operation. The increasing trend of alkalinity can be observed from Fig. 2.18-2.22.

The steady-state condition has been observed from 48th d onward of recovery phase operation and the summary of steady-state data have been presented in Table 2.12. The minimum variation in performance related parameters have been observed during 48-54 d of recovery phase operation as presented in Table 2.12. The COD removal for reactor R_{Cu0} has shown similar trend throughout, while reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 82.1%, 72.0%, 58.5%, and 41.3% of COD removal at steady-state condition in recovery phase operation. COD removal at steady-state condition in stressed phase operation for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 74.9%, 61.9%, 49.1%, and 38.5%, respectively. The data obtained during steady-state conditions in both stressed and recovery phase operations have suggested that reactors R_{Cu10} , R_{Cu20} , and R_{Cu40} biomasses have regained around 9-10% additional organic removal capabilities, however, reactor R_{Cu80} has shown around 2-3% additional recovery during the recovery phase operation.

2.3.2.2 Biomass related general parameters

Aerobic sludge biomass characteristics

It has been observed that MLSS and MLVSS have started to increase when Cu(II) in the feed has been discontinued during the recovery phase operation, as shown in Fig. 2.23 and summarized data is presented in Table 2.13. This has indicated that heterogeneous aerobic sludge biomass microorganisms have started to grow again. The reactor R_{Cu10} and R_{Cu20} biomasses have shown recovery in terms of increased MLSS and MLVSS, whereas reactor R_{Cu40} and R_{Cu80} biomasses have shown slow recovery compared to reactor R_{Cu10} and R_{Cu20} biomasses. On 32nd d of operation, the MLVSS/MLSS ratio have been obtained as 0.92, 0.94,

Table 2.12 Summary of environmental and performance related parameters for reactors operated during steady-state of recovery phase operation (48-54 d) along with data for stressed phase operation (19-25 d).

Parameters	Day-wise	19-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	10	20	40	80
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	23.29±2.36 (7)	23.29±2.36 (7)	23.29±2.36 (7)	23.29±2.36 (7)
		Room temperature (°C) at 8.55 pm	23.57±2.51 (7)	23.57±2.51 (7)	23.57±2.51 (7)	23.57±2.51 (7)
		Initial pH	7.24±0.09 (14)	7.20±0.09 (14)	6.46±0.10 (14)	5.89±0.19 (14)
		Initial Cu(II) (mg/L)	0	9.77±0.30 (14)	19.70±1.56 (14)	39.70±1.93 (14)
	Performance	Effluent pH	7.07±0.09 (14)	6.79±0.10 (14)	6.50±0.07 (14)	6.04±0.12 (14)
		Effluent Cu(II) (mg/L)	0	5.68±0.28 (14)	14.61±1.05 (14)	35.60±1.15 (14)
		Cu(II) retention (%)	0	41.79±3.22 (14)	25.60±5.10 (14)	10.16±4.90 (14)
		Background Cu(II) (mg/L)	0	0.71±0.04 (14)	1.75±0.13 (14)	4.00±0.14 (14)
		Initial soluble COD (mg/L)	528.57±21.10 (14)	508.57±19.00 (14)	522.86±22.42 (14)	508.57±27.28 (14)
		Effluent soluble COD (mg/L)	48.57±14.23 (14)	127.14±13.14 (14)	198.57±18.11 (14)	258.57±20.68 (14)
		COD removal (%)	90.78±2.78 (14)	74.94±2.77 (14)	61.92±4.21 (14)	49.17±3.21 (14)
		Initial alkalinity (mg CaCO ₃ /L)	59.29±3.96 (14)	40.71±5.29 (14)	30.71±4.68 (14)	17.57±2.67 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	57.14±2.16 (14)	39.29±3.33 (14)	20.36±2.39 (14)	9.64±1.59 (14)
Parameters	Day-wise	48-54 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	0	0	0	0
Recovery phase	Environmental	Room temperature (°C) at 9.30 am	22.86±1.35 (7)	22.86±1.35 (7)	22.86±1.35 (7)	22.86±1.35 (7)
		Room temperature (°C) at 8.55 pm	29.71±2.06 (7)	29.71±2.06 (7)	29.71±2.06 (7)	29.71±2.06 (7)
		Initial pH	7.14±0.21 (14)	7.19±0.14 (14)	6.93±0.20 (14)	7.08±0.09 (14)
		Effluent pH	7.13±0.08 (14)	7.08±0.12 (14)	7.17±0.15 (14)	7.08±0.09 (14)
	Performance	Initial soluble COD (mg/L)	515.71±21.32 (14)	517.14±26.79 (14)	511.43±25.31 (14)	514.29±21.31 (14)
		Effluent soluble COD (mg/L)	45.71±14.96 (14)	92.86±24.12 (14)	142.86±17.08 (14)	212.86±21.21 (14)
		COD removal (%)	91.10±2.93 (14)	82.10±4.46 (14)	72.00±3.63 (14)	58.59±4.38 (14)
		Initial alkalinity (mg CaCO ₃ /L)	67.86±3.19 (14)	61.07±5.70 (14)	50.00±10.09 (14)	56.30±16.44 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	58.57±4.47 (14)	58.57±7.57 (14)	58.21±5.78 (14)	41.70±13.51 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

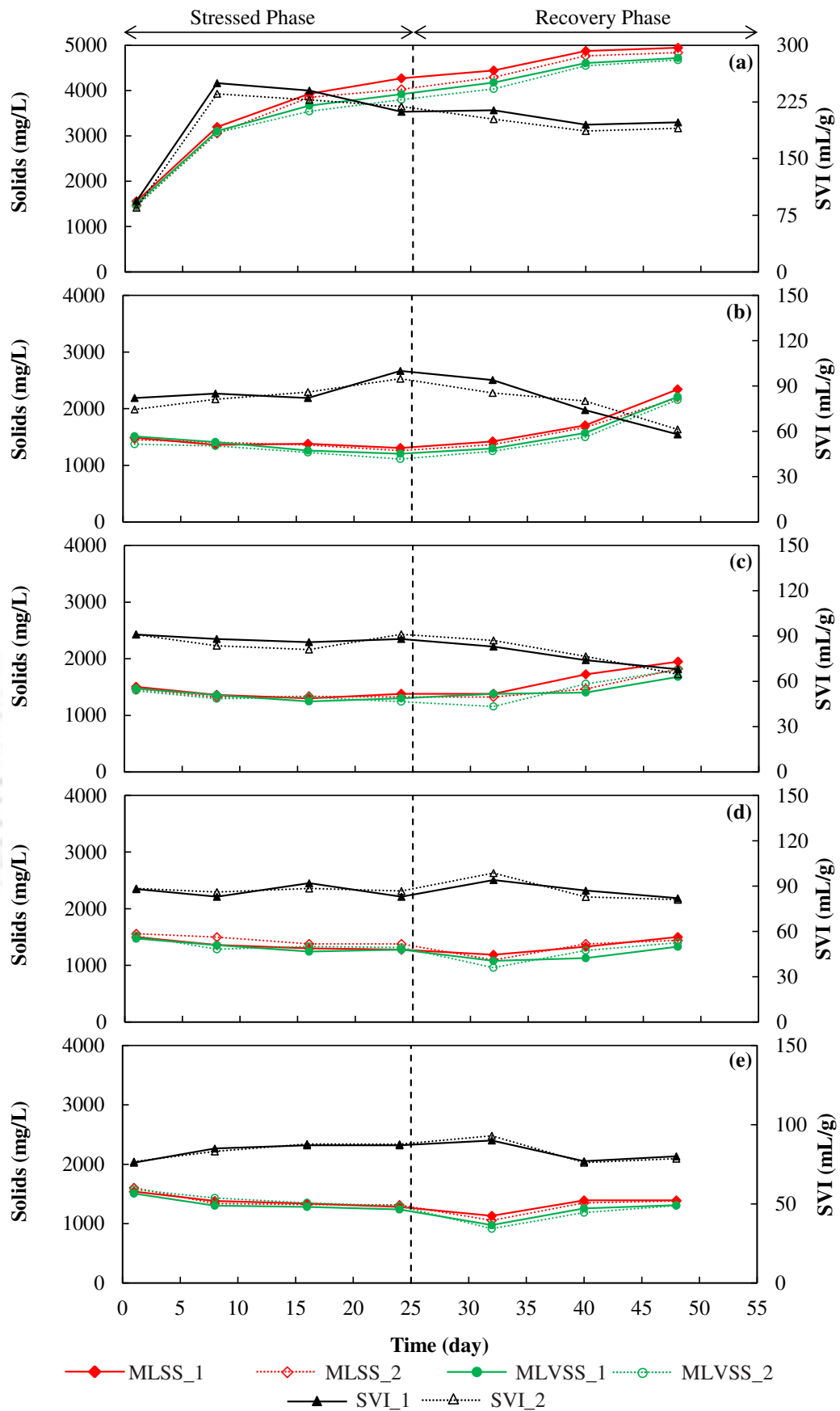


Fig. 2.23 Variation in MLSS, MLVSS, and SVI values for reactor biomass from (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} during 26-54 d under recovery phase operation along with data for stressed phase operation.

Table 2.13 Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.

Gross parameters	Stressed phase operation					
	Day-wise	0-25 d				
	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
MLSS (mg/L)		3171.06±1188.55 (8)	1376.50±79.55 (8)	1373.50±77.28 (8)	1407.50±94.67 (8)	1389.50±125.85 (8)
MLVSS (mg/L)		3010.47±1068.55 (8)	1304.41±129.42 (8)	1333.28±89.74 (8)	1351.85±104.89 (8)	1372.64 ±120.03 (8)
MLVSS/MLSS ratio		0.96±0.03 (8)	0.95±0.05 (8)	0.97±0.03 (8)	0.95±0.04 (8)	0.93±0.07 (8)
SVI (mL/g)		195.40±71.71 (8)	85.68±8.60 (8)	87.45±3.61 (8)	98.18±2.78 (8)	83.76±5.23 (8)
ISV (m/h)		2.87±5.80 (14)	17.22±0.55 (14)	17.89±0.85 (14)	17.64±0.85 (14)	18.63±0.93 (14)
Gross parameters	Recovery phase operation					
	Day-wise	44-78 d				
MLSS (mg/L)		4695.67±282.67 (6)	1781.33±443.10 (6)	1610.08±270.91 (6)	1322.67±170.66 (6)	1284.33±166.08 (6)
MLVSS (mg/L)		4462.62±311.33 (6)	1664.09±467.79 (6)	1495.33±246.10 (6)	1193.67±178.66 (6)	1157.33±188.54 (6)
MLVSS/MLSS ratio		0.95±0.01 (6)	0.93±0.03 (6)	0.93±0.01 (6)	0.90±0.02 (6)	0.90±0.04 (6)
SVI (mL/g)		197.69±9.19 (6)	75.47±15.38 (6)	75.56±9.38 (6)	87.59±7.85 (6)	82.42±7.98 (6)
ISV (m/h)		0.11±0.08 (12)	16.44±0.67 (12)	17.31±0.80 (12)	17.71±0.64 (12)	18.31±0.46 (12)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

0.89, and 0.87 for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively, however the same is 0.96, 0.92, 0.93, and 0.94, respectively, towards the end of recovery phase operation. In recovery phase, faster aerobic sludge biomass growth in reactor R_{Cu10} and R_{Cu20} biomasses have been observed as compared to reactor R_{Cu40} and R_{Cu80} biomasses. The SVI have significantly decreased for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, due to appearance of granular biomass. [Mcdermott et al. \(1963\)](#) have reported that heavy metal reduces filaments population, which results in a lower SVI. The presence of Cu(II) in reactors has inhibited the growth of filamentous microorganisms under stressed phase operation. However, discontinuation of Cu(II) in feed has resulted in the appearance of granular biomass; hence the settling velocity has been observed to be similar to the stressed phase operation.

Typical settling test results for reactor R_{Cu0} and R_{Cu10} biomasses during 26-54 d of recovery phase operation have been shown in [Fig. 2.24](#). The settling behaviors of aerobic biomass in reactor R_{Cu0} has deteriorated over the time under recovery phase operation due to overgrowth of filaments biomass. However, the reactor R_{Cu10} biomass has shown no change in settling behavior during recovery phase as observed in stressed phase operation. Similar observations have obtained for other reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses. The results have showed that there has not been much change in settling velocities of the biomass after discontinuation of Cu(II) in the feed ([Fig. 2.25](#)). However, reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and

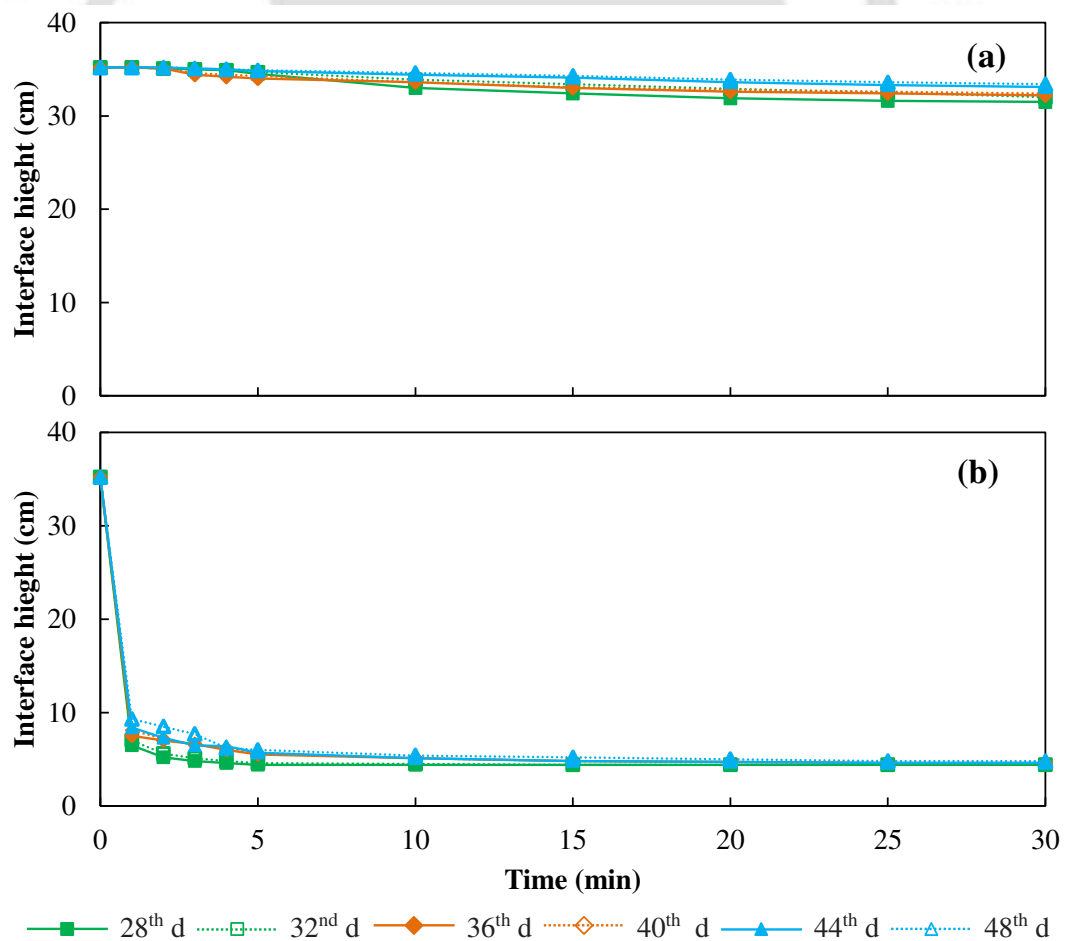


Fig. 2.24 Typical settling test results for reactor (a) R_{Cu0} and (b) R_{Cu10} biomasses during 26-54 d of recovery phase operation.

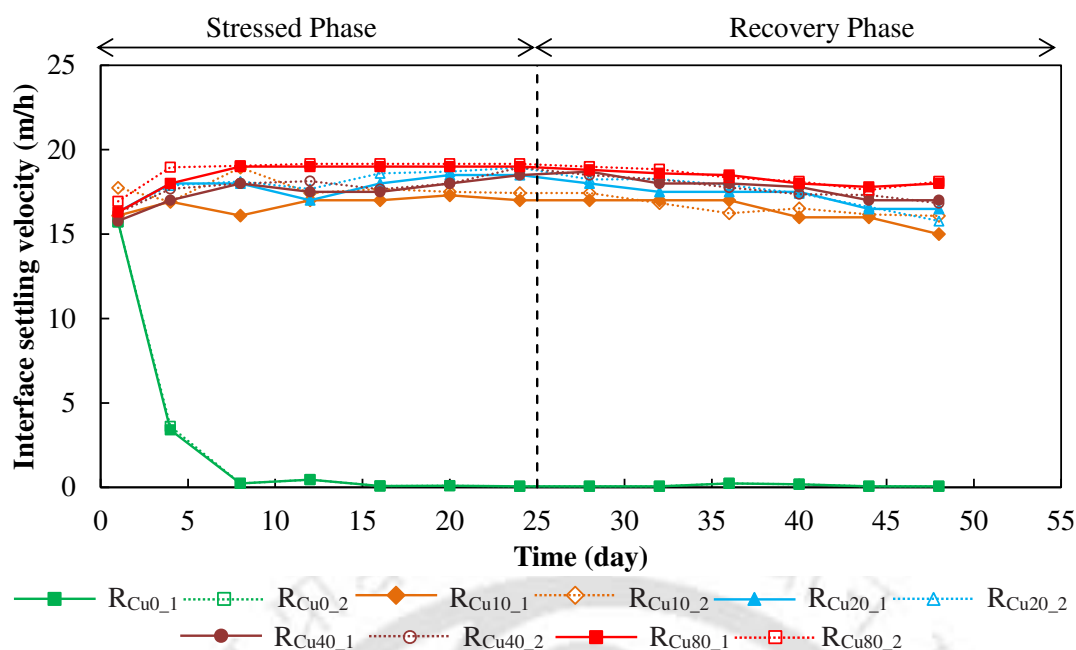


Fig. 2.25 Variation in interface settling velocities of reactor biomasses during 26-54 d under recovery phase operation along with data for stressed phase operation.

R_{Cu80} biomasses under stressed and recovery phases have shown higher settling velocities than reactor R_{Cu0} biomass due to reduction in filamentous microorganisms in the reactor. In this study, the reactors R_{Cu10} and R_{Cu20} have shown 80% and 68% COD removal along with an SVI of 75.4 and 75.5 mL/g and ISV 16.44 and 17.31 m/h, respectively, under recovery phase operation.

Aerobic sludge biomass activity test

The aerobic sludge biomass has been withdrawn from all the reactors at steady-state condition under the recovery phase operation (on 51st d), and ASBA tests have been performed. The results obtained under stressed phase from ASBA tests are shown in Fig. 2.26 and data is presented in Table 2.14. During the recovery phase operation, the aerobic sludge biomass has shown an increase in biomass activity. However, the activity values have been observed to be much lower compared to reactor R_{Cu0} biomass even towards the end of recovery phase, indicating the severe impact of Cu(II) on the aerobic sludge biomass. The ASBA values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been observed as 3.10, 2.40, 1.91, and 1.78 mg COD removed/mg VSS d, respectively, under recovery phase (Table 2.14). However, ASBA values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been observed as 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d, respectively, under stressed phase operation. The ASBA results of stressed and recovery phases have clearly indicated that reactor R_{Cu10} , R_{Cu20} , and R_{Cu40} biomasses have shown improved activity; however, reactor R_{Cu80} biomass has shown partial recovery for microbial activity. There is no literature available to support the recovery phase ASBA test. It can be observed from the result that reactor R_{Cu10} and R_{Cu20} biomasses have shown recovery, whereas reactor R_{Cu40} and R_{Cu80} biomasses have shown slight increment in ASBA values as compared to stressed phase. The aerobic sludge biomass has tried to recover from the stress caused by Cu(II). The high dose of Cu(II) concentration (40

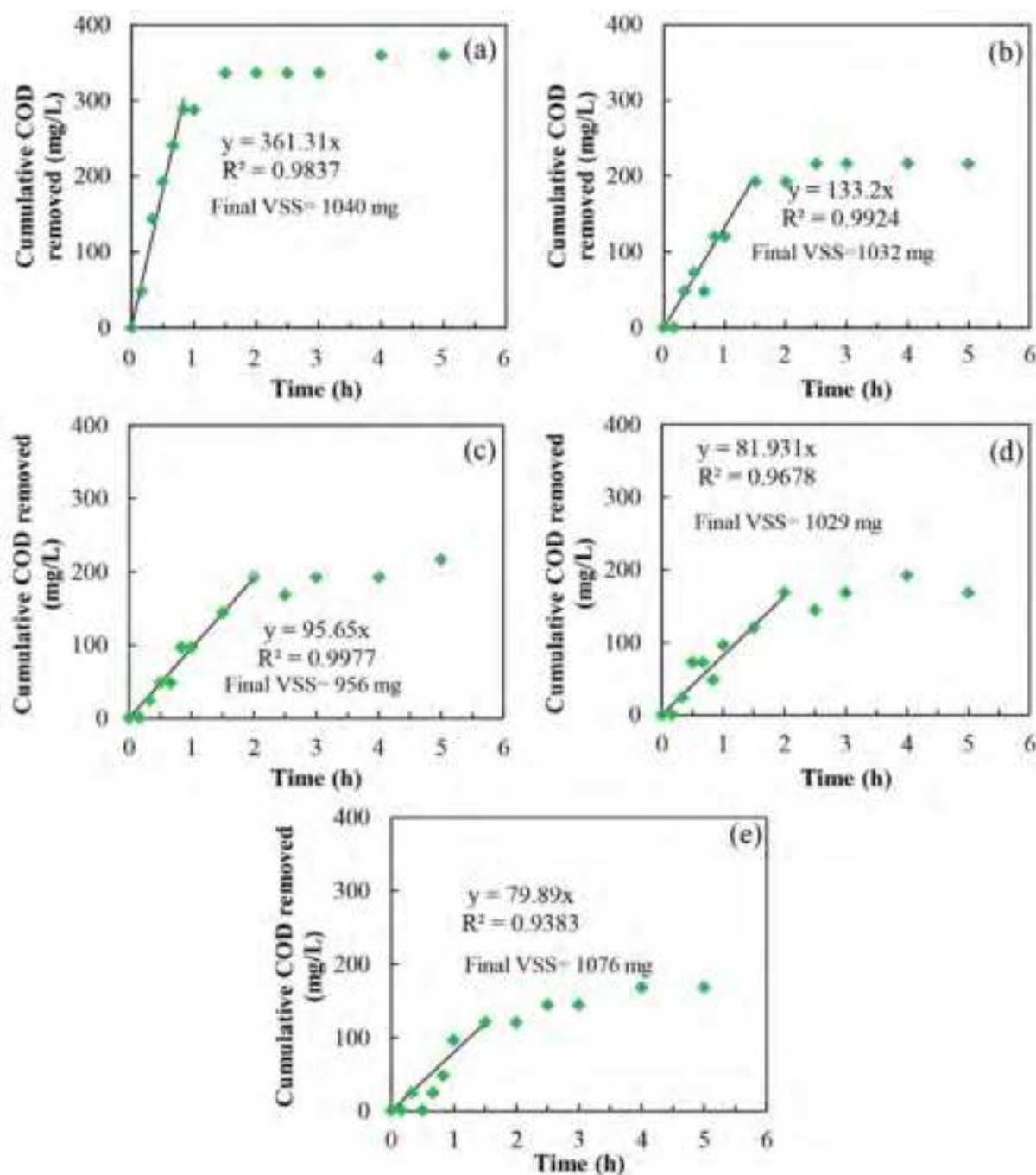


Fig. 2.26 Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactors (a) R_{Cu0} , (b) R_{Cu10} , (c) R_{Cu20} , (d) R_{Cu40} , and (e) R_{Cu80} on 51st d towards the end of recovery phase operation.

Table 2.14 Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.

Stressed phase operation					
Biomass withdrawn on			20 th d		
Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
ASBA (mg COD removed/mg VSS d)	7.00	2.28	1.89	1.55	1.43
Recovery phase operation					
Biomass withdrawn on			51 st d		
ASBA (mg COD removed/mg VSS d)	8.34	3.10	2.40	1.91	1.78

and 80 mg/L) has severely affected the aerobic sludge biomass activity; hence they have shown much slower recovery. The presence of Cu(II) in reactors have damaged the cellular structure and denatured the cellular enzymes of aerobic sludge biomass resulting in decreased ASBA. Microbial cells have been shown to repair their cellular machinery and enzymes during the recovery phase operation to degrade the organic matter. The recovery time can depend on the level of impact and Cu(II) dose. The ASBA test can be correlated with reactor performance. The reactors R_{Cu10} , R_{Cu20} , R_{Cu40} and R_{Cu80} have shown improved COD removal around 82.1%, 72.0%, 58.5%, and 41.3%, respectively, under steady-state condition of recovery phase. Similarly, ASBA values have also improved in comparison to the stressed phase. The increased Cu(II) concentration has severely affected the activity as well as the performance of the respective reactors. The ASBA test is quick and reliable to be used as a tool to measure food utilization rate and microbial activity of the aerobic sludge biomass in a system. The important operational decisions can be made based on test values if required.

Biomass related general parameters such as MLSS, MLVSS, SVI, ISV, and ASBA have been estimated during recovery phase operation. The MLSS and MLVSS results reflect that reactor R_{Cu10} and R_{Cu20} biomasses growth have slightly increased compared to reactor R_{Cu40} and R_{Cu80} biomass. The SVI for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have ranged between 75-87 mL/g, which is lower than the stressed phase SVI. As it has already been mentioned that an activated sludge that settles very rapidly (SVI of less than approximately 70 mL/g) can leave behind a turbid supernatant (Palm et al., 1980), which is undesirable for a treatment process. Even ISV values have also not been changed during recovery phase operation. The ASBA values for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been 8.34, 3.10, 2.40, 1.91, and 1.78 mg COD removed/mg VSS d, respectively, during the recovery phase. This means that the organic degrading capability of biomasses in reactor R_{Cu10} and R_{Cu20} have improved to a certain level, whereas reactor R_{Cu40} and R_{Cu80} biomasses have shown a slight improvement. Similarly, COD removal for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} are 82%, 72%, 59%, and 41%, whereas reactor R_{Cu0} has shown 91% of COD removal during the steady-state condition of stressed phase operation. The control reactor R_{Cu0} and other reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have ASBA values that are significantly different, which reflect that ASBA values are an indicator of active aerobic sludge biomass.

2.3.2.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology

During stressed phase operation, the filaments have decreased gradually over the time for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses (from 5th to 25th d) due to Cu(II) in feed (Table 2.15). Considering reactor R_{Cu10} biomass, the reappearance of filaments have been observed from 35th d and it has gradually increased over the time (clearly visible at 100× magnification). The microscopic images of Gram's stained samples taken at 5× magnification have shown the flocs morphology during the operation. Similar observations have been obtained for other reactor R_{Cu20} and R_{Cu40} biomasses. However, the reappearance of filaments

is slower for reactor R_{Cu80} biomass and filaments density is very less as compared to other reactors. The possible reason could be post-effect of Cu(II) stress that has prevented the filamentous growth after Cu(II) discontinuation. After discontinuation of Cu(II), filaments have reappeared in reactor R_{Cu10} and R_{Cu20} biomasses with granular shape of biomass, while sharp granular biomasses have been observed for reactor R_{Cu40} and R_{Cu80} .

The photographic images of aerobic sludge biomass taken during recovery phase operation along with stressed phase images have been presented in Table 2.16. Considering reactor R_{Cu10} biomass during recovery phase operation (from 35th to 50th d), the flocs structure

Table 2.15 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} in recovery phase operation along with microscopic images for stressed phase operation.

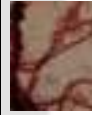
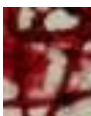
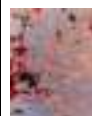
Reactor	Mag.	Aerobic sludge biomass samples withdrawn on							
		Stressed phase				Recovery phase			
		5 th d	10 th d	20 th d	25 th d	30 th d	35 th d	40 th d	50 th d
R_{Cu0}	5×								
	100×								
R_{Cu10}	5×								
	100×								
R_{Cu20}	5×								
	100×								
R_{Cu40}	5×								
	100×								
R_{Cu80}	5×								
	100×								

Table 2.16 Photographic images of aerobic sludge biomasses withdrawn from reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} in recovery phase operation along with images for stressed phase operation.

Sample withdrawn on	Aerobic sludge biomass withdrawn from reactors				
	R_{Cu0}	R_{Cu10}	R_{Cu20}	R_{Cu40}	R_{Cu80}
Stressed Phase	1 st d				
	5 th d				
	10 th d				
	20 th d				
	25 th d				
Recovery Phase	30 th d				
	35 th d				
	40 th d				
	50 th d				

have appeared to change from pin-point to granular shape, and flocs size appear to increase in the range of 3-6 mm. Similar observations have been obtained for reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses.

The discontinuation of Cu(II) in feed has resulted in increase in FI value during recovery phase operation for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses. FI Value for reactor R_{Cu0} biomass has remained the same as stressed phase, however FI value has reached 5 towards the end of the recovery phase operation for reactor R_{Cu10} biomass due to reappearance of filamentous microorganism as presented in Table 2.17. Similarly, the FI value for reactor R_{Cu20} biomass has increased from 1 to 4 at the end of the recovery phase. The FI values for reactor R_{Cu40} and R_{Cu80} biomasses have increased to 4 and 3, respectively, toward the end of the recovery phase. Tables 2.15, 2.16, and 2.17 have shown the day-wise morphology of aerobic sludge biomass, reappearance of filamentous microorganism and filament index of the reactor biomasses.

Table 2.17 Filament index of aerobic sludge biomass estimated during recovery phase operation along with data for stressed phase operation.

Biomass withdrawn on	Stressed phase operation				
Day	R_{Cu0}	R_{Cu10}	R_{Cu20}	R_{Cu40}	R_{Cu80}
1 st d	5	5	5	5	5
5 th d	5	5	2	5	1
10 th d	5	5	3	1	1
20 th d	5	2	1	1	1
Recovery phase operation					
25 th d	5	2	1	1	1
30 th d	5	3	3	2	1
35 th d	5	4	3	2	1
40 th d	5	5	4	3	2
45 th d	5	5	4	3	2
50 th d	5	5	4	4	3

Enzyme activity

$E_{Catalase}$, $E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease} values for all the reactors during recovery phase operation have been shown in Fig. 2.27 and data have been summarized in Table 2.18. $E_{Catalase}$ for reactor R_{Cu10} and R_{Cu20} biomasses have shown little improvement (from 0.09 to 0.13 mg/g MLSS h for reactor R_{Cu10} biomass and from 0.05 to 0.07 mg/g MLSS h for reactor R_{Cu20} biomass) in recovery phase operation compared with stressed phase operation [Fig. 2.27(a)]. However, reactor R_{Cu40} and R_{Cu80} biomasses have shown slight improvement in $E_{Catalase}$ from 0.01 to 0.03 mg/g MLSS h. However, reactor R_{Cu40} and R_{Cu80} biomasses have not shown recovery in $E_{Catalase}$ in the recovery phase operation as compared to reactor R_{Cu0} biomass.

$E_{Dehydrogenase}$ values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses in stressed phase have been 17.34, 14.05, 8.75, and 4.02 μ g/g MLSS h, respectively. The reactor R_{Cu10} , R_{Cu20} , and R_{Cu40} biomasses have shown recovery in $E_{Dehydrogenase}$ as compared to reactor R_{Cu0} biomass and values obtained are 23.01, 19.17 and 13.78 μ g/g MLSS h, respectively [as

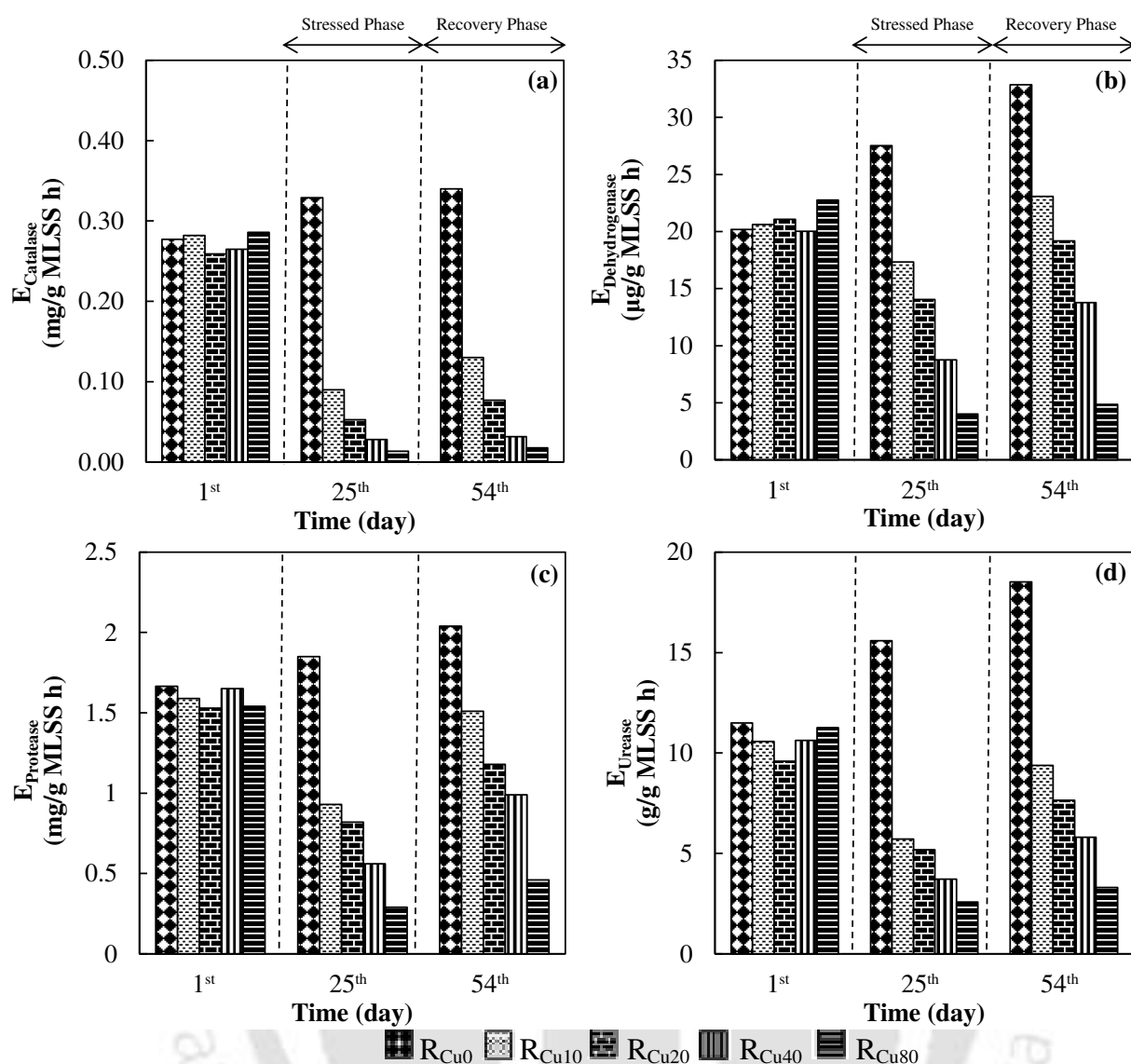


Fig. 2.27 Enzyme activities of aerobic sludge biomass assessed during recovery phase operation (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} along with data for stressed phase operation.

shown in Fig. 2.27(b)], while reactor R_{Cu80} biomass (4.85 µg/g MLSS h) have not shown improvement under recovery phase operation (Table 2.18). The results have suggested that the reactors fed with lower Cu(II) doses namely reactors R_{Cu10}, R_{Cu20}, and R_{Cu40} biomasses during stressed phase operation could recover under recovery phase, however, high Cu(II) doses (80 mg/L) have caused irreversible changes in $E_{\text{Dehydrogenase}}$ that could not recover during recovery phase operation.

After discontinuation of Cu(II) in feed, there has been a slight increase in E_{Protease} as compared to stressed phase operation. The summary of E_{Protease} for all the reactor biomasses during recovery phase operation are given in Table 2.18 and are graphically shown in Fig. 2.27(c). E_{Protease} has been obtained as 2.04, 1.50, 1.18, 0.98, and 0.45 mg/g MLSS h for reactor R_{Cu0}, R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses, respectively, towards the end of the recovery phase operation. The reactor R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses have had lower E_{Protease} values of 0.93, 0.82, 0.56, and 0.29 mg/g MLSS h, respectively, during stressed

Table 2.18 Summary of enzyme activities of aerobic sludge biomass assessed during recovery phase operation along with data for stressed phase operation.

Biomass withdrawn on	Reactor biomass	E _{Catalase} (mg/g MLSS h)	E _{Dehydrogenase} (μg/g MLSS h)	E _{Protease} (mg/g MLSS h)	E _{Urease} (g/g MLSS h)
Stressed phase operation					
1st d	R_{Cu0}	0.28±0.03	20.19±1.76	1.67±0.07	11.50±1.13
	R_{Cu10}	0.28±0.03	20.61±1.76	1.59±0.04	10.57±2.12
	R_{Cu20}	0.26±0.04	21.08±1.82	1.53±0.07	9.59±0.95
	R_{Cu40}	0.27±0.02	20.02±1.92	1.65±0.09	10.63±0.95
	R_{Cu80}	0.29±0.04	22.77±2.15	1.54±0.05	11.26±1.32
25th d	R_{Cu0}	0.33±0.12	27.52±1.85	1.85±0.05	15.59±2.15
	R_{Cu10}	0.09±0.01	17.34±2.54	0.93±0.07	5.71±0.83
	R_{Cu20}	0.05±0.01	14.05±1.41	0.82±0.04	5.19±0.97
	R_{Cu40}	0.03±0.01	8.75±1.28	0.56±0.07	3.72±0.68
	R_{Cu80}	0.01±0.01	4.02±0.87	0.29±0.05	2.58±0.74
Recovery phase operation					
54th d	R_{Cu0}	0.34±0.08	32.87±2.85	2.04±0.04	18.52±3.14
	R_{Cu10}	0.13±0.05	23.08±3.58	1.51±0.07	9.38±1.57
	R_{Cu20}	0.08±0.02	19.18±1.86	1.18±0.06	7.65±1.61
	R_{Cu40}	0.03±0.01	13.78±2.14	0.99±0.03	5.80±0.83
	R_{Cu80}	0.02±0.01	4.85±1.07	0.46±0.01	3.30±0.97

[The data is presented in xx±yy format where xx is the average value and yy is the standard deviation.]

phase operation. The differences in stressed and recovery phase values clearly suggest that reactors have shown improvement in E_{Protease}. The biomass stressed with lower Cu(II) doses of 10, 20, and 40 mg/L have shown capability to regain its E_{Protease} under recovery phase operation, however the recovery rate is much slower for the biomass stressed to high Cu(II) dose of 80 mg/L.

The summary of E_{Urease} for all the reactor biomasses during recovery phase operation is given in Table 2.18 and are graphically shown in Fig. 2.27(d). E_{Urease} has been obtained as 18.51, 9.37, 7.64, 5.80, and 3.29 g/g MLSS h for reactor R_{Cu0}, R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses, respectively, towards the end of the recovery phase operation. E_{Urease} has had lower values of 5.71, 5.19, 3.72, and 2.58 g/g MLSS h for reactor R_{Cu0}, R_{Cu10}, R_{Cu20}, R_{Cu40}, and R_{Cu80} biomasses, respectively, under the stressed phase. The recovery phase operation have shown increased E_{Urease} values for reactor R_{Cu10}, R_{Cu20}, and R_{Cu40} biomasses; however, partial recovery has been observed for reactor R_{Cu80} biomass. The enzyme activity results have suggested that E_{Catalase} and E_{Dehydrogenase} could be recovered if biomass have been stressed to lower doses of Cu(II), i.e., 10, 20, and 40 mg/L, whereas, at higher Cu(II) concentration (80 mg/L), the recovery of E_{Catalase} and E_{Dehydrogenase} might be too slow. In case of E_{Protease} and E_{Urease}, biomass can significantly recover from the adverse impact of lower doses of Cu(II) (10, 20, and 40 mg/L), however, recovery for higher Cu(II) dose (80 mg/L) still can't be achieved. During the recovery phase, Cu(II) remains inside the cell bonded with the active site of the enzyme. This may be the possible reason for no recovery in enzyme activities.

Amplified ribosomal DNA restriction analysis (ARDRA)

The restriction enzymes *AluI* and *MspI* have produced a differential pattern of fragments for all the aerobic sludge biomass samples (withdrawn towards end of the recovery phase operations) as shown in Fig. 2.28. The similarity matrix for all recovery phase aerobic sludge biomass samples digested with *AluI* are presented in Table 2.19. The dendrogram generated with the digestion of *AluI* [shown in Fig. 2.28(b)] and *MspI* [shown in Fig. 2.28(d)] restriction enzyme during recovery phase have suggested the relationship between reactor R_{Cu0} and other reactor biomasses. It clearly shows that reactor R_{Cu0} biomass has much similarity to reactor R_{Cu10} biomass and similarity has decreased for the biomass stressed to higher doses of Cu(II) during stressed phase operation. The reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown similarity of 85.9%, 79.8%, 76.2%, and 61.4%, respectively, under recovery phase operation, as compared to reactor R_{Cu0} biomass, which is significantly higher than the similarity values obtained in stressed phase of 71.4%, 64.7%, 56.8%, and 48.1% for reactor

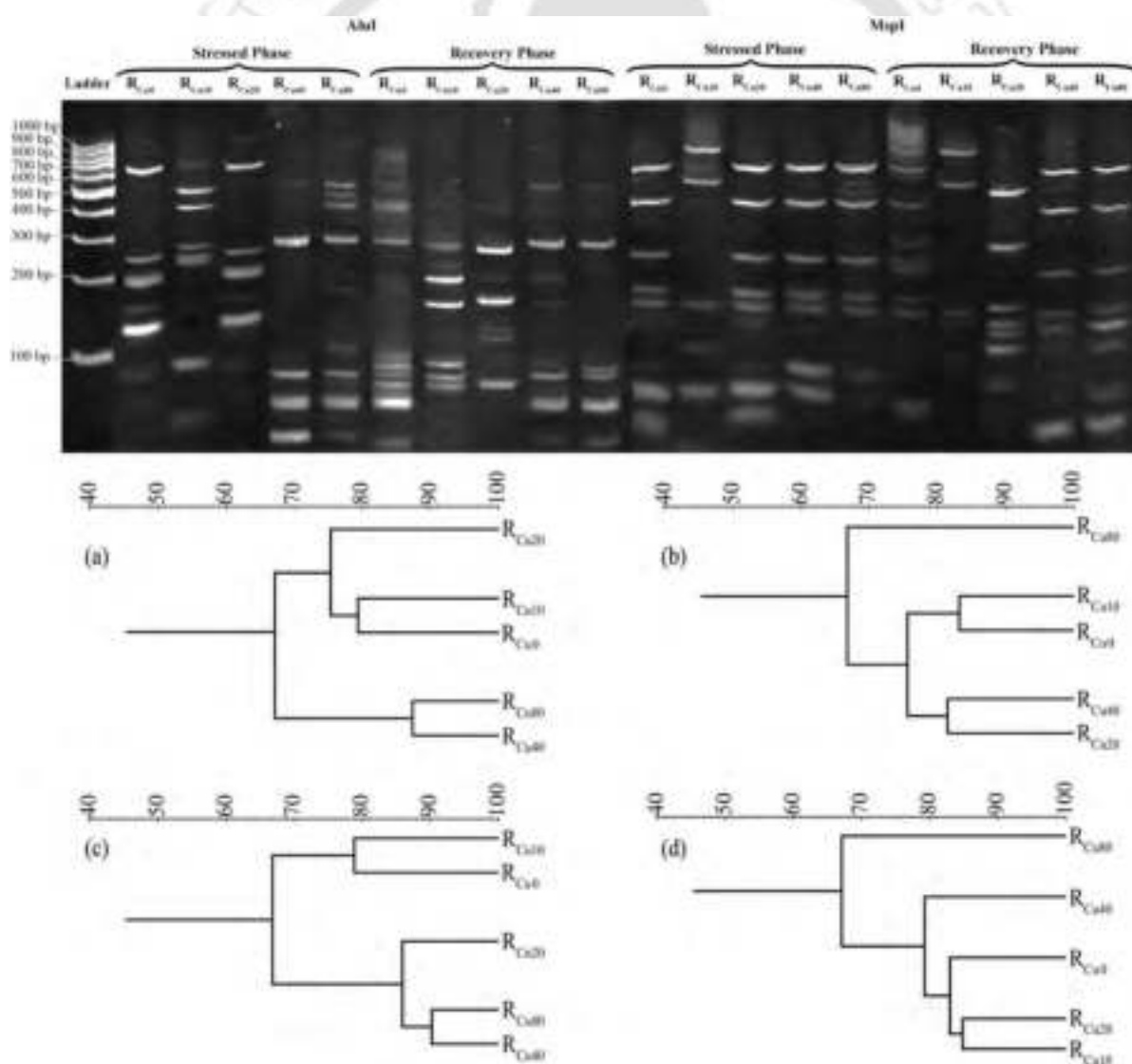


Fig. 2.28 Digestion pattern and dendrogram of aerobic sludge biomass treated with restriction enzyme *AluI*: (a) under stressed phase and (b) under recovery phase; and *MspI*: (c) under stressed phase (d) under recovery phase.

Table 2.19 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase operation.

Stressed phase operation										
Reactor	<i>AluI</i>					<i>MspI</i>				
	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
R _{Cu0}	1	0.7142	0.6471	0.5682	0.4818	1	0.6584	0.5156	0.4287	0.3797
R _{Cu10}	0.7142	1	0.6715	0.5449	0.4987	0.6585	1	0.5783	0.4972	0.4142
R _{Cu20}	0.6471	0.6715	1	0.6486	0.5544	0.5157	0.5781	1	0.8141	0.7264
R _{Cu40}	0.5682	0.5449	0.6486	1	0.8210	0.4284	0.4974	0.8141	1	0.8378
R _{Cu80}	0.4818	0.4987	0.5544	0.8210	1	0.3797	0.4142	0.7264	0.8378	1
Recovery phase operation										
Reactor	<i>AluI</i>					<i>MspI</i>				
	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
R _{Cu0}	1	0.8594	0.7985	0.7624	0.6144	1	0.8357	0.8214	0.7485	0.6421
R _{Cu10}	0.8594	1	0.8341	0.7984	0.7051	0.8357	1	0.8471	0.8129	0.6841
R _{Cu20}	0.7985	0.8341	1	0.8459	0.7585	0.8214	0.8471	1	0.8154	0.7485
R _{Cu40}	0.7624	0.7984	0.8459	1	0.8247	0.7485	0.8129	0.8154	1	0.6214
R _{Cu80}	0.6144	0.7051	0.7585	0.8247	1	0.6421	0.6841	0.7485	0.6214	1

R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. On the other hand, reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown similarity of 83.5%, 82.1%, 74.8%, and 64.2%, respectively, when biomass has been digested with *MspI*, under recovery phase operation. Whereas, lower similarities have been observed in stressed phase for reactors R_{Cu10} (65.8%), R_{Cu20} (51.6%), R_{Cu40} (42.9%), and R_{Cu80} (38.0%) biomasses. The increase in similarity values have suggested that aerobic sludge biomass of different reactors have tried to regain microbial communities under recovery phase operation. The biomass stressed to lower doses of Cu(II) during stressed phase have shown more efforts in recovering the microbial community structure during recovery phase. Whereas, aerobic sludge biomass stressed to 80 mg/L (during stressed phase) could partially be recovered in terms of microbial community structure during recovery phase.

The microscopic examination has shown the reappearance of filaments in reactor R_{Cu10} and R_{Cu20} biomasses from 35th d, while sharp granular biomass has been observed for reactors R_{Cu40} and R_{Cu80} biomasses. Similarly, reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have appeared to change from pinpoint to granular shape, and flocs size seems to increase in the range of 3.0-6.0 mm gradually increased over time. The FI values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have risen from 2, 1, 1, and 1 (on 25th d) to 5, 4, 4, and 3 (on 50th d), respectively. The FI has indicated the reappearance of filamentous biomass in reactors. Hence, flocs morphology and FI are the techniques that may clearly distinguish the difference between stressed phase and recovery phase aerobic sludge biomass morphology. Considering enzyme activities values during recovery phase operation, a little bit of improvement in all four enzymes has been observed for reactor R_{Cu10} and R_{Cu20} biomasses compared to stressed phase operation. However, reactor R_{Cu40} and R_{Cu80} biomasses have shown minor improvement during the recovery phase. The recovery phase results have suggested that enzyme activity may be recovered, but it may take a longer time. In this situation, enzyme activity test may not be suitable for assessing the quality of aerobic sludge biomass during recovery phase operation. The recovery phase microbial community changes have been reflected by ARDRA, which has shown increased similarity compared to stressed phase data. *AluI* has suggested 61.4-85.9% similarity, whereas *MspI* has revealed 64.2-83.5% similarity with control reactor and Cu(II) fed reactors. The aerobic sludge biomass has tried to regain microbial community.

2.4 Summary

The stressed phase operation has revealed that the addition of Cu(II) in feed has severely affected the reactor performance, and its impacts have increased with the hike in Cu(II) doses. The COD removal for Cu(II) fed reactors have deteriorated due to the presence of Cu(II), and it has been drastically inhibited by 80 mg/L of Cu(II) dose. The reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 91%, 75%, 62%, 49%, and 39% of COD removal, respectively, during steady-state conditions of stressed phase operation. The Cu(II) retention by aerobic sludge biomass of reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 42%, 26%, 10%, and 3%, respectively, at steady-state conditions. In addition, the occurrence

of Cu(II) in feed majorly affected the SVI and ISV values for aerobic sludge biomass. The reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown SVI values of 195, 86, 87, 98, and 84 mL/g; and ISV values of 2.87, 17.22, 17.89, 17.64, and 18.63 m/h, respectively. The organic degradation capability has also declined due to Cu(II), which has been reflected by ASBA values of 7.00, 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. The morphological studies have showed that lower Cu(II) doses have inhibited the filamentous growth and formed granular shaped biomass, whereas high concentrations (80 mg/L) of Cu(II) have caused complete flocs disintegration. Moreover, filament index values have been significantly affected due to Cu(II) stress, and 5, 2, 1, 1, and 1 of FI values have been observed for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively, towards the end of the stressed phase. The enzyme activity values have decreased with an increase in Cu(II) concentration. The enzyme activity values of Cu(II) fed reactors have ranged between 0.01-0.09 mg/g MLSS h for $E_{Catalase}$; 4.02-17.34 μ g/g MLSS h for $E_{Dehydrogenase}$; 0.29-0.93 mg/g MLSS h for $E_{Protease}$; and 2.58-5.71 g/g MLSS h for E_{Urease} . Lower doses of Cu(II) (10 and 20 mg/L) have the capability to change the microbial community structure (51-71% similarity with control reactor), while higher doses have revealed 38-43% similarity with control reactor in microbial community structure due to Cu(II) stress.

When the recovery phase has started after 25 d, reactors R_{Cu10} and R_{Cu20} have shown improvement in COD removals; however, no recovery symptoms have been observed for the high dose of Cu(II) for biomass of reactors R_{Cu40} and R_{Cu80} . The reactors R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 91%, 82%, 72%, 59%, and 41% of COD removal, respectively, during the steady-state condition of recovery phase operation. A low SVI and high ISV value with good COD removal have been obtained for reactor R_{Cu10} and R_{Cu20} biomasses. The reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown SVI values of 198, 75, 76, 88, and 82 mL/g; and ISV values of 0.11, 16.44, 17.31, 17.71, and 18.31 m/h, respectively. The reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have changed from pinpoint to granular shape, and flocs size increased in the range of 3.0-6.0 mm. The FI values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have increased from 2, 1, 1, and 1 (on 25th d) to 5, 4, 4, and 3 (on 50th d), respectively. The ASBA values have recovered for reactor R_{Cu10} and R_{Cu20} biomasses, whereas a slow recovery has been observed for reactor R_{Cu40} and R_{Cu80} biomasses. ASBA values of 8.34, 3.10, 2.40, 1.91, and 1.78 mg COD removed/mg VSS d have been obtained for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. All four enzymes have shown recovery for reactor R_{Cu10} and R_{Cu20} biomasses, whereas $E_{Catalase}$ and E_{Urease} have not recovered for reactor R_{Cu40} and R_{Cu80} biomasses. The reactors R_{Cu10} and R_{Cu20} biomasses have shown recovery in microbial community structure. On the other hand, reactor R_{Cu40} and R_{Cu80} biomasses have shown slow and partial recovery in the microbial community. The conclusions can be drawn from the experiment that stress caused by Cu(II) have resulted in incomplete recovery of biomass and Cu(II) at 40 and 80 mg/L have caused a severe impact on reactor performance and biomass morphology and imparting irreversible

changes. The biomass evaluation techniques such as COD, microscopic and photographic examination, SVI, ISV, and ASBA have been used to demonstrate the differentiation between stressed and recovery phase conditions of the reactor biomasses.

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Effects of Antibiotic (Amoxicillin IV) Stress on Aerobic Sludge Biomass

Urban wastewater treatment plants (UWTPs) have a pivotal role in protecting the environment, particularly the natural water bodies. The removal of organic matter and other pollutants from urban sewage, using combinations of physico-chemical and biological treatments, have been a major technological achievement of the last century, allowing the discharge of treated wastewater with good quality to the environment ([Manaia et al., 2018](#)). Nowadays, extensive use of pharmaceutical products has increased their occurrence in sewage that ends up in the sewage treatment plant. Hence, this chapter discusses the effect of antibiotics on aerobic sludge biomass morphology, behavior, and their activities. It briefly describes the recovery capability of aerobic sludge biomass exposed to antibiotic after its discontinuation.

3.1 Background

Antibiotics are organic substances produced through secondary metabolism of living microorganisms or synthesized chemically that can kill other microorganisms or inhibit their growth or metabolic activity via biochemical actions ([Du and Liu, 2012](#); [Oliveira et al., 2013](#)). Nowadays, a wide variety of antibiotics are extensively applied worldwide as drugs for preventing or treating human, animal, and plant infections, or as feed additives for animals and fishes to prevent or treat diseases as well as growth promoter. Rampant use of antibiotics in hospitals, livestock, poultry, aquaculture, and animal farms, and pharmaceutical industries discharge a huge amount of antibiotics in environment ([Iranzo et al., 2018](#); [Li and Zhang, 2010](#); [Sivagami et al., 2020](#)). The human or animal body consumes only 10-20% of antibiotics, and the residual amount (80-90%) is excreted with urine and feces ([Gothwal and Shashidhar, 2015](#); [Githinji et al., 2011](#)). Excreted antibiotics enter wastewater treatment plants (WTPs), disrupting sludge microorganisms and lowering treatment efficiency ([Balakrishna et al., 2017](#); [Kümmerer, 2008](#); [Tang et al., 2017](#); [Sivagami et al., 2020](#); [Zhang et al., 2017](#)).

Several researchers have reported the impact of different antibiotics on the performance of various biological reactors. [Meng et al. \(2017\)](#) have investigated the long-term effect of cefalexin (CFX) on the performance and microbial community structure of an expanded granular sludge bed (EGSB) system. Moreover, the concentration of CFX in influent have been kept around 200 mg/L through successive phases to observe the long-term effect of CFX on the performance of EGSB for 224 d. As a result, the addition of CFX to the reactor has affected the COD removal efficiency, and the reactor has shown to recover after 122 d of operation. In another study, [Guo et al. \(2015\)](#) have investigated the amoxicillin (AMX) removal efficiency by three processes: (i) Fenton process, (ii) activated sludge process, and (iii) combined Fenton-activated sludge system. The removal of AMX has been achieved up to 80% in 70 min. Similarly, when AMX concentration has been less than 350 mg/L, 69.0-88.8% of the antibiotic has been removed. Considerable attention has also been given to the sludge biomass characteristics and characterization of its components. Recently, [Yu et al. \(2019\)](#) have studied the performance, nitrogen removal rate, extracellular polymeric substances (EPS), microbial community, and enzymatic activity of activated sludge in a sequencing batch reactor (SBR) under ampicillin stress and observed that 0-30 mg/L of ampicillin has shown no detrimental impact on the organic matter and nitrogen removals.

The biological process uses a diverse population of microorganisms to transform organic matter from the wastewater. The bio-transformation of organic matter depends on enzymes produced by activated sludge. The microbial cells also agglomerate themselves to form flocs, containing a variety of intracellular and extracellular enzymes. Settling is also a critical parameter in assessing the treatment plant performance, significantly influenced by the morphology of activated sludge flocs. The floc morphology covers the effectiveness of biomass separation and quality of treated effluent ([Jin et al., 2003](#); [Liwarska-Bizukojc et al., 2015](#)). Therefore, it is desired to study enzyme activities and floc morphology of activated sludge in-depth when exposed to antibiotics.

A large volume of published studies describes the occurrences and concentrations of antibiotics in influent, effluent, and sludge of treatment plants. The literature has been occupied with reactor performance and pollutant removal efficiency, but a few investigations have focused on the behavior and morphology of aerobic sludge biomass exposed to antibiotics. Most of the literature describe the fate and elimination of different antibiotics from wastewater at sewage treatment plants. Literature have also revealed the impact of AMX on the performance of the activated sludge process; however, limited attention has been paid to relate the reactor performance with the sludge morphology in presence of antibiotics in influent. On the other hand, it is also essential to estimate the recovery capabilities of aerobic sludge biomass when antibiotics is discontinued in the influent.

Therefore, the primary aims of this study are (i) to assess the impact of amoxicillin (AMX) on reactor performance and morphology of aerobic sludge biomass and (ii) to evaluate aerobic sludge biomass recovery capabilities when AMX is discontinued in the feed. This work focuses on the morphology and behavior of aerobic sludge biomass, including the enzymes

and biomass activity. In addition, the probability of changes in microbial community and extracellular polymeric substance (EPS) due to AMX is also estimated.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

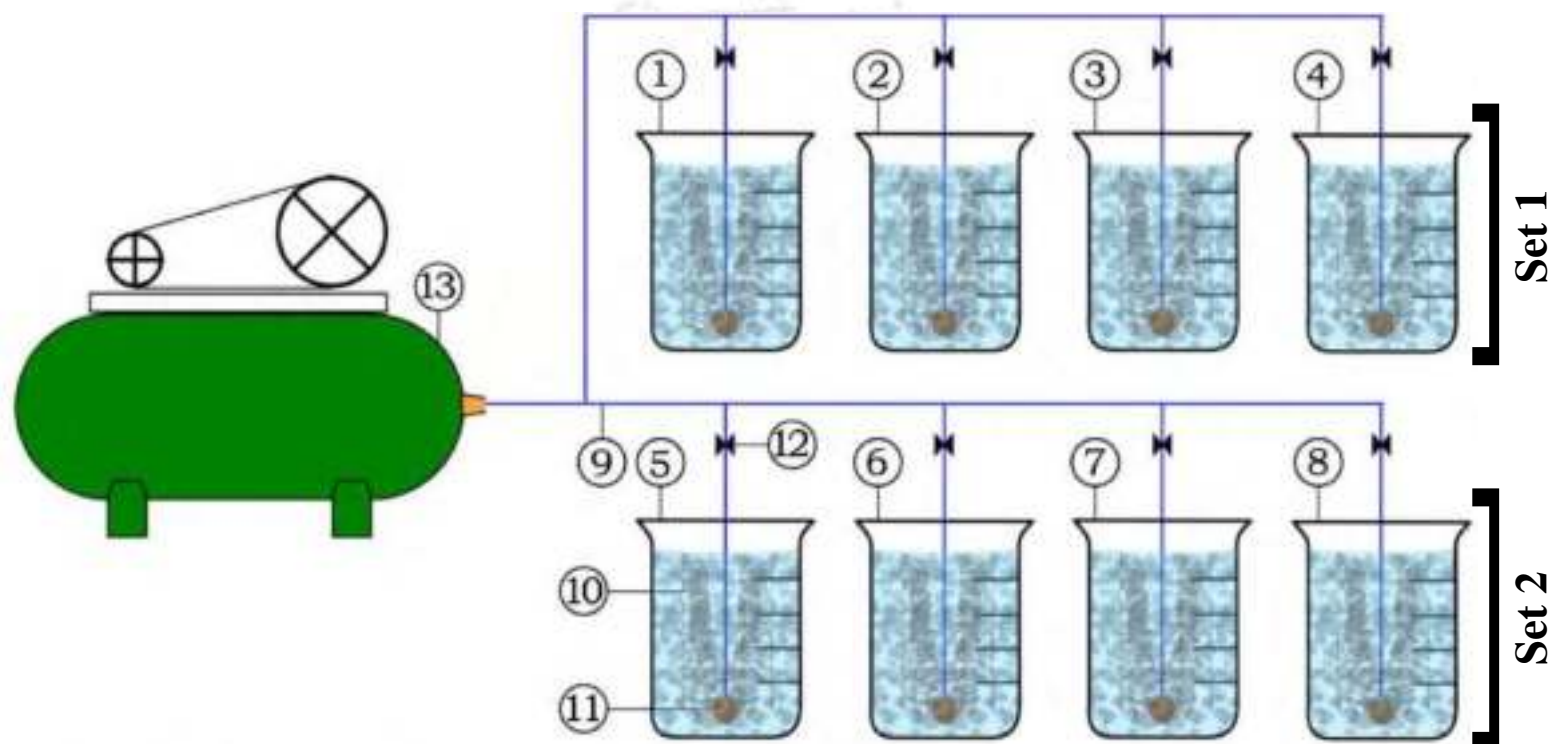
All chemicals and reagents used in this study are of analytical or HPLC grade, manufactured by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals is presented in *Appendix-I* (Table A-I.1). The selection of antibiotics for experiment has been briefly described in *Appendix-III*. Hipen-Clav intravenous (IV) manufactured by Zydus Cadila, India has been purchased from a local pharmacy shop in Guwahati, Assam and used as source of AMX. A vial of Hipen-Clav (1.2 g) contains amoxicillin (1000 mg) and potassium clavulanate (200 mg).

3.2.2 Aerobic sludge biomass

The aerobic sludge biomass, withdrawn from the mother reactor (MR), has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in detail in *Appendix-II*.

3.2.3 Reactor setup and operation

The experimental set-up consists of two sets of four identical reactors (height 14.5 cm×diameter 12.5 cm) with a working volume of 1 L (Fig. 3.1). The reactors have been designated as R_{amx0_1} and R_{amx0_2} (control reactors with no AMX in the feed), R_{amx500_1} and R_{amx500_2} (500 mg/L AMX in the feed), $R_{amx1000\_1}$ and $R_{amx1000\_2}$ (1000 mg/L AMX in the feed), $R_{amx2000\_1}$ and $R_{amx2000\_2}$ (2000 mg/L AMX in the feed). The reactors have been fed with AMX along with Lactogen 3 as a source of carbon and nutrients. The composition of Lactogen 3 powder is presented in Table A-II.2. The reactors have been operated at ambient conditions without temperature and pH control for two cycles per day with a fill time of 10 min, a react time of 665 min, a settling time of 10 min, a decant time of 15 min and a wash time (two times) of 20 min (as shown in Fig. 3.2). To prevent the progressive build-up of increasing AMX concentrations (residuals from previous feedings and fresh addition in the subsequent feeding) and subsequent exposure of biomass to more than the planned AMX load, the retained settled mixed liquor in the reactor after decantation is washed twice with tap water before starting the next feeding. The washing is carried out by refilling the reactors with tap water up to 1 L volume and followed by gentle mixing the content for 1-2 min. After that, the content is allowed to settle for a period of 5 min before the supernatant is decanted. This process has been repeated two times. After washing, the reactor is again filled with tap water up to a 1 L volume, and the clear liquid is sampled for the estimation of background (or residual) AMX concentration left after the washing— designated as ' BG_{amx} '. The loss in the liquid volume created due to the withdrawal of the sample has to be make up by the new feed. The settling time has been kept short (10 min) in order to decant dispersed and slow-settling flocs. Each reactor has been initially filled with ~570 mL of the mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~3000 mg/L] withdrawn from mother



1. R_{amx0_1} 2. R_{amx500_1} 3. $R_{amx1000\_1}$ 4. $R_{amx2000\_1}$ 5. R_{amx0_2} 6. R_{amx500_2} 7. $R_{amx1000\_2}$ 8. $R_{amx2000\_2}$
 9. Flexible PVC pipe 10. Aerobic sludge biomass 11. Diffused aeration 12. Air valve 13. Air compressor

Fig. 3.1 Schematic diagram of experimental set-up.

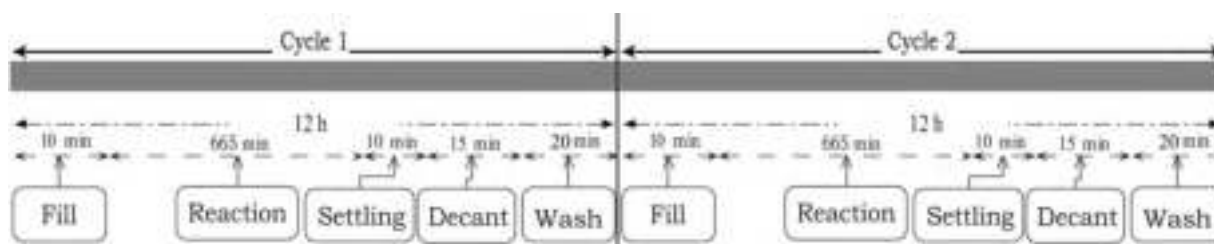


Fig. 3.2 Schematic representation of cycle time for reactors.

reactor (MR). The synthetic feed has been prepared using Lactogen 3 (5.50 g for reactor $R_{\text{amx}0}$, 4.25 g for reactor $R_{\text{amx}500}$, 3.00 g for reactor $R_{\text{amx}1000}$, and 0.50 g for reactor $R_{\text{amx}2000}$) and lukewarm water (~ 100 mL) followed by proper mixing using magnetic stirrer. Hipen-Clav has been used as the source of amoxicillin (AMX) in the feed. The AMX solution has been freshly prepared by injecting 10 mL milli-Q water in to Hipen-Clav vial, which contained 1000 mg of powdered AMX. Then vial has been shaken by hand for proper mixing and AMX solution has then aspirated using 10 mL syringe. Total 7 vials of Hipen-Clav have been used to feed the reactors in one cycle. The reactors $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been fed with 0, 5, 10, and 20 mL of AMX solution and 5.50, 4.25, 3.00, and 0.50 g of feed suspended in 100 mL, respectively, in each cycle. After addition of AMX solution, the final volume of the reactors have been made to 1 L with tap water. The COD provided by Hipen-Clav has been estimated in this study by dissolving 12 mg of powered AMX (Hipen-Clav) into 10 mL of milli-Q water. The COD of the prepared solution has been determined by Standard Methods (APHA et al., 2005). The results suggest that 1 mg/L Hipen-Clav gives COD of 2.51 mg/L. Hence, feed (different amount of Lactogen 3 and AMX) gives a COD concentration of 5500-5600 mg/L in each reactor. For keeping the biomass in suspension and imparting mixing, air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffuser.

The 1st cycle has been started around 9.30 am in the morning and initial room temperature has been record at that time, while room temperature during decanting has been recorded at 8.45 pm in the night and then 2nd cycle started around 9.30 pm. Since, the reactors have been fed with AMX solution which is likely to cause stress on aerobic sludge biomass, hence the operation of these reactors have been termed as *stressed phase operation*. The stressed phase operation has lasted for 43 d in which the steady-state condition with respect to COD removal (%) has been achieved after 30-35 d of operation from the start and steady-state condition data obtained till 43rd d of operation. Thereafter, the reactors have been operated without AMX in the feed for the next 35 d. This phase of operation has been termed as *recovery phase operation*. The steady-state condition has been achieved after 17-20 d of operation from the start of the recovery phase with respect to COD removal (%).

The initial liquid samples have been withdrawn after the fill stage (proper mixing the feed and AMX solution ensured), and effluent samples have been collected during the decant step at the end of a cycle. During a settling time of 10 min, the settled mixed liquor (sludge biomass + liquid) has occupied up to 610 mL for poorly settling biomass and 350 mL for

rapidly settling biomass. The volume exchange ratio for all reactors have been in the range of 39-65% throughout the study. The aerobic sludge biomass has been washed thrice with tap water at the end of the stressed phase operation but before starting the recovery phase operation.

3.2.4 Analytical methods

The frequencies of initial and effluent sample analysis are presented in Table 3.1. The analytical parameters have been grouped into four different categories as explained earlier. The analytical techniques for estimation of temperature, pH, alkalinity, COD, MLSS, MLVSS, interface settling velocity, sludge volume index (SVI), photographic observation, microscopic analysis, aerobic sludge biomass activity (ASBA) test, enzymes activity and ARDRA, used in this study, have already been explained in Chapter 2 in detail. The cell viability test, estimation of polysaccharide (PS), protein (PN), extracellular polymeric substance (EPS) contents and determination of AMX concentration used in this investigation are presented in the following subsections.

Table 3.1 Analysis frequency of initial and effluent sample parameters.

Category	Parameters	Frequency
Environmental related parameters	Room temperature	Daily
	Initial pH	
	Initial AMX concentration	
Reactor related performance parameters	Effluent pH	
	Effluent AMX concentration	
	Chemical oxygen demand (COD)	
	Alkalinity	
Biomass related general parameters	Mixed liquor suspended solids (MLSS)	Every 7 th day
	Mixed liquor volatile suspended solids (MLVSS)	
	Sludge volume index (SVI)	
	Interface settling velocity (ISV)	
Biomass related microbiological parameters	Aerobic sludge biomass activity (ASBA)	Towards end of the stressed and recovery phase operations
	Microscopic observation after Gram's staining	Every 7 th day
	Photographic observation	
	Filament index (FI)	
	Cell viability test	Towards end of the stressed and recovery phase operations
	Polysaccharides (PS), proteins (PN) and extracellular-polymeric substances (EPS)	
	Catalase activity	
	Dehydrogenase activity	
	Protease activity	
	Urease activity	
	Amplified ribosomal DNA restriction analysis (ARDRA)	

3.2.4.1 Cell viability test

The cell viability test of aerobic sludge biomass has been performed as per the method suggested by [Jones and Senft \(1985\)](#) using fluorescein diacetate (FDA) and propidium iodide (PI) dyes for staining the live and dead cells. A stock solution of FDA (Sigma, St. Louis, MO) has been prepared by dissolving 5 mg/mL in acetone. The FDA working solution has been freshly prepared by adding 0.04 mL of stock solution to 10 mL of phosphate buffer. Propidium iodide (1 mg) has been dissolved in 50 mL of phosphate buffer. The aerobic sludge biomass has been washed with phosphate buffer and then stained with FDA-PI [0.1 mL (2 µg) of FDA working solution and 0.03 mL (0.6 µg) of PI have been added directly to the aerobic sludge biomass]. The samples are placed on ice after being stained for 3 min at room temperature. After staining, samples have been transferred to glass slides and observed under fluorescence microscope (Nikon TiS, Japan).

3.2.4.2 Extracellular polymeric substance

The extracellular polymeric substance (EPS) estimation in aerobic sludge biomass has been carried out by the modified heat extraction procedure ([Tomar and Chakraborty, 2018](#); [Li and Yang, 2007](#)). In this method, 15 mL aerobic sludge biomass has been withdrawn from reactors and centrifuged at 4000 rpm for 5 min. Then sludge pellet in the tube has been re-suspended into 15 mL of 0.02% NaCl solution having similar salinity as in the reactors. The suspension has been kept in water bath for 30 min at 60°C. After heat treatment, suspension has been centrifuged at 4000 rpm for 15 min. The collected supernatant has been considered as EPS and further used for estimation of polysaccharides (PS) and proteins (PN) contents. PS concentration has been measured by the anthrone-sulphuric acid method using glucose as the standard ([Frølund et al., 1996](#)). The carbohydrate and anthrone reagents react in presence of strong sulphuric acid which gives a green colour (in diluted samples) and a blue color (in concentrated samples), which is then determined colorimetrically. The EPS extract (2 mL) mixed with 4 mL of 0.125% anthrone is heated for 17 min at 90°C in water bath followed by cooling down at 4°C for 5 min and then absorbance at 625 nm has been measured. The polysaccharides are hydrolyzed by the strong acid to monosaccharides during heating, which are dehydrated to furfural derivatives after reacting with anthrone and then develops green color.

The protein (PN) content has been estimated by Lowry assay with bovine serum albumin as a standard ([Lowry et al., 1951](#)). In this method, 1 mL EPS extract has been mixed with 0.9 mL of Reagent A (consists of 2 g sodium potassium tartrate tetrahydrate, 100 g sodium carbonate, and 0.5N NaOH in 1 L distilled water) and incubated for 10 min at 50°C in a water bath and then allowed to cool down at room temperature. Further, 0.1 mL of Reagent B (2 g sodium potassium tartrate, 1 g copper sulfate and 10 mL 1N NaOH in 90 mL distilled water) is added to each tube, mixed and incubated for 10 min at room temperature. Afterwards, 3 mL of Reagent C (1 volume Folin-Ciocalteu reagent diluted with 15 volume of water) has been added to each tube and mixed properly. Further tubes have been incubated

at 50°C for 10 min and cooled to room temperature. The phenolic group of tyrosine and tryptophan residues (amino acid) in the protein will produce a blue purple color complex and then the absorbance has been measured at 650 nm. The standard curve of PS and PN are presented in *Appendix-I* (Fig. A-I.5 and A-I.6).

3.2.4.3 Determination of AMX

The initial and effluent samples have been centrifuged at 12,000 rpm, and supernatant have been collected. Then the collected supernatant have been syringe filtered through a 0.22 μm nylon membrane filter to remove biomass. Samples have been stored at 4°C until analyzed. The AMX concentration has been determined by HPLC (Prostar 210, Varian, USA) equipped with a micro-vacuum degasser, a dual pump, UV detector (working wavelength of 208 nm), and Agilent 5 TC-C18 analytical column (250×4.5 mm, particle size 5 μm). The data have been recorded by chemstation software. The mobile phase has been prepared using 0.02M NaH_2PO_4 and acetonitrile, with the volume ratio of 65:35. The flow rate of mobile phase has been set to 0.8 mL/min (Weng et al., 2018). The retention time of amoxicillin has been observed at 13.4±0.7 min and the total run time of the HPLC-DAD method has been 30 min. The standard curve has been presented in *Appendix-I* (Fig. A-I.7).

3.3 Results and Discussion

The reactors have been operated during 0-43 d with AMX in the feed — termed as stressed phase operation and afterward for 44-78 d without AMX in the feed — termed as recovery phase operation. The results obtained are discussed below in subsequent subsections. The average values of duplicate reactors (average of R_{amx0_1} and R_{amx0_2} have been represented as R_{amx0} and similarly for other reactors as R_{amx500} , R_{amx1000} , and R_{amx2000}) along with the standard deviation in the respective tables.

3.3.1 Stressed phase operation

3.3.1.1 Environmental and performance parameters

The reactors have been operated for 43 d under stressed phase operation. Variation in environmental and performance-related parameters is shown in Fig. 3.3 to 3.6 and summary of data are presented in Table 3.2. The reactors have been operated under ambient conditions throughout the operation; hence pH and reactor temperature have not been adjusted. The room temperature variation has been mainly observed due to weather changes. The temperature variation has been shown in Fig. 3.3 and data has been summarized in Table 3.2. Since all the reactors have been operated at room temperature, variation in room temperature recorded at 9.30 am, and 8.45 pm have been presented in Fig. 3.3.

Variations in the initial and effluent pH for reactors have been presented in Fig. 3.3 to 3.6. The average initial and effluent pH for reactor R_{amx0} during 0-43 d of operation has been obtained as 7.17±0.14 and 7.17±0.15, respectively. Fig. 3.3 shows uniform pH for reactor R_{amx0} throughout the experiment. However, pH for other reactors R_{amx500} , R_{amx1000} , and R_{amx2000} have slightly decreased as compare to the reactor R_{amx0} during stressed phase

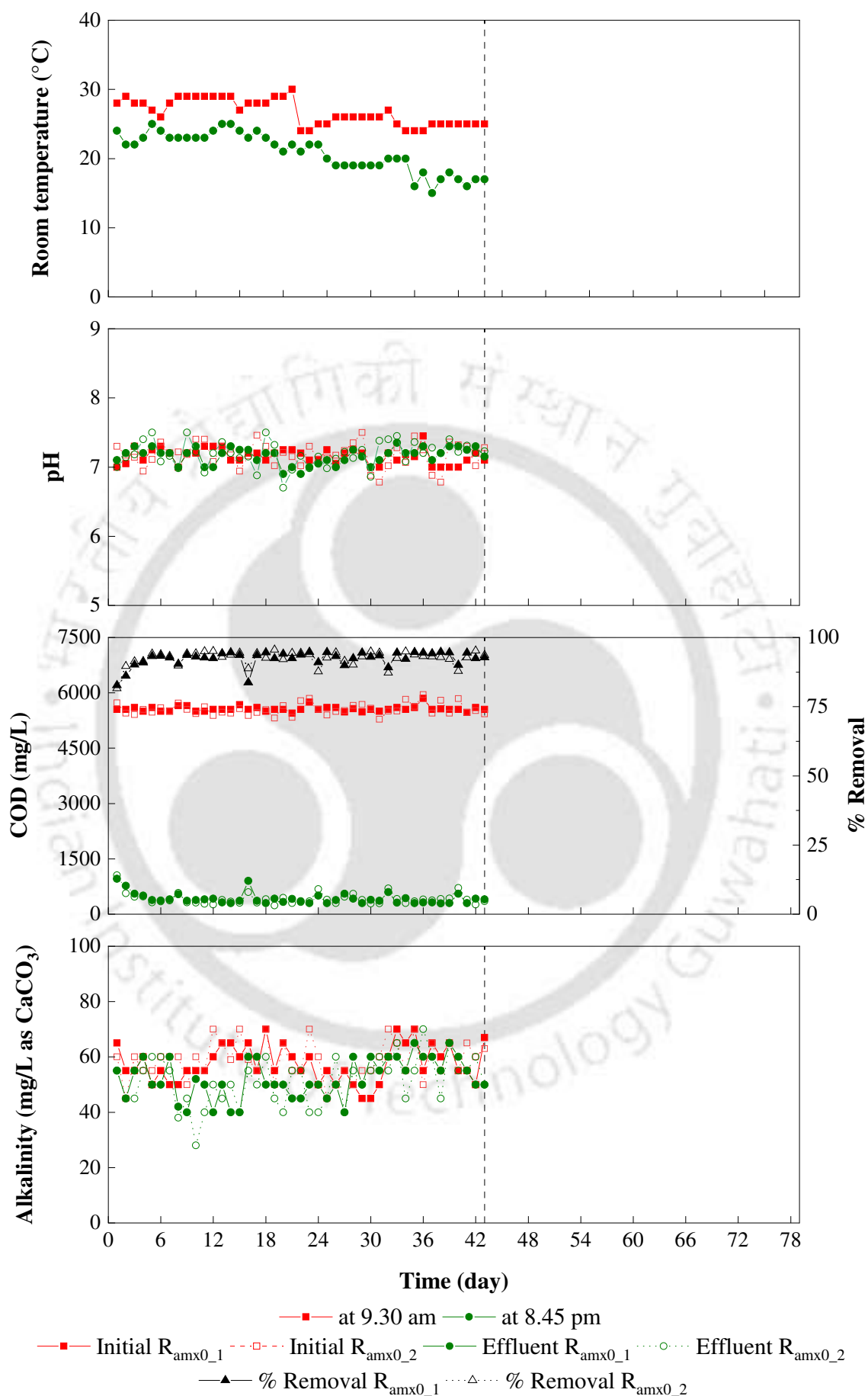


Fig. 3.3 Variation in environmental and performance related parameters for reactor R_{amx0} during 0-43 d of operation.

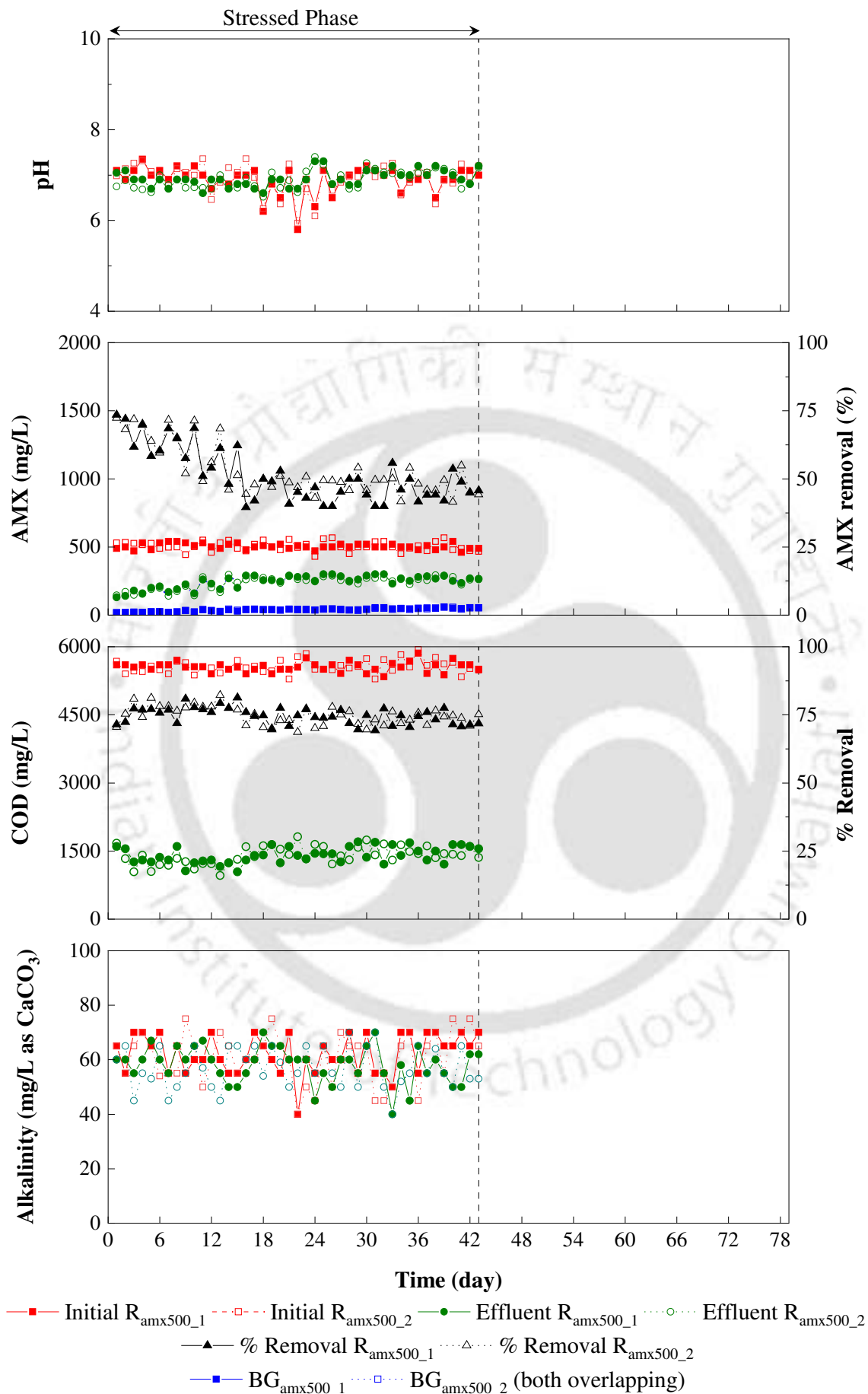


Fig. 3.4 Variation in environmental and performance related parameters for reactor R_{amx500} during 0-43 d of operation.

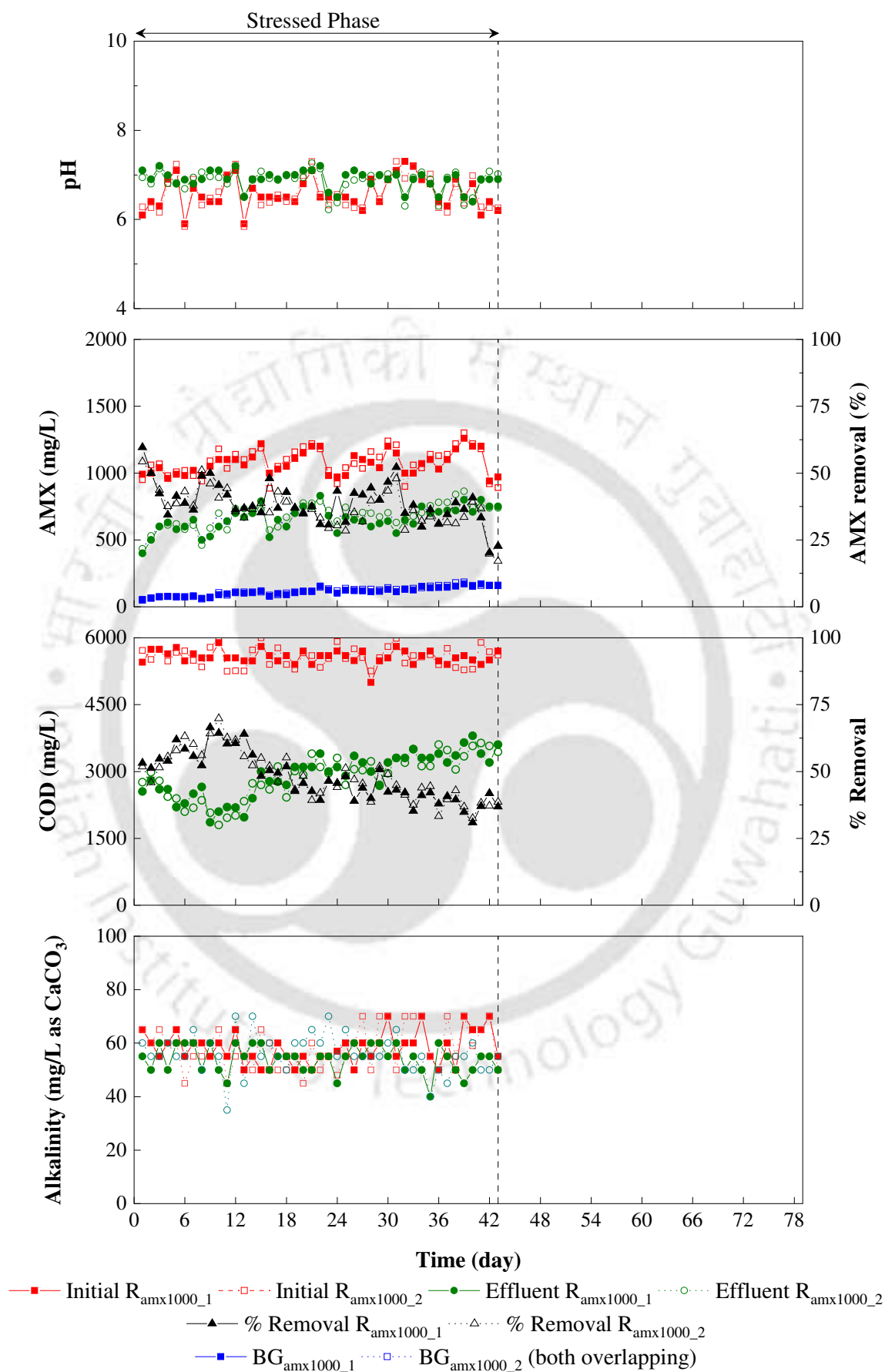


Fig. 3.5 Variation in environmental and performance related parameters for reactor $R_{amx1000}$ during 0-43 d of operation.

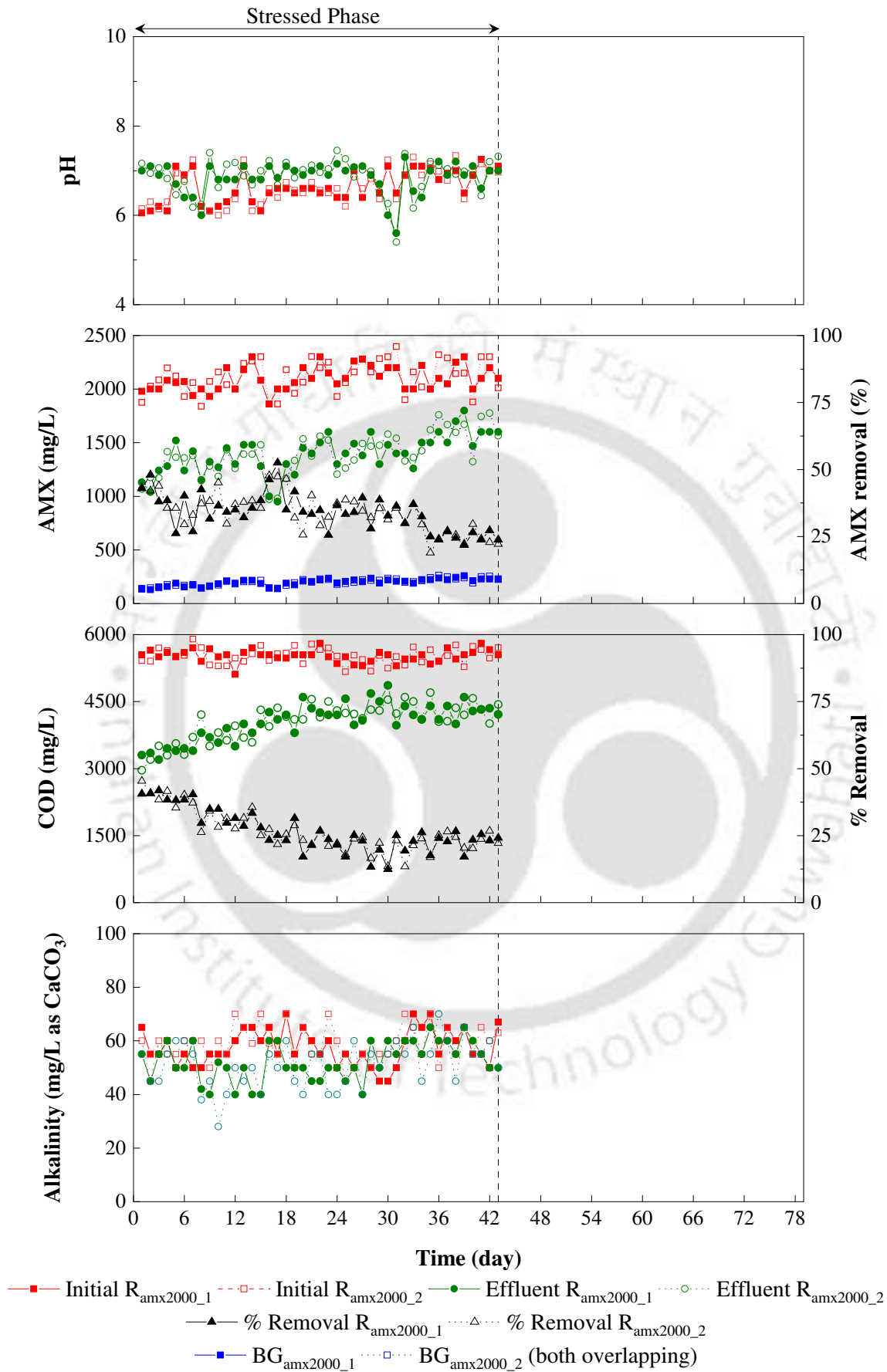


Fig. 3.6 Variation in environmental and performance related parameters for reactor $R_{amx2000}$ during 0-43 d of operation.

Table 3.2 Summary of environmental and performance related parameters for reactors operated during 0-43 d of stressed phase operation.

Parameters	Day-wise	0-43			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	4.25	3.00	0.50
	AMX (mg/L)	0	500	1000	2000
Stressed phase	Environmental				
	Room temperature (°C) at 9.30 am	26.7±1.8 (43)	26.7±1.8 (43)	26.7±1.8 (43)	26.7±1.8 (43)
	Room temperature (°C) at 8.45 pm	20.8±2.7 (43)	20.8±2.7 (43)	20.8±2.7 (43)	20.8±2.7 (43)
	Initial pH	7.17±0.14 (86)	6.91±0.32 (86)	6.59±0.37 (86)	6.64±0.38 (86)
	Initial AMX (mg/L)	0	505.37±27.39 (86)	1078.77±95.60 (86)	2106.60±133.24 (86)
	Effluent pH	7.17±0.15 (86)	6.91±0.19 (86)	6.88±0.23 (86)	6.86±0.38 (86)
	Effluent AMX (mg/L)	0	240.96±47.03 (86)	669.00±91.92 (86)	1395.74±190.25 (86)
	AMX removal (%)	0	52.26±9.53 (86)	37.93±7.43 (86)	33.88±7.18 (86)
	Background AMX (mg/L)	0	38.40±11.38 (86)	115.53±32.98 (86)	198.23±33.35 (86)
	Performance				
	Initial soluble COD (mg/L)	5558.51±114.22 (86)	5558.51±127.89 (86)	5572.78±186.01 (86)	5523.05±158.72 (86)
	Effluent soluble COD (mg/L)	417.56±153.74 (86)	1408.94±189.07 (86)	2902.02±492.55 (86)	4047.29±416.31 (86)
	COD removal (%)	92.49±2.73 (86)	74.64±3.24 (86)	47.87±9.00 (86)	26.67±7.63 (86)
	Initial alkalinity (mg CaCO ₃ /L)	64.24±7.67 (86)	61.56±8.27 (86)	58.24±7.04 (86)	58.23±6.78 (86)
	Effluent alkalinity (mg CaCO ₃ /L)	59.03±6.96 (86)	57.57±7.10 (86)	55.00±6.24 (86)	51.63±7.97 (86)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

operation due to addition of AMX in the feed. The average initial pH for reactors R_{amx500} , R_{amx1000} , and R_{amx2000} have been observed as 6.91 ± 0.32 , 6.59 ± 0.37 , and 6.64 ± 0.38 , respectively, while the average effluent pH of the reactors have been obtained as 6.91 ± 0.19 , 6.88 ± 0.23 , and 6.86 ± 0.38 , respectively.

The variation in AMX removal has been shown in Fig. 3.4 to 3.6 and data has been summarized in Table 3.2. Initially, reactor R_{amx500} has shown significant AMX removal under the stressed phase operation and later on it has not been able to sustain the stress imparted by AMX and hence resulting in poor removal efficiency. On day 1st, 21st, and 43rd of operation, AMX removal for reactor R_{amx500} has been obtained as 72.9%, 45.1%, and 46.2%, respectively. Moreover, reactors R_{amx1000} and R_{amx2000} have shown poor AMX removal with gradual decrease over time. Increased AMX concentration has imparted stress to the aerobic sludge biomass, resulting in the reduction in AMX removal efficiency. The reactors R_{amx500} , R_{amx1000} , and R_{amx2000} have been fed with a high concentration of AMX in the feed; therefore, to prevent the accumulation of AMX in reactors, the aerobic sludge biomass has been washed before subsequent feedings. The background AMX (BG_{amx}) concentration has also been estimated after washing (Fig. 3.4 to 3.6). Initially, a very less amount of AMX has remained in the reactors but has increased gradually over time towards the end of the stressed phase. The reactor R_{amx500} has retained 16.92 mg/L and 53.45 mg/L of AMX on 1st and 43rd d of operation, respectively. Similarly, reactor R_{amx1000} has retained 51.82 mg/L and 158.84 mg/L of AMX, while 136.55 mg/L and 227.07 mg/L of AMX has been retained by reactor R_{amx2000} on 1st and 43rd d of operation, respectively. The average variation in BG_{amx} concentration for reactors R_{amx500} , R_{amx1000} , and R_{amx2000} have been observed as 38.40, 115.53, and 198.23 mg/L, respectively, during stressed phase operation (Table 3.2).

The possible AMX removal mechanism by activated sludge are (i) bio-degradation and (ii) adsorption (Zhao et al., 2015). The major removal route for AMX is adsorption rather than bio-degradation (Li and Zhang, 2010). It is reported that lower antibiotics concentrations have no significant impact on the biological process. In addition to that, Jendrzewska and Karwowska (2018) have reported that the removal of organic matter content by activated sludge reactor had not been affected by the presence of 100-300 $\mu\text{g/L}$ of AMX. Yu et al. (2019) have reported that lower doses of ampicillin (0, 5, 10, 20, and 30 mg/L) has not affected the organic matter removal and ammonia oxidation efficiencies. These studies have indicated that the effect of antibiotics depends on the antibiotic concentration; as the concentration of antibiotics used for the studies has been extremely low.

The variations in COD removal during the stressed phase for all the reactors have been shown in Fig. 3.3 to 3.6 and data has been summarized in Table 3.2. Initially, a lower COD removal has been observed for reactor R_{amx0} due to the transfer of aerobic sludge biomass from MR to reactors and sudden increase in organic content in the feed. MR has been fed with 500-550 mg/L of COD (Appendix-II), while the reactors have been fed with a COD of ~ 5500 mg/L in this study. The aerobic sludge biomass has taken 6 d to adapt to the new level of COD in the feed and thereafter has started to degrade the organic matter significantly and

shown uniform COD removal throughout the operation. On the 1st and 6th d of operation, the COD removal for reactor $R_{\text{amx}0}$ has been observed as 82.1% and 93.6%, respectively, while the average % COD removal for entire duration of operation of reactor $R_{\text{amx}0}$ has been observed as 92.5% (Table 3.2). The reactors fed with 500, 1000, and 2000 mg/L of AMX have shown significant variation in COD removal. The AMX concentration at 500 mg/L in the reactor $R_{\text{amx}500}$ has caused a slight decreased in COD removal as shown in Fig. 3.4. On 1st, 21st, and 43rd d of stressed phase operation, the COD removal for reactor $R_{\text{amx}500}$ has been observed as 70.4%, 72.9%, and 74.7%, respectively. The COD removal for AMX fed reactors has been affected by the increased concentration of AMX in the feed. The COD removal for reactors $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ on 1st, 21st, and 43rd d of stressed phase operation have been observed as 52.5%, 40.9% and 37.7%; 42.9%, 21.5%, and 23.2%, respectively (Fig. 3.5-3.6). The reactor fed with 1000 mg/L of AMX has shown almost 50% of COD removal on the 1st d and thereafter has decreased gradually after 12th d of operation. The possible reason may be the continuous addition of AMX, which has affected the aerobic sludge biomass. The COD removal for reactor $R_{\text{amx}2000}$ has been severely affected from the 1st d itself and has further decreased as the stressed phase progressed. The AMX is a complex antibiotic that is not easily biodegradable. Hence, adaptation time has been taken by aerobic sludge biomass to develop its cellular machinery to degrade AMX. The continuous addition of AMX has a negative effect on aerobic sludge biomass that has inhibited its microbial activity and resulted in a gradual decrease in COD removal (Fig. 3.4 to 3.6). The average % COD removal for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been 74.6%, 47.9%, and 26.7%, respectively, during the stressed phase operation.

A slight variation in alkalinity has been observed for all the reactors. The average initial and effluent alkalinity has been obtained in the range of 51.63 ± 7.97 – 64.24 ± 7.67 mg CaCO_3/L , respectively, during stressed phase operation.

The COD removal has appeared to be stabilized after 30-35 d of operation during stressed phase for AMX fed reactors and thereafter has yielded steady performance. Summary of environmental and performance related parameters for reactors operated during steady-state (37-43 d) of stressed phase operation are presented in Table 3.3. The data suggests minimum variation in reactors performance. During the steady-state condition, the average AMX removal for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been observed as 46.5%, 31.1%, and 24.9%, respectively, during 37-43 d of operation. The average BG_{amx} for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been observed as 52.30, 162.05, and 232.95 mg/L, respectively, during steady-state condition of stressed phase operation. The average COD removal for reactors $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been observed as 93.0%, 73.5%, 37.7%, and 23.4%, respectively, during steady-state of stressed phase operation. The variation in alkalinity for all the reactors have been observed in the range of 52.14–67.86 mg CaCO_3/L .

Table 3.3 Summary of environmental and performance related parameters for reactors operated during 37-43 d of steady-state condition of stressed phase operation.

Parameters	Day-wise	37-43			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	4.25	3.00	0.50
	AMX (mg/L)	0	500	1000	2000
Environmental	Room temperature (°C) at 9.30 am	25.0±0.0 (7)	25.0±0.0 (7)	25.0±0.0 (7)	25.0±0.0 (7)
	Room temperature (°C) at 8.45 pm	16.7±0.9 (7)	16.7±0.9 (7)	16.7±0.9 (7)	16.71±0.9 (7)
	Initial pH	7.10±0.16 (14)	6.93±0.25 (14)	6.45±0.31 (14)	6.94±0.27 (14)
	Initial AMX (mg/L)	0	497.18±32.20 (14)	1122.86±140.51 (14)	2148.00±135.73 (14)
	Effluent pH	7.25±0.08 (14)	7.02±0.17 (14)	6.81±0.27 (14)	6.96±0.23 (14)
	Effluent AMX (mg/L)	0	265.75±21.71 (14)	764.27±45.09 (14)	1615.03±131.54 (14)
	AMX removal (%)	0	46.47±4.11 (14)	31.14±8.14 (14)	24.87±2.41 (14)
	Background AMX (mg/L)	0	52.30±4.39 (14)	162.05±10.51 (14)	232.95±18.71 (14)
	Initial soluble COD (mg/L)	5556.10±108.64 (14)	5556.10±131.19 (14)	5535.71±178.07 (14)	5604.05±145.60 (14)
	Effluent soluble COD (mg/L)	393.19±117.52 (14)	1473.67±139.15 (14)	3449.57±217.01 (14)	4295.38±192.60 (14)
Performance	COD removal (%)	92.95±1.99 (14)	73.49±2.33 (14)	37.68±3.45 (14)	23.35±2.84 (14)
	Initial alkalinity (mg CaCO ₃ /L)	67.14±5.08 (14)	67.86±4.83 (14)	62.43±7.86 (14)	60.36±5.49 (14)
	Effluent alkalinity (mg CaCO ₃ /L)	55.71±6.81 (14)	56.36±5.48 (14)	52.14±4.33 (14)	56.07±6.14 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

3.3.1.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The impact of AMX on aerobic sludge biomass characteristics has been assessed grossly through MLSS and MLVSS, interface settling velocity (ISV), SVI, and ASBA test. The variations in solid characteristics have been shown in Fig. 3.7 and data has been summarized in Table 3.4. The zig-zag pattern observed in solids characteristics for all the reactors are due to sludge wastage to maintain MLSS ~ 3000 mg/L or withdrawal for other tests from each reactor every week as shown in Fig. 3.7. The reactor $R_{\text{amx}0}$ has shown continuous growth of aerobic sludge biomass and an increased SVI as illustrated in Fig. 3.7(a). The MLSS concentration in reactor $R_{\text{amx}0}$ has increased from 3098 mg/L (1st d) to 4276 mg/L (7th d) in a week and has maintained the same growth trend throughout the operation. Similarly, reactor $R_{\text{amx}500}$ has shown significant biomass growth in a week (from 3108 mg/L to 3932 mg/L) [Fig. 3.7(b)]. On the other hand, the reactors $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ fed with higher doses of AMX have shown slower biomass growth as compared to reactors $R_{\text{amx}0}$ and $R_{\text{amx}500}$. The reactor $R_{\text{amx}2000}$ has shown reduced biomass growth (from 3008 mg/L to 3354 mg/L in a week) [Fig. 3.7(d)] as compared to reactor $R_{\text{amx}0}$. The biomass growth has reduced with increase in AMX concentration in feed and further reduced over time towards the end of the stressed phase operation. The AMX stress has not much affected the MLVSS/MLSS ratio. The average MLVSS/MLSS ratio have been observed as 0.88, 0.88, and 0.83 for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses as compared to reactor $R_{\text{amx}0}$ biomass (0.90) (Table 3.4). The SRT for reactors $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ has been observed in the range of 22-53 d during stressed phase operation.

The estimated SVI for all the reactors during stressed phase operation have been shown in Fig. 3.7 and data has been presented in Table 3.4. On the 1st and 37th of operation, the SVI for reactor $R_{\text{amx}0}$ biomass has been observed as 113 and 130 mL/g, respectively, as illustrated in Fig. 3.7(a), and the average SVI has been obtained as 140 mL/g during stressed phase operation. The possible reason for increased SVI for reactor $R_{\text{amx}0}$ biomass could be the continuous growth of filamentous microorganisms in the reactor. Also, reactor $R_{\text{amx}0}$ has been fed with high organic content (~ 5500 mg/L), which is favorable for filamentous growth. The reactor $R_{\text{amx}500}$ has shown a decreasing trend in biomass growth and has exhibited increasing SVI (114 and 159 mL/g on 1 and 43rd d of operation, respectively) as shown in Fig. 3.7(b), with an average SVI value of 145 mL/g. The presence of 500 mg/L of AMX in feed has slowed down the aerobic sludge biomass growth due to AMX stress. A further increase in AMX concentration (from 500 to 2000) has significantly decreased aerobic sludge biomass growth during the stressed phase operation. The SVI for reactor $R_{\text{amx}1000}$ biomass on 1st and 37th d of operation has been observed as 116 and 114 mL/g, respectively (Fig. 3.7(c)), with an average SVI value of 121 mL/g. The reactor fed with 2000 mg/L of AMX has shown very limited aerobic sludge biomass growth during stressed phase operation with SVI of 116 and 128 mL/g on 1st and 37th d, respectively [Fig. 3.7(d)]. The average SVI value for reactor $R_{\text{amx}2000}$ biomass has been obtained as 126 mL/g. The reactors fed with high doses of AMX

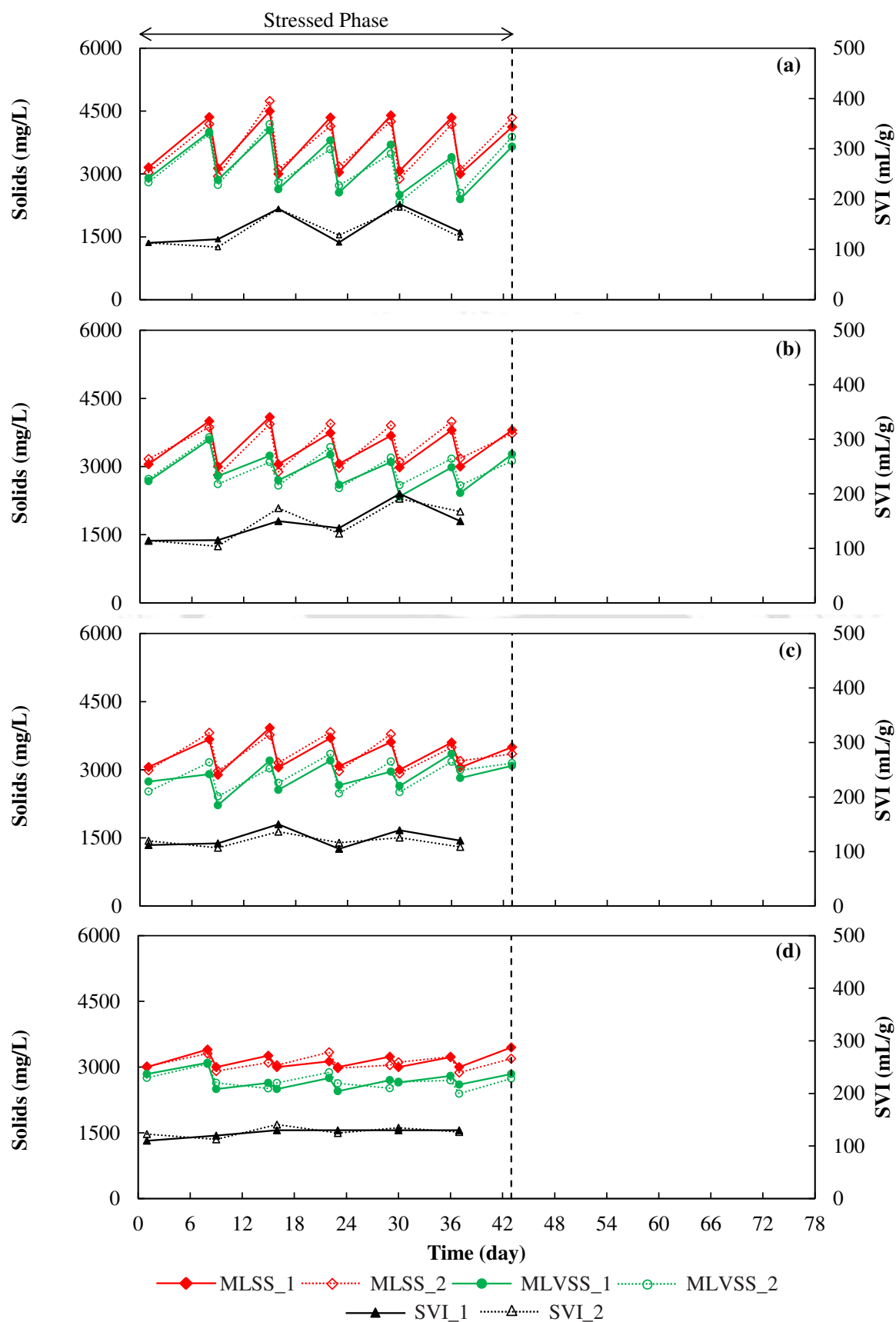


Fig. 3.7 Variation in MLSS, MLVSS, and SVI values for reactors (a) R_{aml0} , (b) R_{aml500} , (c) $R_{aml1000}$, and (d) $R_{aml2000}$ during 0-43 d under stressed phase operation.

Table 3.4 Summary of solids characteristics estimated during stressed phase operation.

Gross parameters	Stressed phase operation				
	Day-wise	0-43			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
MLSS (mg/L)		3056.00±89.42 (12)	3021.67±94.25 (12)	3027.00±91.81 (12)	2994.33±43.66 (12)
MLVSS (mg/L)		2649.40±193.21 (12)	2596.40±120.60 (12)	2605.21±211.50 (12)	2605.32±131.39 (12)
MLVSS/MLSS ratio		0.90±0.02 (12)	0.88±0.05 (12)	0.88±0.05 (12)	0.83±0.04 (12)
SVI (mL/g)		140.54±34.44 (12)	145.28±33.68 (12)	121.14±14.22 (12)	125.99±9.09 (12)
ISV (m/h)		1.50±0.32 (12)	1.49±0.37 (12)	1.87±0.12 (12)	1.93±0.08 (12)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

(1000 and 2000 mg/L) have shown decreased SVI during stressed phase as compared to reactors fed with lower doses of AMX (500 and 1000 mg/L). The presence of AMX has affected aerobic sludge biomass growth by converting a fraction into inactive biomass without affecting the flocs structure. This could be the possible reason for increased SVI for reactor R_{amx1000} and R_{amx2000} biomasses.

The settling tests for all the reactors have been carried out on every 7th d throughout the operation, as shown in Fig. 3.8 and ISV for all the reactors have also been estimated on every 7th d, as shown in Fig. 3.9 and summarized data is presented in Table 3.4. Fig. 3.8(a) has shown the settling behavior for reactor R_{amx0} biomass, which has deteriorated after 16th d of operation. However, the settling behavior for reactor R_{amx2000} biomass has changed from 9th d and remained almost the same throughout the stressed phase operation as shown in Fig. 3.8(b).

The reactor R_{amx0} and R_{amx500} biomasses have shown decreased ISV over the time during stressed phase operation as shown in Fig. 3.9. The possible reason could be abundance of filamentous microorganisms in the reactors. The reactor R_{amx1000} and R_{amx2000} biomasses have shown faster settling velocity than reactor R_{amx0} and R_{amx500} biomasses (Fig. 3.9). On the 1st d of operation, the ISV for all the reactor biomasses have been observed close to

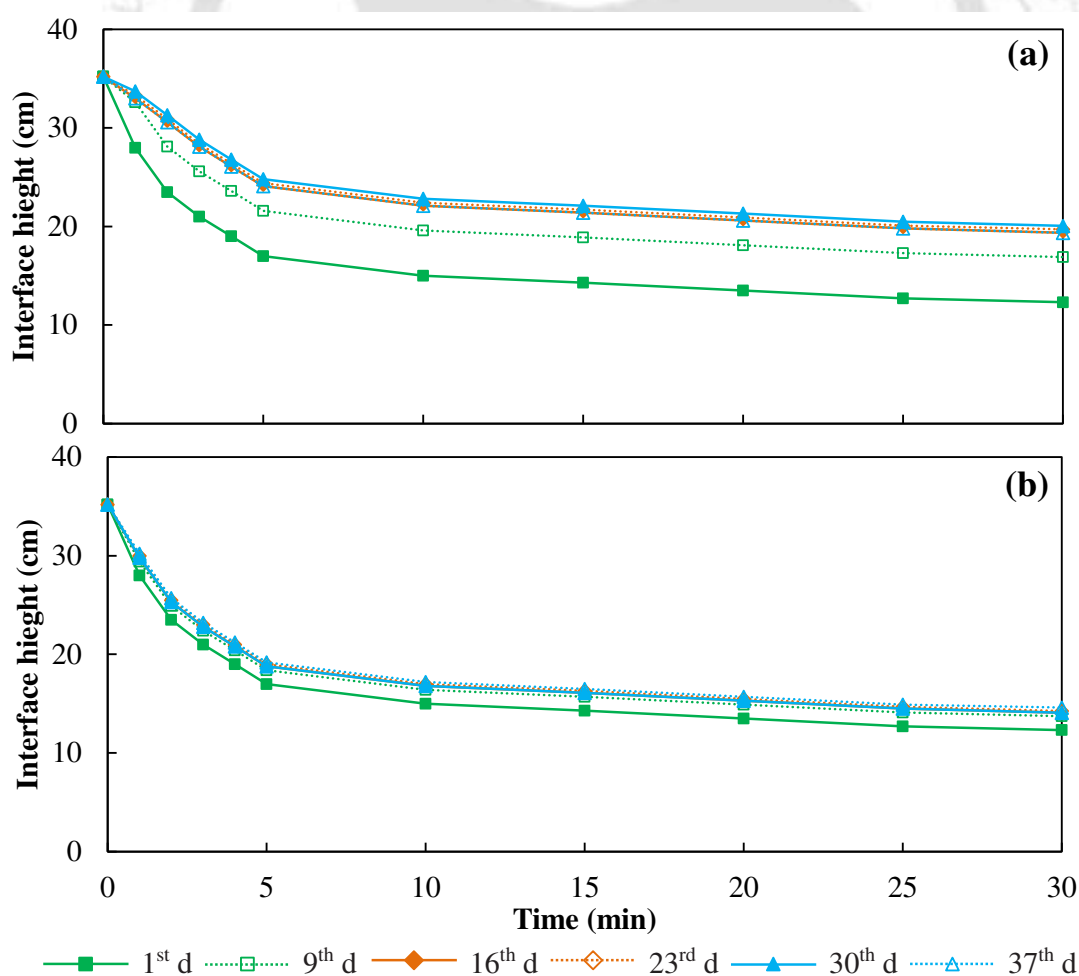


Fig. 3.8 Typical settling test results for reactor (a) R_{amx0} and (b) R_{amx2000} biomasses during 0-43 d under stressed phase operation.

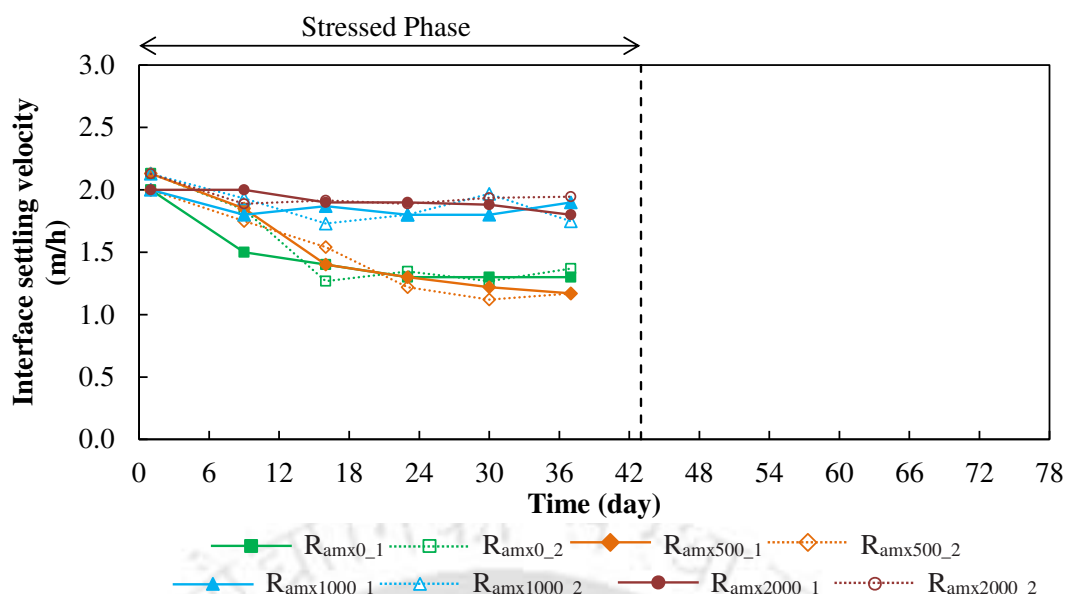


Fig. 3.9 Variation in interface settling velocities of reactor biomasses during 0-43 d during stressed phase operation.

2.06 m/h. Moreover, average ISV for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have been observed as 1.50, 1.49, 1.87, and 1.93 m/h, respectively, during stressed phase (Table 3.4). The decreased ISV are due to abundance of filamentous biomass in reactors while higher ISV are probably due to reduced filaments in reactors.

Aerobic sludge biomass activity test

ASBA test has been carried out at the end of the stressed phase operation (on 43rd d), when reactors have achieved the steady-state condition. The test results have been shown in Fig. 3.10, while activity values has been represented in Table 3.5. The results obtained from the ASBA test suggest that aerobic sludge biomasses have been severely affected by AMX stress. The ASBA for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have been obtained as 7.65, 3.84 and 2.22 mg COD removed/mg VSS d during stressed phase, which is lower than reactor R_{amx0} biomass (9.44 mg COD removed/mg VSS d). The results have clearly indicated inhibition of the organic degradation capability of aerobic microbes due to AMX. This could be the possible reason behind decreased COD removal for AMX fed reactors during stressed phase operation. Yu et al. (2019) have reported a reduction of 27.46% in soluble oxygen uptake rate (sOUR) at 30 mg/L ampicillin compared with 0 mg/L of ampicillin. Wang et al. (2018) have also reported that the addition of tetracycline (at 50 mg/L) has decreased the specific ammonium oxidation rate (SAOR), specific nitrite oxidation rate (SNOR), and specific nitrate reduction rate (SNRR) and have suggested that decrease in aerobic sludge biomass activity is due to a high concentration of antibiotics in the feed that could slow down the process through damaging the integrity of cell membrane and inhibiting the synthesis of some enzymes. The AMX acts on a broad spectrum of microbes and inhibits cell synthesis. It can be assumed that the presence of AMX in the reactor has slowed down the activity of aerobic sludge biomass, and its level of impact varies with AMX concentration. The higher doses of AMX has caused more adverse impacts on biomasses with lower ASBA.

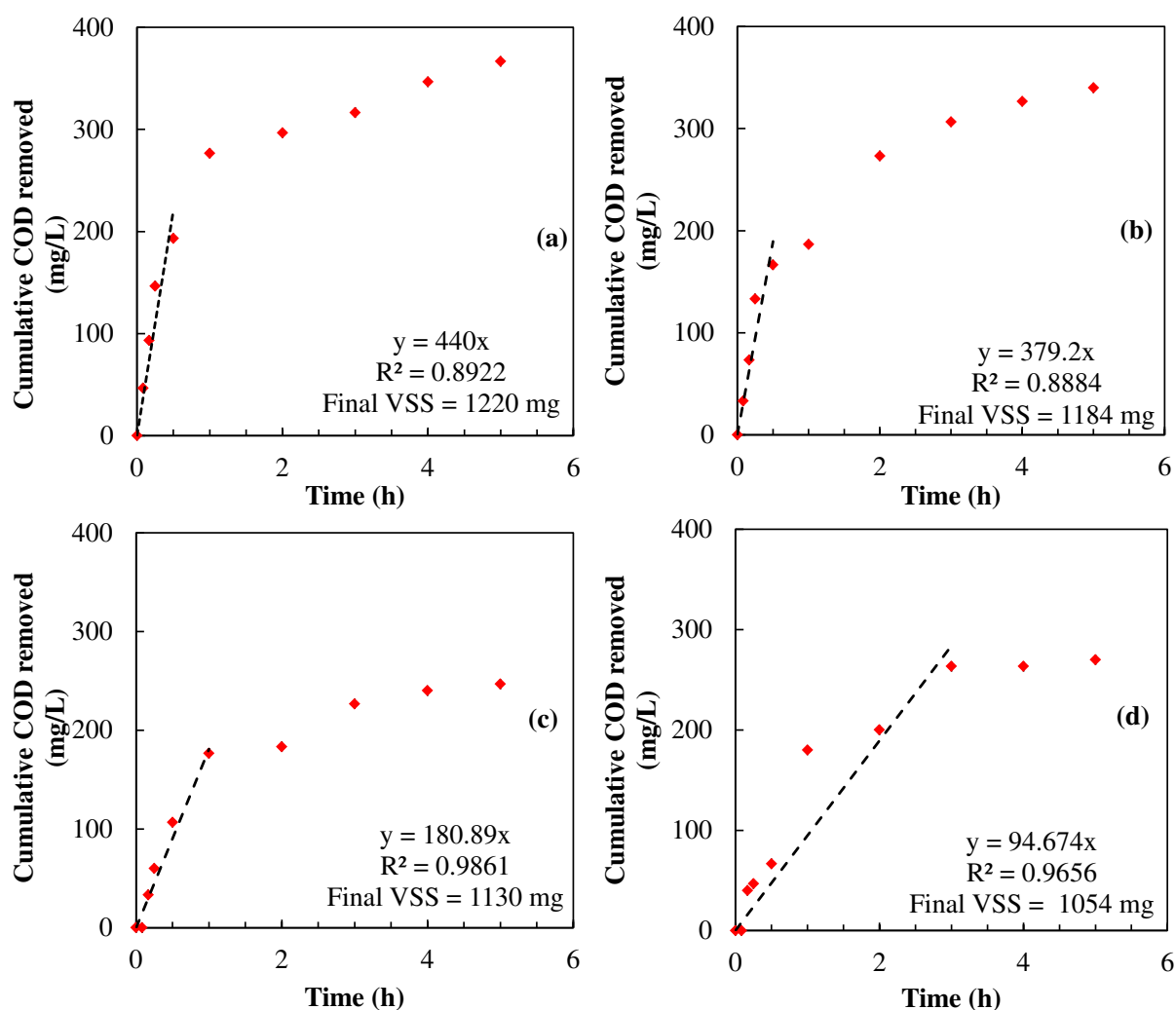


Fig. 3.10 Aerobic sludge biomass activity test results with sludge biomasses withdrawn from reactors (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ on 43rd d of stressed phase operation.

Table 3.5 Aerobic sludge biomass activity test results carried out at the end of stressed phase operation.

Stressed phase operation				
Biomass withdrawn on	43 rd d			
Reactor	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
ASBA (mg COD removed/mg VSS d)	9.43	7.65	3.84	2.22

Focusing on biomass related general parameters, MLSS and MLVSS do not appear to provide much information about biomass growth with AMX concentration in the reactors. The MLVSS/MLSS ratio for AMX fed reactors have fluctuated within a range of 0.83 to 0.88. A slight variation in SVI and ISV has been observed for AMX fed reactors compared to the control reactor. The average SVI for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged between 121-145 mL/g, whereas ISV have been observed in the range of 1.49-1.93 m/h during stressed phase operation. AMX concentrations up to 1000 mg/L has no impact on aerobic sludge biomass, according to the results of the study. However, higher AMX concentrations (at 2000 mg/L) have induced minor changes in SVI and ISV due to its

stress. On the other hand, AMX has imparted a negative impact on aerobic sludge biomass, suggested by the ASBA test. The ASBA for reactor $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses are 7.65, 3.84, and 2.22 mg COD removed/mg VSS d, respectively, which is on a lower side compared to 9.43 mg COD removed/mg VSS d for control reactor $R_{\text{amx}0}$ biomass. Similarly, COD removal for reactor $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ are 74%, 38%, and 23%, respectively, whereas reactor $R_{\text{amx}0}$ has shown 93% of COD removal during the steady-state condition of stressed phase operation. The ASBA test showed that organic degradation capability has been affected due to the presence of AMX in the reactor. As a result, the ASBA test can identify the influence of AMX on aerobic sludge biomass, while other approaches such as MLSS, MLVSS, SVI, and ISV are less successful.

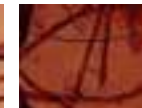
3.3.1.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

The flocs structure and morphology of the sludge have been examined through microscopic observation. The images of aerobic sludge biomass samples have been taken at $5\times$ and $100\times$ magnifications using the digital camera attached to the microscope after being stained (Gram's staining), and the result has been presented in Table 3.6. The changes in biomass morphology during stressed phase operation has also been observed through the photographic observation as shown in Table 3.7. The cell viability test differentiate the active (live) and inactive (dead) portion of aerobic sludge biomass. The aerobic sludge biomass has been stained and observed under a fluorescence microscope, and results have been presented in Table 3.8. The filament index (FI) has also been estimated during stressed phase operation, and data are summarized in Table 3.9.

The results suggest that increased concentration of AMX in reactors has affected aerobic sludge biomass growth during stressed phase operation. However, no significant impact of AMX has been observed on the structure and morphology of aerobic sludge biomass as shown in Table 3.6. The microscopic images taken at $5\times$ magnification has shown the overall flocs morphology, while $100\times$ magnification images have illustrated the density of filaments of aerobic sludge biomass. Microscopic observation suggests that there has been continuous growth of filamentous microorganisms and fairly dense flocs in reactor $R_{\text{amx}0}$ throughout the experiment and flocs size has also been continuously increasing; similar morphology of aerobic sludge biomass has been observed for reactor $R_{\text{amx}500}$ during stressed phase operation (Table 3.6). On 1st d, very less amount of filaments could be distinguished, whereas density of filaments appear to have increased on 8th d of operation for all the reactors (Table 3.6). Filaments are clearly visible in the images captured at $100\times$ magnification. The addition of AMX at higher doses in reactors $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ has resulted in weak and loose strands of filaments (on 43rd d, Table 3.6). The reactor $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ biomasses have shown limited growth and reduced size during 1-15 d of operation (Table 3.6), but later on biomasses have started to grow with slight increase in floc sizes (22-43 d of operation).

Table 3.6 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ during stressed phase operation.

Reactor Mag.	Aerobic sludge biomass samples withdrawn on Stressed phase operation						
	1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d	43 rd d
R_{amx0}	5× 						
	100× 						
R_{amx500}	5× 						
	100× 						
$R_{amx1000}$	5× 						
	100× 						
$R_{amx2000}$	5× 						
	100× 						

The photographic observation presented in Table 3.7 suggests that initially, the flocs size of aerobic sludge biomass for all the reactors have ranged between 1.0-3.0 mm on the 1st d. The reactor R_{amx0} and R_{amx500} biomasses have shown almost similar flocs morphology (flocs size in the range of 1.0-7.0 mm on 43rd d); however, higher AMX doses in reactor $R_{amx1000}$ and $R_{amx2000}$ biomasses have slightly changed the flocs morphology (1.0-5.0 mm on 43rd d). The flocs size in the reactor $R_{amx1000}$ and $R_{amx2000}$ biomasses have increased only slightly, but the flocs structure have been observed as weak and loose in reactor $R_{amx1000}$ and $R_{amx2000}$ biomasses as compared to reactor R_{amx0} and R_{amx500} biomasses during stressed phase (Table 3.7).

FI values have been presented in Table 3.8. It demonstrates the abundance of filaments in the reactors. Initially, all the reactors have lesser count of filamentous microorganisms, hence the FI values for all reactor have been assigned as 1 on 1st d of the operation. FI values have increased from 1 to 3 on 8th d of operation and reached to 5 on 15th d of operation for all reactors. At the end of stressed phase (43rd d), the FI value for reactor R_{amx0} , R_{amx500} , and $R_{amx1000}$ biomasses have been obtained as 5, while FI value 4 has been obtained for reactor $R_{amx2000}$ biomass (Table 3.8). The slightly lower in FI value for reactor $R_{amx2000}$ biomass could be due to the stress of higher doses of AMX. From the results, it is clear that filamentous biomass has always existed in the reactor throughout the stressed phase operation. In fact, it appears that AMX has encouraged increase in number of filaments.

Fluorescence microscopic results distinguish the live and dead portion of aerobic sludge biomass as presented in Table 3.9. The bright-field images show overall flocs structure. The red color represents the inactive/dead biomass portion, while the green color exhibits the active/live biomass portion of aerobic sludge biomass. On 1st d of operation, the live and dead portion of biomass have been observed as almost same for all the reactors; however, there has been a severe impact of AMX on aerobic sludge biomass at the end of the stressed phase. The active biomass portion remained same for reactor R_{amx0} biomass on 43rd d. Whereas the active biomass part has decreased with increased concentration of AMX in the reactor feed, which is clearly observed from Table 3.9 during stressed phase. A major portion of inactive biomass has been observed for reactor $R_{amx1000}$ biomass and a large portion of inactive aerobic has been observed for reactor $R_{amx2000}$ biomass at the end of the stressed phase operation. The increase in the inactive portion of aerobic biomass could be due to stress imparted by the higher dose of AMX. The fluorescence microscopic results have shown 97.7%, 87.2%, 39.2%, and 17.3% of live cells and 0.4%, 30.8%, 58.9%, and 76.4% of dead cells for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, at steady-state condition of stressed phase operation.

It could be concluded from Table 3.6 to 3.9 that the AMX stress to aerobic sludge biomass does not reduce filaments in the biomass in the reactor, and it also does not affect the flocs morphology. The aerobic sludge biomasses have appeared to be entangled strongly with filament strands. However, imposed AMX stress might have weakened the entanglement of filament strands thereby allowing them to separate in loose filaments. This might have

Table 3.7 Photographic images of aerobic sludge biomasses withdrawn from reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ during stressed phase operation.

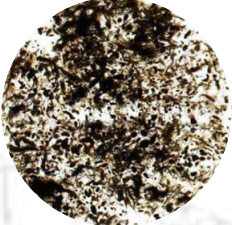
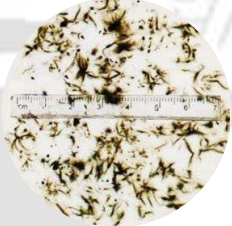
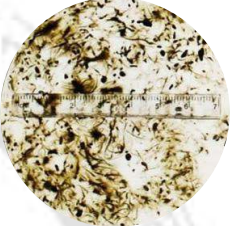
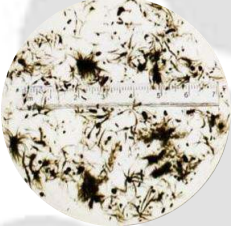
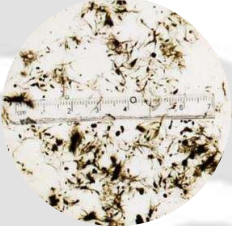
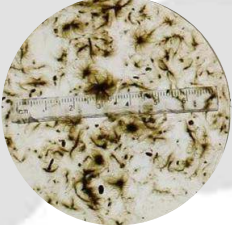
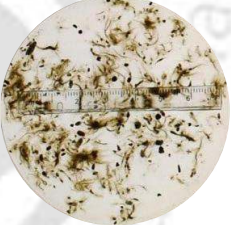
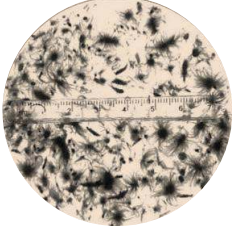
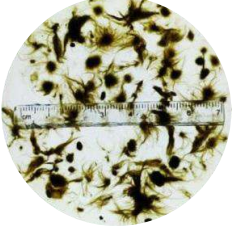
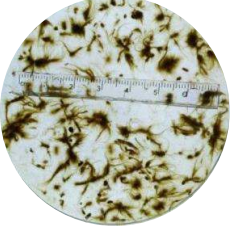
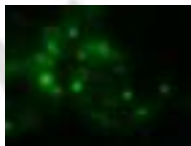
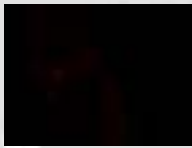
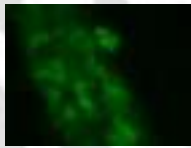
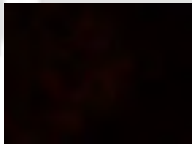
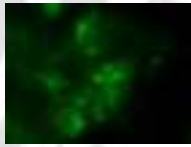
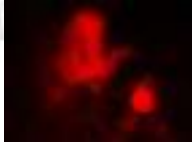
Sample withdrawn on	Stressed phase operation			
	Aerobic sludge biomass withdrawn from reactors			
	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
1 st d				
8 th d				
15 th d				
22 nd d				
29 th d				
36 th d				
43 rd d				

Table 3.8 Filament index of aerobic sludge biomass estimated during stressed phase operation.

Biomass withdrawn on	Stressed phase operation			
Day	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
1 st d	1	1	1	1
8 th d	3	3	3	3
15 th d	5	5	5	5
22 nd d	5	5	5	5
29 th d	5	5	5	5
36 th d	5	5	5	5
43 rd d	5	5	5	4

Table 3.9 Fluorescence microscopic images of stained aerobic sludge biomasses withdrawn from reactors R_{amx0}, R_{amx500}, R_{amx1000}, and R_{amx2000} during stressed phase operation.

Sample withdrawn on	Rector	Bright field (20×)	Dead Cells (20×)	Live Cells (20×)
1 st d	R _{amx0}			
	R _{amx500}			
	R _{amx1000}			
	R _{amx2000}			
43 rd d	R _{amx0}			
	R _{amx500}			
	R _{amx1000}			
	R _{amx2000}			

caused increase in FI value with time. However, this aspect needed to be investigated. Moreover, the cell viability test has suggested adverse impact of AMX stress on active portion of aerobic sludge biomass.

Polysaccharide, protein and extracellular polymeric substance

PS, PN, and EPS are secreted by microbial cells, which help them adhere to each other. It also forms a layer over the bacterial cell surface that protect them from the toxicity of lethal compounds. PS, PN, and EPS contents in aerobic sludge biomass have been estimated on 1st and 43rd d of the operation. The results have been shown in Fig. 3.11 and data has been presented in Table 3.10. On 1st and 43rd d of operation, PS content for reactor R_{amx0} biomass has been obtained as 12.32 and 14.20 mg/g VSS, respectively, simultaneously, PN content has been obtained as 36.23 and 37.43 mg/g VSS, respectively, and EPS contents has been obtained as 48.55 and 51.63 mg/g VSS, respectively. AMX fed reactors have shown increasing trends in PS, PN, and EPS contents in aerobic sludge biomass. The PS content for AMX fed reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses have been observed as 14.23, 15.23, and 16.53 mg/g VSS, respectively, on 43rd d of operation. Similarly, PN content for AMX fed reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses have been observed as 39.43, 40.20, and 41.23 mg/g VSS, respectively. While, 53.66, 55.43, and 57.76 mg/g VSS of EPS have been obtained for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively, on 43rd d of operation. The literature suggests that the PS, PN, and EPS contents in aerobic sludge biomass increase with increase in AMX concentration, which helps microbes to protect from AMX toxicity (Gao et al., 2018). It is also reported that antibiotics including ofloxacin, sulfadiazine, and tetracycline could also promote EPS production by activated sludge (Yu et al., 2019). The high concentration of PS, PN, and EPS layer on the sludge surface might absorb more AMX, preventing it from going into the interior of microbes and alleviating AMX stress to microbes. The secretion of PS, PN, and EPS by aerobic sludge biomass also help in biosorption of AMX (Shi et al., 2013; Yu et al., 2019).

Enzyme activity

The impact of AMX on catalase, dehydrogenase, protease and urease activities have been estimated on 1st and 43rd d of the operation. The results have been shown in Fig. 3.12 and summary of enzyme activities have been presented in Table 3.11. On 1st d of operation, catalase activity (E_{Catalase}) for all reactors has ranged between 0.33-0.36 mg/g MLSS h. Towards the end of the stressed phase (on 43rd d), E_{Catalase} has been observed as 0.37, 0.18, 0.14, and 0.06 mg/g MLSS h for reactor R_{amx0}, R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively [Fig. 3.12(a)]. The obtained results suggest that the addition of AMX has lowered the E_{Catalase} of aerobic sludge biomass and AMX concentration at 500 mg/L has reduced E_{Catalase} almost half as compared to reactor R_{amx0} biomass. Moreover, higher AMX concentrations (1000 and 2000 mg/L) have severely affected E_{Catalase} . It can be observed that E_{Catalase} has decreased with increase in AMX concentration in reactor biomasses. There is no literature available which could explain the effect of AMX on E_{Catalase} . The % inhibition of E_{Catalase} for

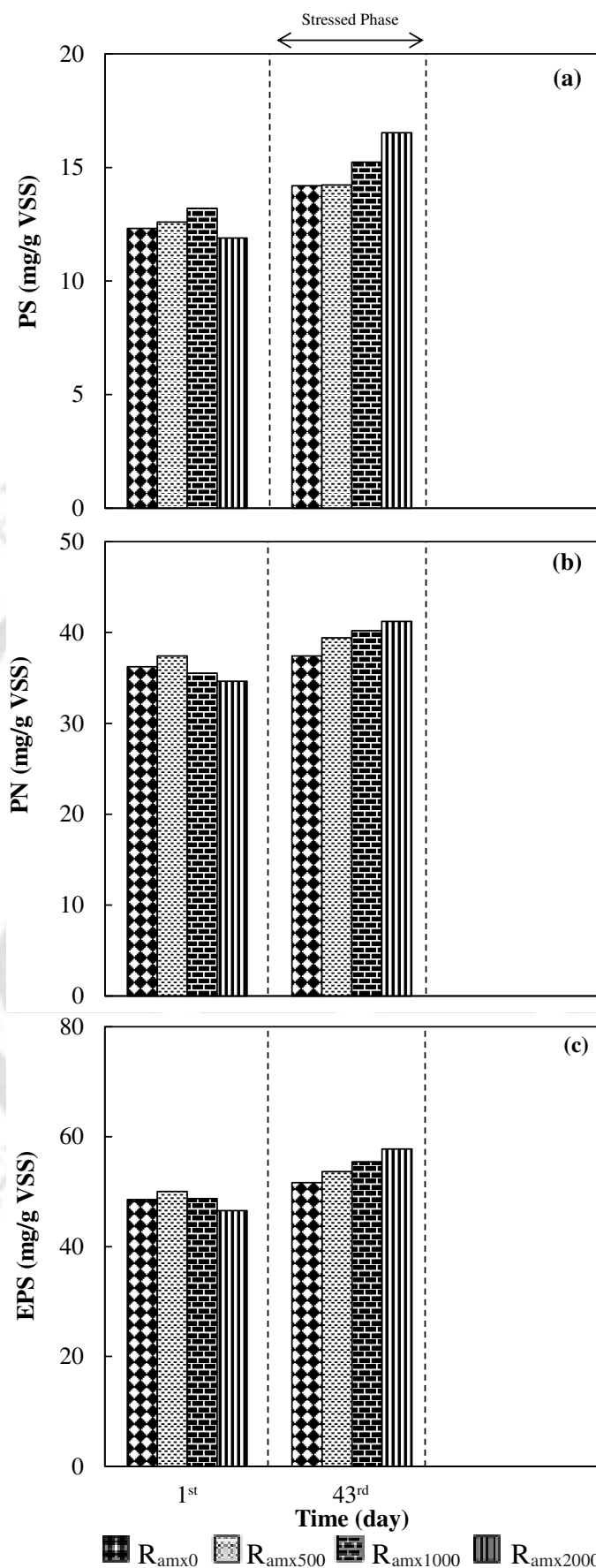


Fig. 3.11 (a) PS, (b) PN, and (c) EPS contents in aerobic sludge biomass estimated during stressed phase operation.

Table 3.10 PS, PN, and EPS contents in aerobic sludge biomass estimated during stressed phase operation.

Biomass withdrawn on	Reactor biomass	PS	PN	EPS
		mg/g VSS		
Stressed phase operation				
1 st d	R _{amx0}	12.32 ±0.62	36.23±0.70	48.55±0.66
	R _{amx500}	12.60 ±1.03	37.43±2.10	50.03±1.56
	R _{amx1000}	13.20±0.92	35.53±2.00	48.73±1.46
	R _{amx2000}	11.89±0.63	34.65±2.20	46.54±1.41
43 rd d	R _{amx0}	14.20±1.07	37.43±0.90	51.63±0.98
	R _{amx500}	14.23±0.46	39.43±1.90	53.66±1.18
	R _{amx1000}	15.23±0.72	40.20±1.40	55.43±1.06
	R _{amx2000}	16.53±0.83	41.23±1.10	57.76±0.96

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

AMX fed reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses has been obtained as 51.4%, 62.0%, and 84.6%, respectively.

The results for dehydrogenase activity ($E_{\text{Dehydrogenase}}$) has been shown in Fig. 3.12(b) and data has been summarized in Table 3.11. $E_{\text{Dehydrogenase}}$ for all reactors has ranged 22.42-24.23 $\mu\text{g/g MLSS h}$ on 1st d of operation. Towards the end of the stressed phase (on 43rd d) operation, $E_{\text{Dehydrogenase}}$ for reactor R_{amx0}, R_{amx500}, R_{amx1000} and R_{amx2000} biomasses has been observed as 27.34, 20.34, 15.23, and 13.43 $\mu\text{g/g MLSS h}$, respectively. Fig. 3.12(b) exhibits that lower concentration of AMX has a slight effect on $E_{\text{Dehydrogenase}}$ while higher dose of AMX (2000 mg/L) has shown significant impact on $E_{\text{Dehydrogenase}}$. The activity for reactor R_{amx2000} biomass has been significantly reduced to almost half as compared to reactor R_{amx0} biomass. The % inhibition of $E_{\text{Dehydrogenase}}$ enzyme for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses has been 25.6%, 44.3%, and 50.9%, respectively, towards the end of the stressed phase operation.

The effect of AMX on protease activity (E_{Protease}) has been shown in Fig. 3.12(c) and data has been summarized in Table 3.11. Initially (1st d of operation), E_{Protease} has been observed between 1.63-1.74 mg/g MLSS h. E_{Protease} has been affected due to AMX stress. On 43rd d of operation, E_{Protease} has been observed as 1.65, 1.32, 0.85, and 0.76 mg/g MLSS h for reactor R_{amx0}, R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively. The % inhibition of E_{Protease} of reactor fed with 500 mg/L, 1000 mg/L and 20000 mg/L of AMX has been 20.1%, 48.3% and 53.8%, respectively. It implies that increased AMX concentration in feed has a negative effect on E_{Protease} . The high dose of AMX has caused up to 53.8% E_{Protease} inhibition towards the end of the stressed phase.

Urease activity (E_{Urease}) has also been affected due to presence of AMX in feed as shown in Fig. 3.12(d) and Table 3.11. The addition of AMX has caused a negative impact on E_{Urease} . There has been minor changes in E_{Urease} for all reactors as the values ranged 9.82-10.73 g/g MLSS h on 1st d of operation. On the other hand, E_{Urease} has significantly

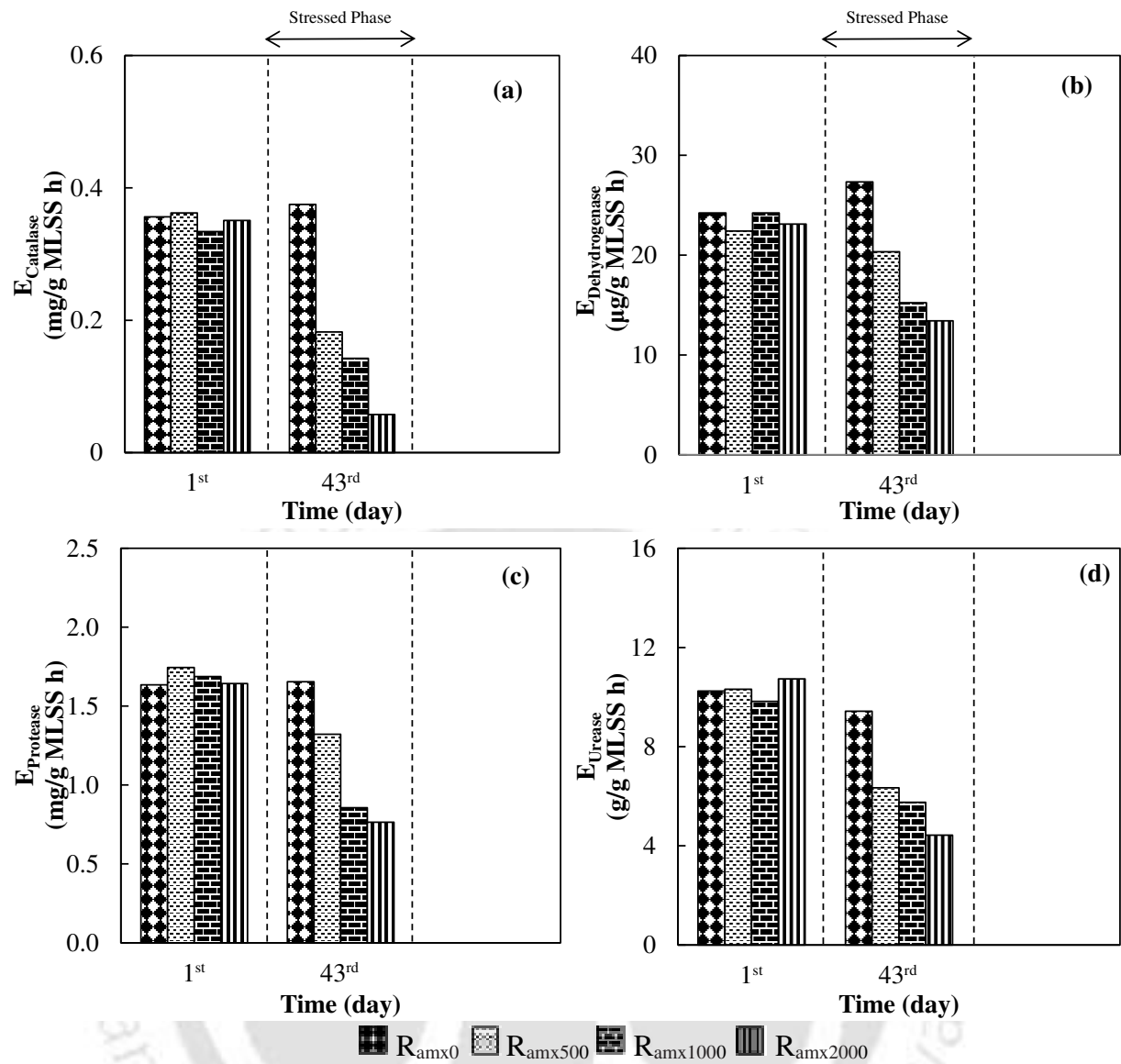


Fig. 3.12 Enzyme activities of aerobic sludge biomass assessed during stressed phase operation (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} .

Table 3.11 Summary of enzyme activities of aerobic sludge biomass during stressed phase operation.

Biomass withdrawn on	Reactor biomas	E_{Catalase} mg/g MLSS h	$E_{\text{Dehydrogenase}}$ μg/g MLSS h	E_{Protease} mg/g MLSS h	E_{Urease} g/g MLSS h
Stressed phase operation					
1 st d	R_{amx0}	0.35 ± 0.03	24.23 ± 2.00	1.63 ± 0.08	10.24 ± 1.12
	R_{amx500}	0.36 ± 0.03	22.42 ± 2.50	1.74 ± 0.05	10.32 ± 1.10
	R_{amx1000}	0.33 ± 0.02	24.23 ± 1.80	1.68 ± 0.15	9.82 ± 1.40
	R_{amx2000}	0.35 ± 0.03	23.13 ± 2.20	1.64 ± 0.07	10.73 ± 0.50
43 rd d	R_{amx0}	0.37 ± 0.02	27.34 ± 2.98	1.65 ± 0.09	9.43 ± 2.32
	R_{amx500}	0.18 ± 0.02	20.34 ± 1.50	1.32 ± 0.06	6.34 ± 0.50
	R_{amx1000}	0.14 ± 0.02	15.23 ± 1.70	0.85 ± 0.13	5.76 ± 0.60
	R_{amx2000}	0.05 ± 0.02	13.43 ± 1.10	0.76 ± 0.17	4.43 ± 1.10

[The data is presented in $xx \pm yy$ format where xx is the average value, yy is the standard deviation.]

decreased towards the end of stressed phase. E_{Urease} has been obtained as 9.43, 6.34, 5.76, and 4.43 g/g MLSS h for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, on 43rd d of operation. It has been observed that E_{Urease} has been inhibited to 32.8%, 38.9%, and 53.0% by 500, 1000, and 2000 mg/L of AMX fed reactors, respectively.

The results indicate that AMX affects the enzyme activities and impacts depend on AMX concentration levels. Nevertheless, 500 mg/L of AMX can cause slight inhibition on enzyme activities, whereas higher doses of AMX can severely affect the enzyme activity. This may also result in slow biotransformation of organic matter or pollutant depending on the enzyme activity produced by aerobic sludge biomass (Jin et al., 2003; Liwarska-Bizukojc et al., 2015). Hence, decrease in enzyme activities could also be one of the reasons for decrease in COD removal during stressed phase operation for AMX fed reactors.

Amplified ribosomal DNA restriction analysis (ARDRA)

The aerobic sludge biomass samples have been withdrawn on 43rd d to examine through ARDRA. The samples have been digested with tetra cutter restriction enzymes *AluI*, *HhaI*, and *MspI* and Fig. 3.13 shows the gel run after the treatment. The similarity matrix generated by GelJ software has been represented in Table 3.12. The results obtained after gel run (Fig. 3.13) implies that the reactor biomasses show different banding pattern for each restriction enzymes. The dendrogram has demonstrated the relationship among all the reactor biomasses. Fig. 3.13(a) exhibits that the biomass from reactor R_{amx500} is closest to the reactor R_{amx0} biomass, whereas biomass from reactor $R_{amx2000}$ exist faraway to reactor R_{amx0} biomass. Similar observations have been made for samples digested with *HhaI* [Fig. 3.13(b)] and *MspI* [Fig. 3.13(c)]. Table 3.12 has shown the similarity among all reactor biomasses for each restriction enzymes obtained on the basis of dendrogram. The aerobic sludge biomass samples have shown 78.6%, 68.7%, and 57.1% of similarity for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, with reactor R_{amx0} biomass, when digested with *AluI*. Similarly, 80.0%, 75.0%, and 74.1% similarity has been observed for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, with reactor R_{amx0} biomass, when digested with *HhaI*. The restriction enzyme *MspI* has shown 72.2%, 59.3%, and 51.9% of similarity for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively.

These findings suggest that increase in AMX concentration in reactors has decreased the similarity between AMX fed reactor as compared to control reactor. Due to higher doses of AMX in feed, it could be possible that only AMX resistant microbes may have persisted in the reactors that could have led to organic removal during stressed phase operation. Hence, it can be concluded that there has been change in microbial community and change depends on the AMX concentration in feed. Disappearance of microbial community could also be a possible reason for decreased COD removal and lowered aerobic sludge biomass activity for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses.

Reassessing the biomass related microbiological parameters have revealed that analyzing the effects of AMX on aerobic sludge biomass using microscopic and photographic

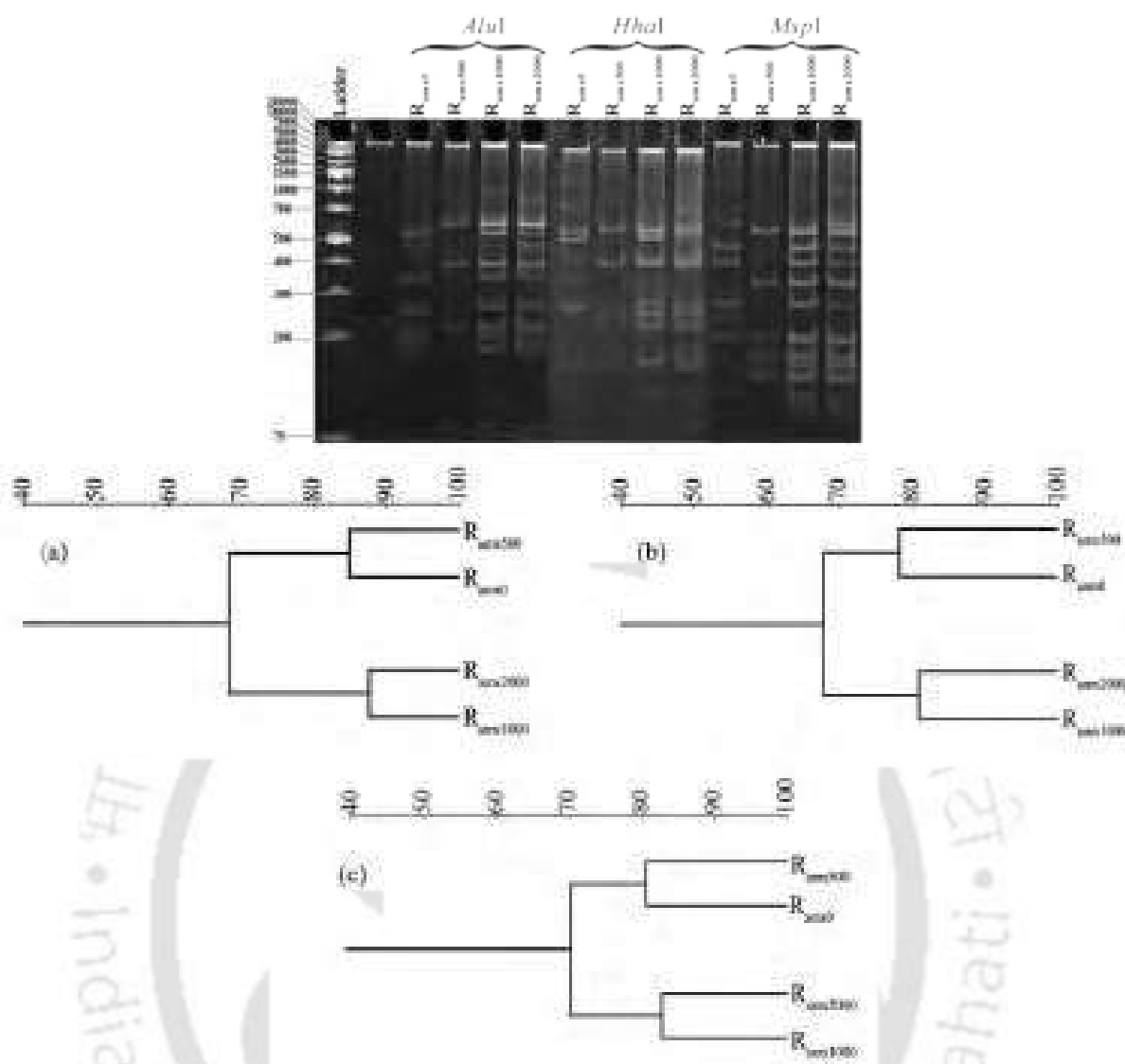


Fig. 3.13 Digestion pattern and dendrogram of aerobic sludge biomass treated with (a) *AluI*, (b) *HhaI*, and (c) *MspI* restriction enzymes under stressed phase operation.

methods is less effective. AMX has not shown substantial impact on the structure and morphology of aerobic sludge biomass. The continuous growth of filamentous microorganisms and dense flocs has been observed in reactor R_{amx500}, and R_{amx1000} biomasses throughout the experiment, and flocs size has also been continuously increasing. In contrast, loose flocs structure has been observed for reactor R_{amx2000} biomass. The flocs size has ranged 1.0-7.0 mm for reactor R_{amx0}, R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses during stressed phase operation. FI values have increased from 1 to 5 for reactor R_{amx0}, R_{amx500}, and R_{amx1000} biomasses, while FI value has reached 4 for reactor R_{amx2000} biomass. The fluorescence microscopic results have shown 97.7%, 87.2%, 39.2%, and 17.3% of live cells and 0.4%, 30.8%, 58.9%, and 76.4% of dead cells for reactor R_{amx0}, R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively, at steady-state condition of stressed phase operation. Increased AMX doses have resulted in a drop in the percentage of live cells and an increase in dead cells. There has been an increase in PS, PN, and EPS with a little increment. The PS content for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses have ranged 14.23-16.53 mg/g VSS, while 14.23 mg/g VSS for reactor

Table 3.12 Similarity matrix for stressed phase aerobic sludge biomass samples resulting from digestion by restriction enzymes.

Reactor	Stressed phase operation											
	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
R_{amx0}	1	0.7857	0.6875	0.5714	1	0.8000	0.7500	0.7407	1	0.7223	0.5934	0.5192
R_{amx500}	0.7857	1	0.5217	0.4444	0.8000	1	0.6897	0.6452	0.7223	1	0.5798	0.6234
$R_{amx1000}$	0.6875	0.5217	1	0.8235	0.7500	0.6897	1	0.8235	0.5934	0.5798	1	0.7534
$R_{amx2000}$	0.5714	0.4444	0.8235	1	0.7407	0.6452	0.8235	1	0.5192	0.6234	0.7534	1

R_{amx0} biomass. Similarly, PN content for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 39.43-41.23 mg/g VSS, while 37.43 mg/g VSS for reactor R_{amx0} biomass. EPS content for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses has ranged 53.66-57.76 mg/g VSS, which is slightly lower than reactor R_{amx0} biomass (51.63 mg/g VSS). Since PS, PN, and EPS contents in the AMX fed reactors and the control reactor have shown close similarity, indicating that PS, PN, and EPS do not contribute to the evaluation of aerobic sludge biomass under stressed conditions. $E_{Catalase}$ activity has most severely affected of the four enzymes tested. $E_{Catalase}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 0.05-0.18 mg/g MLSS h, while 0.37 mg/g MLSS h for reactor R_{amx0} biomass. $E_{Dehydrogenase}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 13.43-20.34 μ g/g MLSS h, while 27.34 μ g/g MLSS h for reactor R_{amx0} biomass. $E_{Protease}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 0.76-1.32 mg/g MLSS h, while 1.65 mg/g MLSS h for reactor R_{amx0} biomass. Similarly, E_{Urease} for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged from 4.43-6.34 g/g MLSS h, while 9.43 g/g MLSS h for reactor R_{amx0} biomass. $E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease} appear to be less effective than $E_{Catalase}$ in evaluating the stresses induced by AMX on aerobic sludge biomass. ARDRA has revealed the changes in microbial community structure. *AluI* has shown 78.6%, 68.7%, and 57.1% of similarity; *HhaI* has shown 80.0%, 75.0%, and 74.0% of similarity; and *MspI* has shown 72.2%, 59.3%, and 51.9% of similarity for reactor biomasses fed with AMX concentration of 500, 1000, and 2000 mg/L, respectively, as compared to reactor R_{amx0} biomass. ARDRA shows a significant change in microbial community structure with increased doses of AMX. It has been observed that fluorescence microscopy and ARDRA significantly preferable than microscopic and photographic examinations and enzyme activity for making on assessment of impact on aerobic sludge biomass. Also, it has been perplexing to note that physical conditions of aerobic sludge biomass has impaired in terms of filamentous growth and flocs sizes under stressed phase operation even-though COD removal, portion of live cells, and ASBA have declined significantly. It calls for in-depth research to delineate increase in biomass and reason for the same.

3.3.2 Recovery phase operation

The addition of AMX has been discontinued when reactors have achieved steady-state condition during the stressed phase (after 43rd d of operation) and thereafter operated for 44-78 d without AMX in the feed terming as recovery phase operation. To highlight the differences between observations and results of recovery phase operation with the stressed phase operation, most of the figures and tables have been reproduced with the recovery phase operation results along with stressed phase operation.

3.3.2.1 Environmental and performance parameters

The reactors have been operated for 35 d during recovery phase operation. The results obtained during recovery phase operation have been shown in Fig. 3.14 to 3.17 and summary of data has been presented in Table 3.13. Similar to stressed phase operation, room temperature and pH have not been controlled during recovery phase operation. The variation

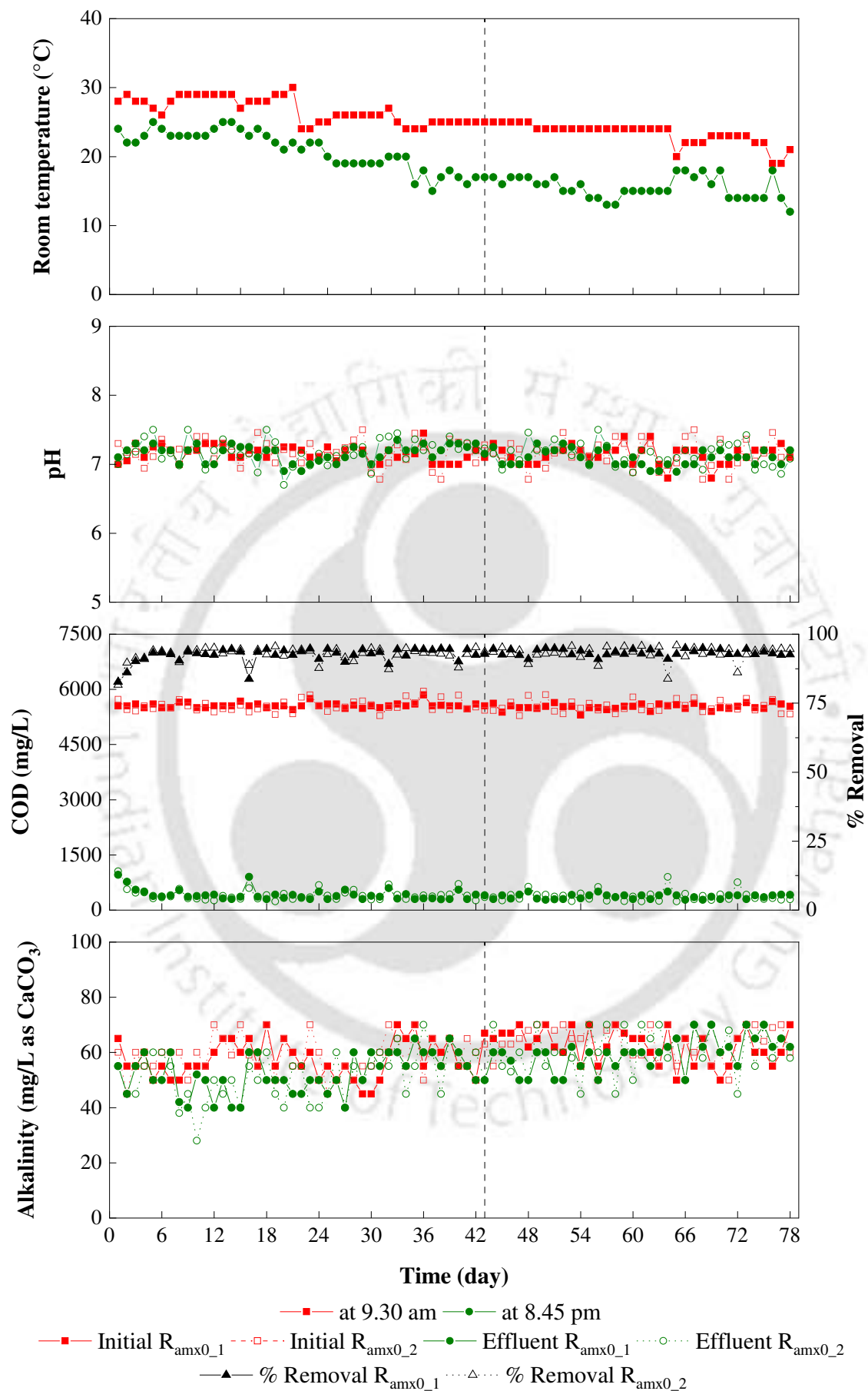


Fig. 3.14 Variation in environmental and performance related parameters for reactor R_{amx0} during 44-78 d of operation along with 0-43 d of operation [without AMX in the feed].

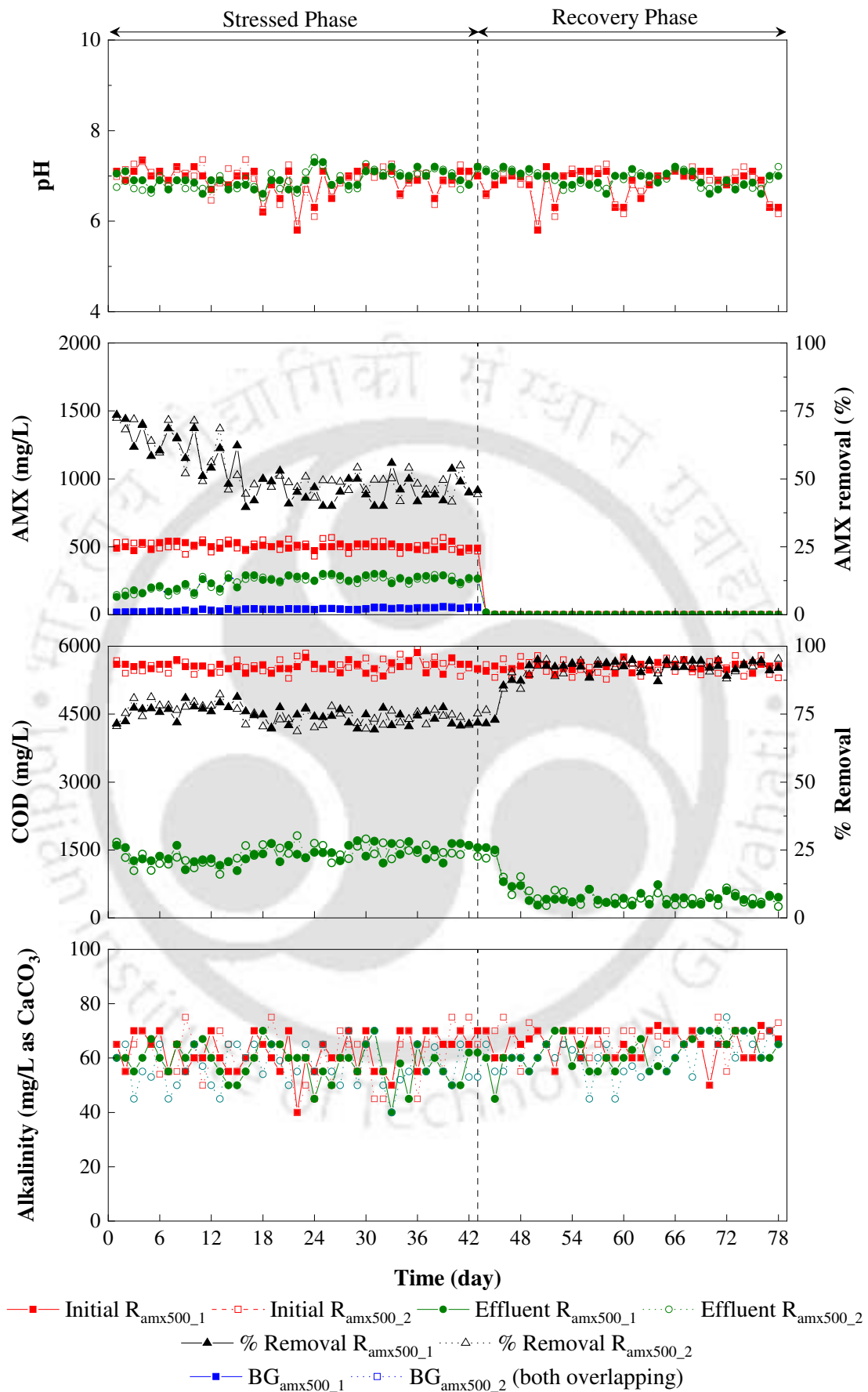


Fig. 3.15 Variation in environmental and performance related parameters for reactor R_{amx500} operated during 44-78 d of recovery phase operation along with data for stressed phase operation.

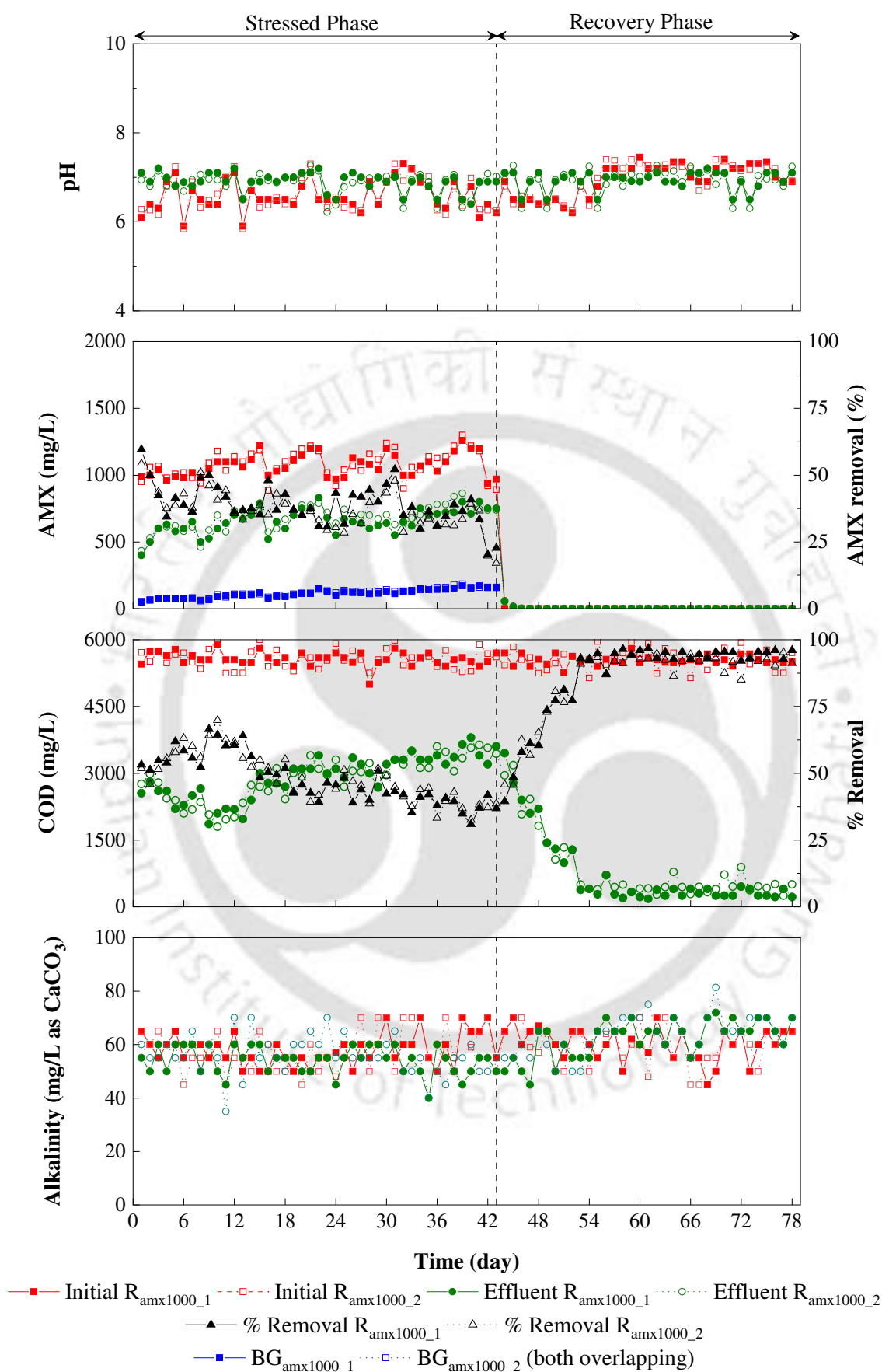


Fig. 3.16 Variation in environmental and performance related parameters for reactor $R_{amx1000}$ operated during 44-78 d of recovery phase operation along with data for stressed phase operation.

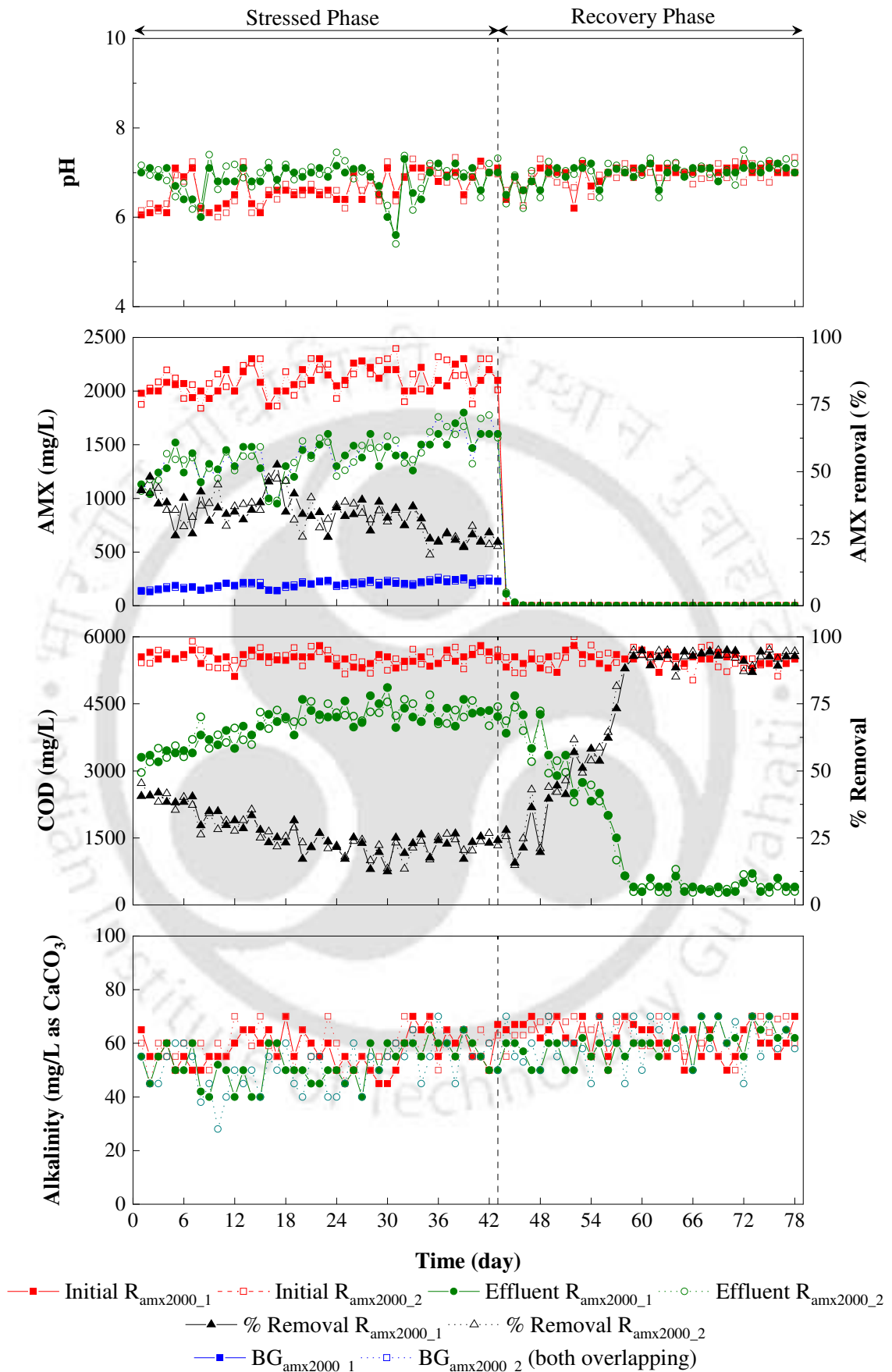


Fig. 3.17 Variation in environmental and performance related parameters for reactor $R_{amx2000}$ operated during 44-78 d of recovery phase operation along with data for stressed phase operation.

Table 3.13 Summary of environmental and performance related parameters for reactors operated during 44-78 d of recovery phase operation along with data for stressed phase operation (0-43 d).

Parameters	Day-wise	0-43 d			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	4.25	3.00	0.50
	AMX (mg/L)	0	500	1000	2000
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	26.7±1.8 (43)	26.7±1.8 (43)	26.7±1.8 (43)
		Room temperature (°C) at 8.45 pm	20.8±2.7 (43)	20.8±2.7 (43)	20.8±2.7 (43)
		Initial pH	7.17±0.14 (86)	6.91±0.32 (86)	6.59±0.37 (86)
		Initial AMX (mg/L)	0	505.37±27.39 (86)	1078.77±95.60 (86)
	Performance	Effluent pH	7.17±0.15 (86)	6.91±0.19 (86)	6.88±0.23 (86)
		Effluent AMX (mg/L)	0	240.96±47.03 (86)	669.00±91.92 (86)
		AMX removal (%)	0	52.26±9.53 (86)	37.93±7.43 (86)
		Background AMX (mg/L)	0	38.40±11.38 (86)	115.53±32.98 (86)
		Initial soluble COD (mg/L)	5558.51±114.22 (86)	5558.51±127.89 (86)	5572.78±186.01 (86)
		Effluent soluble COD (mg/L)	417.56±153.74 (86)	1408.94±189.07 (86)	2902.02±492.55 (86)
		COD removal (%)	92.49±2.73 (86)	74.64±3.24 (86)	47.87±9.00 (86)
		Initial alkalinity (mg CaCO ₃ /L)	64.24±7.67 (86)	61.56±8.27 (86)	58.24±7.04 (86)
		Effluent alkalinity (mg CaCO ₃ /L)	59.03±6.96 (86)	57.57±7.10 (86)	55.00±6.24 (86)
Parameters	Day-wise	44-78 d			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	5.50	5.50	5.50
	AMX (mg/L)	0	0	0	0
Recovery phase	Environmental	Room temperature (°C) at 9.30 am	23.2±1.5 (35)	23.2±1.5 (35)	23.2±1.5 (35)
		Room temperature (°C) at 8.45 pm	15.4±1.6 (35)	15.4±1.6 (35)	15.4±1.6 (35)
		Initial pH	7.14±0.17 (70)	6.83±0.33 (70)	6.96±0.37 (70)
		Effluent pH	7.12±0.14 (70)	6.93±0.16 (70)	6.92±0.25 (70)
	Performance	Effluent AMX (mg/L)	0	0.55±2.52 (70)	2.12±9.56 (70)
		Initial soluble COD (mg/L)	5531.38±118.28 (70)	5525.70±131.07 (70)	5555.90±173.02 (70)
		Effluent soluble COD (mg/L)	372.82±106.65 (70)	501.53±280.87 (70)	797.97±806.73 (70)
		COD removal (%)	93.26±1.92 (70)	90.91±5.11 (70)	85.62±14.76 (70)
		Initial alkalinity (mg CaCO ₃ /L)	61.79±9.95 (70)	65.93±5.77 (70)	60.37±6.73 (70)
		Effluent alkalinity (mg CaCO ₃ /L)	61.64±6.71 (70)	61.36±6.43 (70)	62.98±7.15 (70)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

in room temperature has been observed due to weather changes. The average variation in room temperature recorded at 9.30 am and 8.45 pm has been observed as 23.2 ± 1.5 and $15.4 \pm 1.6^\circ\text{C}$, respectively (Table 3.13). The average initial and effluent pH for reactor $R_{\text{amx}0}$ have been obtained as 7.14 ± 0.17 and 7.12 ± 0.14 during recovery phase operation (Table 3.13). The reactor $R_{\text{amx}0}$ has shown uniform pH throughout the operation (Fig. 3.14). However, pH for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have reached to neutral during recovery phase operation. The average pH variation during recovery phase operation has slightly changed from stressed phase operation. During stressed phase operation, average initial pH for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been obtained as 6.91 ± 0.32 , 6.59 ± 0.37 , and 6.64 ± 0.38 , respectively, whereas effluent pH has been 6.91 ± 0.19 , 6.88 ± 0.23 , and 6.86 ± 0.38 , respectively (Table 3.13). During recovery phase operation, the average initial pH for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been obtained as 6.83 ± 0.33 , 6.96 ± 0.37 , and 6.95 ± 0.23 , respectively, whereas effluent pH has been observed as 6.93 ± 0.16 , 6.92 ± 0.25 , and 6.98 ± 0.25 , respectively (Table 3.13).

When reactors have been achieved steady-state condition in terms of COD removal during stressed phase operation and operated further to obtain steady-state data, then AMX addition has been discontinued in the feed and the recovery phase study has been started. The residual AMX in effluent has been detected during initial days of recovery phase operation (Fig. 3.15 to 3.17). On 44th d, the effluent AMX concentration has been 15.2, 55.6, and 117.3 mg/L for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$, respectively, whereas it has decreased with in two days (on 46th d) to 0.10 mg/L of AMX for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ as shown in Fig. 3.15 to 3.17.

The variations in COD removal have been shown in Fig. 3.14 to 3.17 and summary of data has been presented in Table 3.13. The reactor $R_{\text{amx}0}$ has exhibited uniform COD removal throughout the operation (Fig. 3.14). While reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have shown gross recovery in COD removal (Fig. 3.15 to 3.17). The reactor $R_{\text{amx}500}$ has shown quick COD removal recovery after discontinuation of AMX (Fig. 3.15). On 44th, 47th, and 78th d of operation, COD removal has been observed as 74.2%, 89.4%, and 93.8% for reactor $R_{\text{amx}500}$. Similarly, COD removal for reactor $R_{\text{amx}1000}$ has been obtained as 42.5%, 92.0%, and 93.5%, respectively, on 44th, 52nd, and 78th d of operation (Fig. 3.16). While, reactor $R_{\text{amx}2000}$ has shown 26.7%, 93.8%, and 93.6% of COD removal on 44th, 53rd, and 78th d, respectively, of operation in recovery phase (Fig. 3.17). During recovery phase operation, COD removal for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have drastically improved as compared to stressed phase operation. During stressed phase operation, the average COD removal for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been observed as 74.6%, 47.9%, and 26.7%, respectively, which further increased to 90.9%, 85.6%, and 73.0%, respectively, during recovery phase operation (Table 3.13). The reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ took 8, 12, and 18 d, respectively, to recover from the adverse impact of AMX. Meng et al. (2017) have also reported that the performance of EGSB has recovered to its normal level after the long-term effect of cefalexin (CFX).

A slight variation in alkalinity has been observed for all the reactors, and it varies in the range of 60–66 mg CaCO_3/L . During recovery phase operation, the alkalinity values for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been observed similar to reactor $R_{\text{amx}0}$.

The steady-state condition has been achieved after 17–20 d of recovery phase operation, and reactors have been operated in steady-state condition during 72–78 d. The summary of data of steady-state performance during recovery phase operation has been presented in Table 3.14. The steady-state operation has shown stabilization in the performance-related parameters. Both initial and effluent pH for all the reactors have been observed in the range of 6.85–7.18; whereas, the initial and effluent pH values have ranged 6.45–7.25 during the steady-state condition of the stressed phase operation (Table 3.14). The steady-state COD removal for reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have been obtained as 92.2%, 92.9%, and 91.7%, respectively, during recovery phase, which has significantly improved from 73.5%, 37.7%, and 23.4%, respectively, of the stressed phase steady-state COD removal (Table 3.14). The COD removal profile suggests that reactors have recovered from AMX impact and have shown significant organic removal. Minimum variation in alkalinity values have been obtained during the steady-state of the recovery phase operation, and it varied in the range of 61–67 mg CaCO_3/L .

3.3.2.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The variation in solids characteristics and SVI has been shown in Fig. 3.18 and data has been summarized in Table 3.15. The presence of AMX in feed has lowered biomass growth during stressed phase operation, whereas discontinuation of AMX in feed during recovery phase operation has allowed reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ to recover in terms of biomass growth. The sudden decrease in solids concentration (just after 43rd d) in all reactors is due to biomass withdrawal for ASBA test during stressed phase operation. During recovery phase operation, reactor $R_{\text{amx}0}$ has shown continuous biomass growth. From 44th d to 50th d, the MLSS concentration in reactor $R_{\text{amx}0}$ has increase from 3198 mg/L to 4104 mg/L.

Similarly, reactor $R_{\text{amx}500}$ has also shown increment in MLSS from 2578 mg/L to 3588 mg/L from 44th d to 50th d, which indicate that the reactor $R_{\text{amx}500}$ has shown accelerated biomass growth with an increased SVI (202 mL/g on 72nd d) [Fig. 3.18(b)]. The MLSS concentration in $R_{\text{amx}1000}$ has been observed as 2334 mg/L on 44th d which has increased to 3255 mg/L on 50th d. On the other hand, reactor $R_{\text{amx}2000}$ has shown slower increment in MLSS as 2134 mg/L on 44th d to 2644 mg/L on 50th d, which indicate that the reactor $R_{\text{amx}1000}$ has shown faster biomass growth than reactor $R_{\text{amx}2000}$. The SVI has been observed as 195 mg/L for reactor $R_{\text{amx}1000}$ biomass [Fig. 3.18(c)] and 133 mL/g for reactor $R_{\text{amx}2000}$ biomass [Fig. 3.18(d)] on 72nd d. The reactors $R_{\text{amx}0}$ and $R_{\text{amx}500}$ have shown continuous growth of biomass, whereas reactors $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ have slower growth during 44–58 d of operation. Later on, the significant biomass growth have been observed for all the reactors during recovery phase operation. The MLVSS/MLSS ratio has been observed close to 0.82 for

Table 3.14 Summary of environmental and performance related parameters for reactors operated during steady-state of recovery phase operation (72-78 d) along with data for stressed phase operation (37-44 d).

Parameters	Day-wise	37-43 d			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	4.25	3.00	0.50
	AMX (mg/L)	0	500	1000	2000
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	25.0±0.0 (7)	25.00±0.00 (7)	25.0±0.0 (7)
		Room temperature (°C) at 8.45 pm	16.7±0.9 (7)	16.7±0.9 (7)	16.7±0.9 (7)
		Initial pH	7.10±0.16 (14)	6.93±0.25 (14)	6.45±0.31 (14)
		Initial AMX (mg/L)	0	497.18±32.20 (14)	1122.86±140.51 (14)
	Performance	Effluent pH	7.25±0.08 (14)	7.02±0.17 (14)	6.81±0.27 (14)
		Effluent AMX (mg/L)	0	265.75±21.71 (14)	764.27±45.09 (14)
		AMX removal (%)	0	46.47±4.11 (14)	31.14±8.14 (14)
		Background AMX (mg/L)	0	52.30±4.39 (14)	162.05±10.51 (14)
		Initial soluble COD (mg/L)	5556.10±108.64 (14)	5556.10±131.19 (14)	5535.71±178.07 (14)
		Effluent soluble COD (mg/L)	393.19±117.52 (14)	1473.67±139.15 (14)	3449.57±217.01 (14)
		COD removal (%)	92.95±1.99 (14)	73.49±2.33 (14)	37.68±3.45 (14)
		Initial alkalinity (mg CaCO ₃ /L)	67.14±5.08 (14)	67.86±4.83 (14)	62.43±7.86 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	55.71±6.81 (14)	56.36±5.48 (14)	52.14±4.33 (14)
Parameters	Day-wise	72-78 d			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	5.50	5.50	5.50
	AMX (mg/L)	0	0	0	0
Recovery phase	Environmental	Room temperature (°C) at 9.30 am	21.3±1.7 (7)	21.29±1.7 (7)	21.3±1.7 (7)
		Room temperature (°C) at 8.45 pm	14.3±1.8 (7)	14.3±1.8 (7)	14.3±1.8 (7)
		Initial pH	7.18±0.12 (14)	6.77±0.36 (14)	7.13±0.18 (14)
		Effluent pH	7.09±0.14 (14)	6.85±0.16 (14)	6.90±0.26 (14)
	Performance	Initial soluble COD (mg/L)	5540.00±122.36 (14)	5540.00±143.54 (14)	5570.14±175.24 (14)
		Effluent soluble COD (mg/L)	384.05±106.86 (14)	433.86±122.91 (14)	399.29±149.23 (14)
		COD removal (%)	93.07±1.95 (14)	92.18±2.19 (14)	92.88±2.47 (14)
		Initial alkalinity (mg CaCO ₃ /L)	67.14±9.11 (14)	65.71±5.80 (14)	61.43±5.56 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	62.14±7.57 (14)	66.07±5.03 (14)	66.79±3.86 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

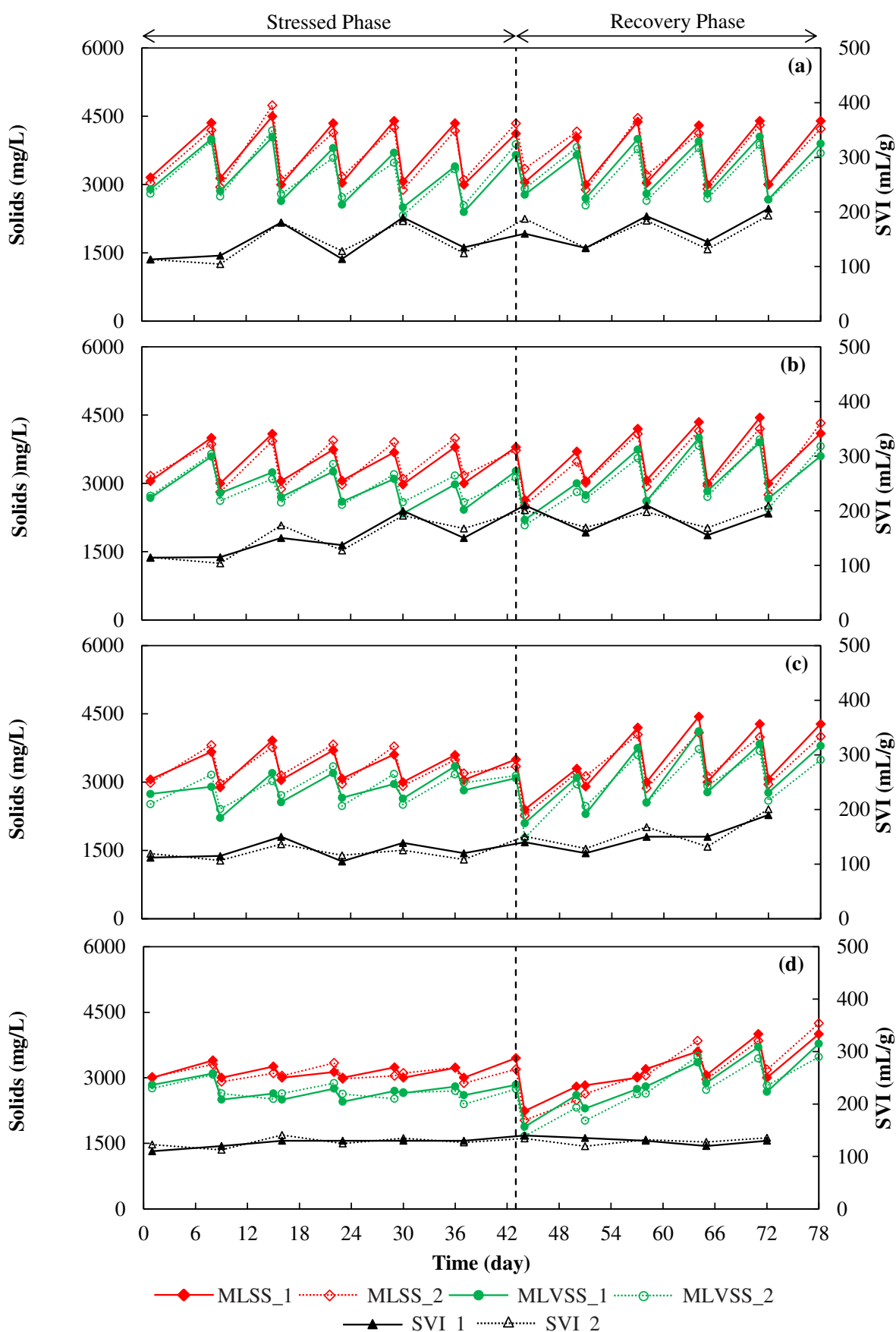


Fig. 3.18 Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ biomasses during 44-78 d of recovery phase operation along with data for stressed phase operation.

Table 3.15 Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.

Gross parameters	Stressed phase operation				
	Day-wise	0-43 d			
	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
MLSS (mg/L)		3056.00±89.42 (12)	3021.67±94.25 (12)	3027.00±91.81 (12)	2994.33±43.66 (12)
MLVSS (mg/L)		2649.40±193.21 (12)	2596.40±120.60 (12)	2605.21±211.50 (12)	2605.32±131.39 (12)
MLVSS/MLSS ratio		0.90±0.02 (12)	0.88±0.05 (12)	0.83±0.04 (12)	0.88±0.05 (12)
SVI (mL/g)		140.54±34.44 (12)	145.28±33.68 (12)	121.14±14.22 (12)	125.99±9.09 (12)
ISV (m/h)		1.50±0.32 (12)	1.49±0.37 (12)	1.87±0.12 (12)	1.93±0.08 (12)
Gross parameters	Recovery phase operation				
	Day-wise	44-78 d			
MLSS (mg/L)		3045.80±112.42 (10)	2892.60±190.03 (10)	2874.20±314.25 (10)	2819.20±418.72 (10)
MLVSS (mg/L)		2721.88±97.84 (10)	2555.27±248.96 (10)	2482.37±359.61 (10)	2440.44±461.38 (10)
MLVSS/MLSS ratio		0.82±0.02 (10)	0.82±0.03 (10)	0.82±0.04 (10)	0.82±0.05 (10)
SVI (mL/g)		166.78±30.64 (10)	187.60±22.87 (10)	153.03±27.49 (10)	130.38±6.96 (10)
ISV (m/h)		1.24±0.14 (10)	0.89±0.90 (10)	1.58±0.24 (10)	1.84±0.04 (10)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

all the reactors during recovery phase operation (Table 3.15). The SRT for reactors $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ has been observed in the range of 24-50 d during recovery phase operation.

The SVI for all the reactor biomasses during recovery phase operation have been summarized in Table 3.15. The average SVI for reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ biomasses have been determined as 167, 188, 153, and 130 mL/g, respectively, during recovery phase operation, which has increased from 141, 145, 121, and 126 mL/g, respectively, during stressed phase operation (Table 3.15). The reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, and $R_{\text{amx}1000}$ biomasses have shown abundance of filaments which has resulted in increase in SVI, whereas the higher AMX doses during stressed phase operation have prevented the filamentous growth in reactor $R_{\text{amx}2000}$ biomass during recovery phase operation.

The settling test has also been performed during recovery phase operation as shown in Fig. 3.19. The results suggest that settling behavior of reactor $R_{\text{amx}0}$ biomass has been hindered due to abundance and overgrowth of filamentous microorganisms [Fig. 3.19(a)]; however, reactor $R_{\text{amx}2000}$ biomass has shown uniform settling behavior throughout the operation during recovery phase [Fig. 3.19(b)]. ISV results have also been shown in Fig. 3.20 and data has been summarized in Table 3.14. The ISV for reactor $R_{\text{amx}0}$ and $R_{\text{amx}500}$ biomasses have been obtained as 1.38 and 1.02 m/h on 44th d; and 1.02 and 0.86 m/h on 72nd d of

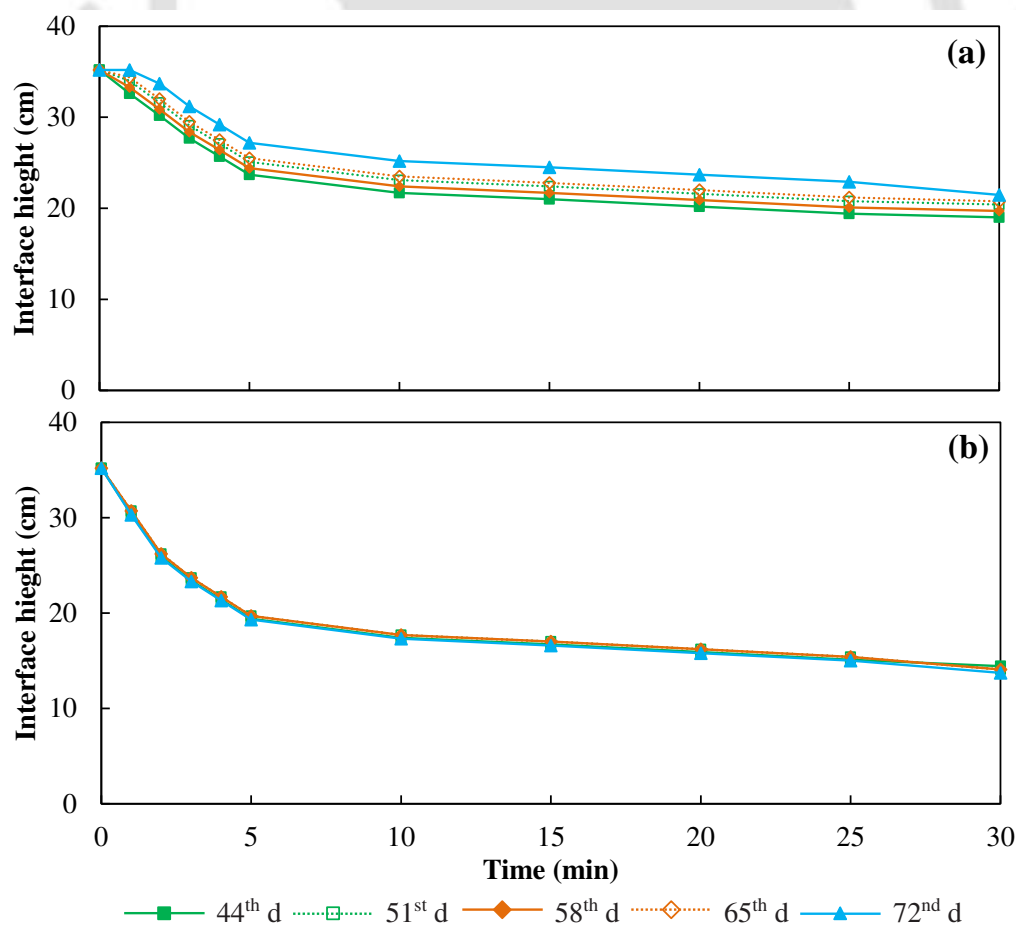


Fig. 3.19 Typical settling test results for reactor (a) $R_{\text{amx}0}$ and (b) $R_{\text{amx}2000}$ biomasses during 44-78 d of recovery phase operation.

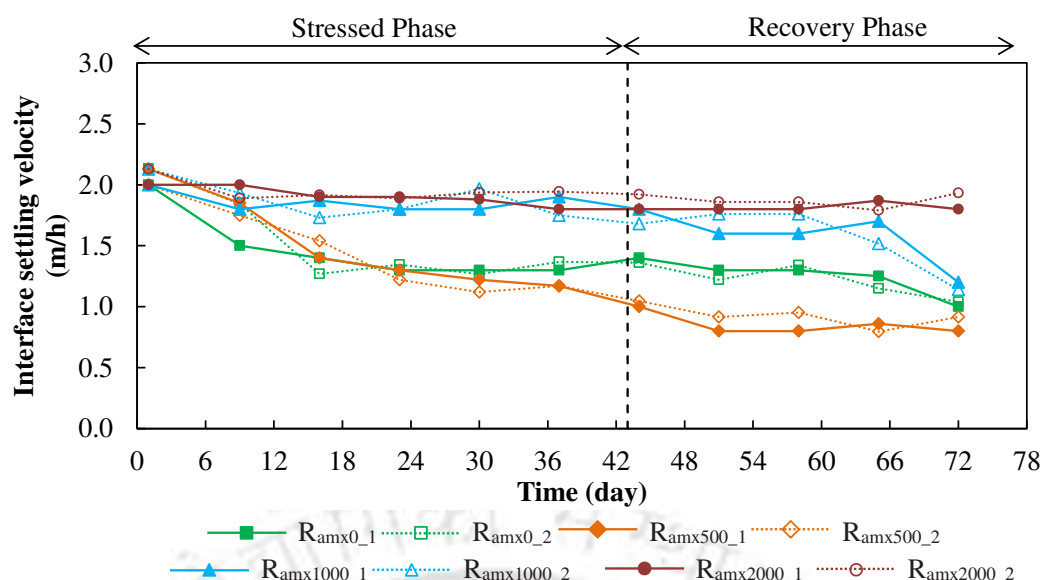


Fig. 3.20 Variation in interface settling velocities of reactor biomasses during 44-78 d of recovery phase operation along with data for stressed phase operation.

operation, respectively (Fig. 3.20). The decrement in settling velocities for reactor $R_{\text{amx}0}$ and $R_{\text{amx}500}$ biomasses might be due to abundance of filamentous microorganism in reactors. Similarly, settling velocities for reactor $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ biomasses have been observed as 1.74 and 1.86 m/h on 44th d; and 1.17 and 1.87 m/h on 72nd d of operation, respectively (Fig. 3.20).

Aerobic sludge biomass activity test

The ASBA test has been carried out to estimate the organic removal capability of aerobic sludge biomass towards the end of the recovery phase operation. When the reactors have achieved steady-state condition in the recovery phase operation, biomass has been withdrawn from all the reactors on 78th d of recovery phase operation and ASBA test has been performed. The test results have been shown in Fig. 3.21, while data has been presented in Table 3.16.

The ASBA results suggest that aerobic sludge biomass from reactors $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ have recovered to a large extent after the termination of AMX in the feed. The ASBA of reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses have been observed as 11.58, 9.22, 8.96, and 7.96 mg COD removed/mg VSS d (Table 3.16), respectively, towards the end of the recovery phase operation, which indicates the reversibility of the inhibition caused by AMX. During the stressed phase operation, ASBA for reactor $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses have been obtained as 7.65, 3.84, and 2.22 mg COD removed/mg VSS d (Table 3.16), respectively, lower than the recovery phase ASBA.

From ASBA results, it is clear that AMX has affected the organic removal capability of aerobic sludge biomass during the stressed phase operation, and the post-effect of AMX has also remained in the recovery phase (especially for higher doses). The reactor $R_{\text{amx}2000}$ biomass has shown slower recovery as compared to reactor $R_{\text{amx}0}$ biomass. The microbial cells repair their enzymes and cellular machinery to metabolize organic matter present in the

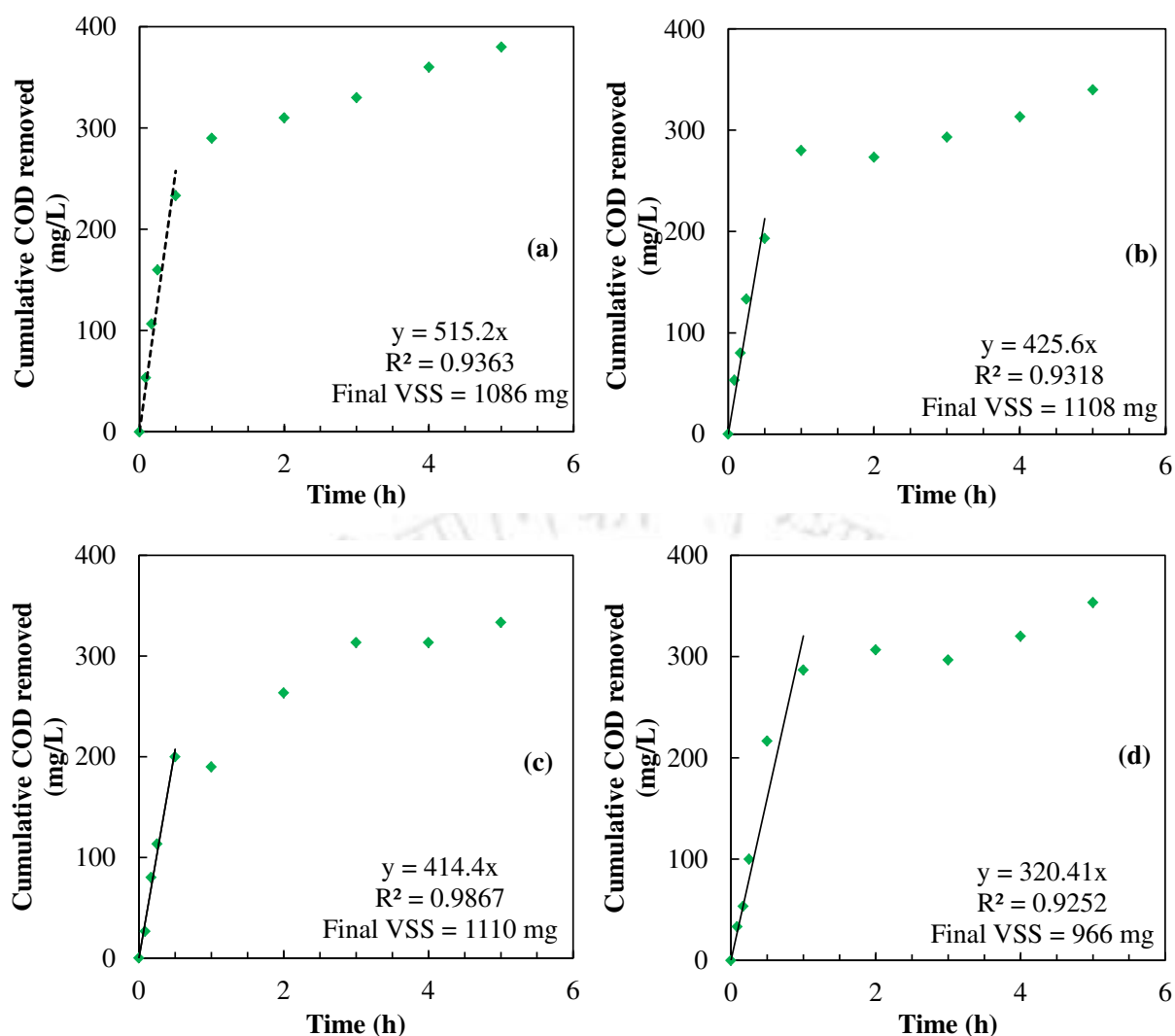


Fig. 3.21 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{amx0} , (b) R_{amx500} , (c) $R_{amx1000}$, and (d) $R_{amx2000}$ on 78th d of recovery phase operation.

Table 3.16 Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.

Stressed phase operation				
Biomass withdrawn on			43 rd d	
Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
ASBA (mg COD removed/mg VSS d)	9.43	7.65	3.84	2.22
Recovery phase operation				
Biomass withdrawn on			78 th d	
ASBA (mg COD removed/mg VSS d)	11.58	9.22	8.96	7.96

feed. The ASBA results have clearly shown that increased concentration of AMX in reactors have caused a reduction in aerobic sludge biomass activity, which could be recovered to a large extent after discontinuation of AMX in the feed.

Biomass related general parameters such as MLSS, MLVSS, MLVSS/MLSS ratio, SVI and ISV during the recovery phase do not provide substantial information regarding changes in the characteristics of aerobic sludge biomass. The average SVI for reactor R_{amx0} , R_{amx500} ,

R_{amx1000} , and R_{amx2000} biomasses have ranged between 130-188 mL/g, whereas ISV have been observed in the range of 0.89-1.84 m/h during recovery phase operation. Comparing both the stressed and recovery phases of operation, it appears that SVI and ISV are quite similar. Biomass related general factors could not be able to differentiate stressed and recovery phase aerobic biomass characteristics. However, ASBA revealed the organic degradation capability of aerobic sludge biomass. The ASBA for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses are 9.22, 8.96, and 7.96 mg COD removed/mg VSS d, which is on a lower side compared to 11.58 mg COD removed/mg VSS d for control reactor R_{amx0} biomass. Similarly, the COD removal for reactors R_{amx0} , R_{amx500} , R_{amx1000} , and R_{amx2000} has been observed $\sim 93\%$, which has indicated the recovery of aerobic sludge biomass.

3.3.2.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

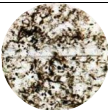
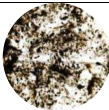
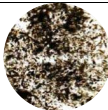
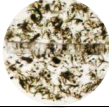
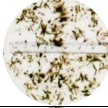
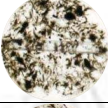
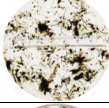
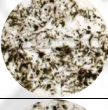
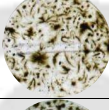
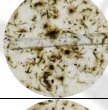
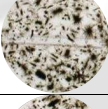
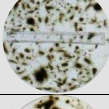
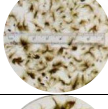
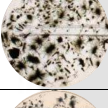
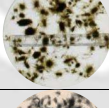
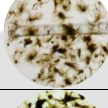
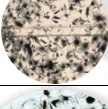
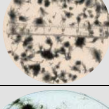
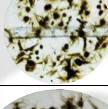
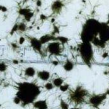
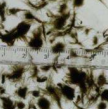
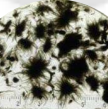
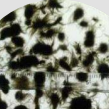
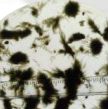
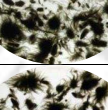
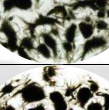
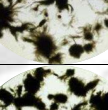
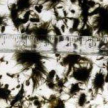
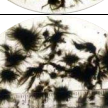
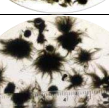
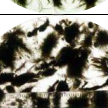
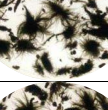
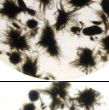
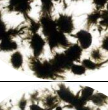
Flocs structure and morphology of aerobic sludge biomass have been examined through microscopic observation after Gram's staining ($5\times$ and $100\times$ magnifications), photographic observation, filament index, and cell viability test. Microscopic images of aerobic biomass have been shown in Table 3.17, which demonstrates that reactor R_{amx0} biomass has shown continuous growth of filamentous microorganisms and fairly dense flocs structure have been observed throughout the experiment (in both stressed and recovery phase operations). The flocs size has also been continuously increasing for reactor R_{amx0} biomass. Similar observations have also been made for reactor R_{amx500} biomass during recovery phase operations (Table 3.17). The floc size of reactor R_{amx500} biomass has been observed unaffected and continuously increasing during recovery phase operation (as well as during stressed phase operation). The discontinuation of AMX in the feed has resulted in increase in flocs size and has also allowed the filamentous growth. As the recovery phase operation has progressed, reactor R_{amx1000} and R_{amx2000} biomasses have shown abundance of filaments (Table 3.17). The microscopic images captured at $5\times$ magnification have shown the dense flocs structure, and $100\times$ magnification images have shown the filament density, which has increased over time for all the reactors. The difference in flocs size and morphology can be clearly observed by comparing the images taken on 50th and 78th d of operation. The images suggest that all four reactors have shown increased flocs size with abundance of strong filaments.

The photographic observations have also been carried out to see the changes in flocs morphology during recovery phase operations, and the images have been presented in Table 3.18. Discontinuation of AMX in recovery phase operation has stimulated the growth of aerobic sludge biomass for reactors R_{amx500} , R_{amx1000} , and R_{amx2000} . Initially (i.e., on 50th d), the biomass morphology and flocs size has remained the same for all the reactors. The flocs sizes of all the reactor biomasses have increased over the time towards the end of the recovery phase operation. However, reactor R_{amx0} and R_{amx500} biomasses have shown uniform flocs structure with time, and flocs size for both reactors have ranged between 2.0-16.0 mm towards the end of the recovery phase operation (Table 3.18). The thick and dense floc

Table 3.17 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with microscopic images of biomasses withdrawn during stressed phase operation.

Reactor	Mag.	Aerobic sludge biomass samples withdrawn on											
		Stressed phase operation						Recovery phase operation					
		1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d	43 rd d	50 th d	57 th d	64 th d	71 st d	78 th d
R_{amx0}	5×												
	100×												
R_{amx500}	5×												
	100×												
$R_{amx1000}$	5×												
	100×												
$R_{amx2000}$	5×												
	100×												

Table 3.18 Photographic images of aerobic sludge biomasses withdrawn from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with images of biomasses withdrawn during stressed phase operation.

Sample withdrawn on	Aerobic sludge biomass withdrawn from reactors			
	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
Stressed phase operation	1 st d			
	8 th d			
	15 th d			
	22 nd d			
	29 th d			
	36 th d			
	43 rd d			
	50 th d			
Recovery phase operation	57 th d			
	64 th d			
	71 th d			
	78 th d			
	78 th d			

structures have been observed towards the end of the recovery phase for reactor $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ biomasses within the range of 2.0-20.0 mm (Table 3.18). Comparing the images obtained on 50th and 78th d for reactor $R_{\text{amx}1000}$ biomass clearly illustrates that higher AMX dose applied during stressed phase operation has probably stimulated the biomass growth and transformed the flocculant biomasses into thicker and denser flocs structure. Similar, observation has also been made for reactor $R_{\text{amx}2000}$ biomass.

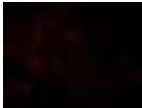
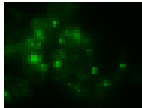
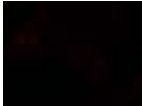
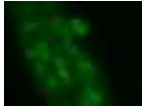
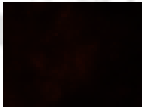
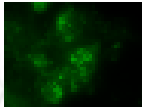
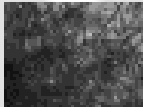
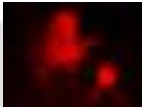
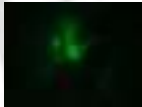
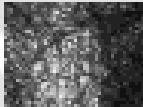
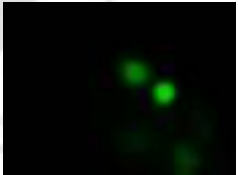
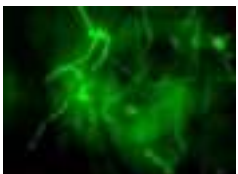
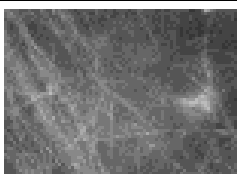
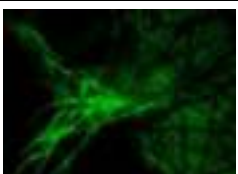
FI values have also been estimated during recovery phase operation, and data has been presented in Table 3.19, which shows the abundance of filaments throughout the operation. FI value has suggested that filaments have never been reduced in any reactor biomasses throughout the operation. The FI value has been observed as 5 for all reactor biomasses during the recovery phase operation. The reactor $R_{\text{amx}0}$ biomass has shown an FI value of 5 (on 50th d), which has similarity to stressed phase operation, and no change in FI values has been observed for the rest of the days of recovery phase operation (57-78 d). Similar, observations have been made for reactor $R_{\text{amx}500}$ and $R_{\text{amx}1000}$ biomasses (Table 3.19). The FI value for reactor $R_{\text{amx}2000}$ biomass has changed from 4 to 5 in the beginning of the recovery phase operation, and remained same throughout. The high FI value for all reactor has been observed due to continuous growth of filamentous biomass after termination of AMX.

The aerobic sludge biomass has been stained and observed under a fluorescence microscope at 20× magnification for active (live) and inactive (dead) portion of aerobic sludge biomass. The microscopic images have illustrated in Table 3.20. During recovery phase operation, no change in the active portion of aerobic sludge biomass has been distinguished for reactor $R_{\text{amx}0}$ biomass as compared to stressed phase images (Table 3.20). However, the

Table 3.19 Filament index of aerobic sludge biomass estimated during stressed and recovery phase operations.

Biomass withdrawn on	Stressed phase operation			
Day	$R_{\text{amx}0}$	$R_{\text{amx}500}$	$R_{\text{amx}1000}$	$R_{\text{amx}2000}$
1 st d	1	1	1	1
8 th d	3	3	3	3
15 th d	5	5	5	5
22 nd d	5	5	5	5
29 th d	5	5	5	5
36 th d	5	5	5	5
43 rd d	5	5	5	4
Recovery phase operation				
50 th d	5	5	5	5
57 th d	5	5	5	5
64 th d	5	5	5	5
71 st d	5	5	5	5
78 th d	5	5	5	5

Table 3.20 Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ in recovery phase operation along with images for stressed phase operation.

Sample withdrawn on	Reactor	Bright field (20×)	Dead Cells (20×)	Live Cells (20×)
1 st d Stressed phase operation	R_{amx0}			
	R_{amx500}			
	$R_{amx1000}$			
	$R_{amx2000}$			
	R_{amx0}			
	R_{amx500}			
	$R_{amx1000}$			
	$R_{amx2000}$			
78 th d Recovery phase operation	R_{amx0}			
	R_{amx500}			
	$R_{amx1000}$			
	$R_{amx2000}$			

discontinuation of AMX in the feed to reactors has increased the active portion of biomass towards the end of the recovery phase operation. All the reactors have shown increased active biomass portion during the recovery phase operation, whereas a small part of inactive biomass has also been observed for all the reactors (Table 3.20). Biomasses from reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ showed 98.1%, 88.2%, 81.3%, and 64.2% live cell concentrations during recovery phase operation, up from 97.7%, 87.2%, 39.2%, and 17.3% during stressed phase operation.

It can be concluded that filaments have not been affected by either presence or absence of AMX in the feed (Table 3.17). However, after AMX stress, biomass growth has been stimulated to form thicker biomass (Table 3.18). The FI values have not been affected by AMX in feed; however, higher doses of AMX slightly reduces FI values during stressed phase operation (Table 3.19). AMX imparts drastic changes in active and inactive biomass portions during the stressed phase operation, but the biomass has regained the equivalent active part during recovery phase operation (Table 3.20).

Polysaccharide, protein and extracellular polymeric substance

The variations in PS, PN, and EPS contents of aerobic sludge biomass during recovery phase operation have been shown in Fig. 3.22, while results have been summarized in Table 3.21. On 78th d of operation, PS, PN, and EPS contents for reactor $R_{\text{amx}0}$ biomass has been obtained as 16.43, 39.54, and 55.97 mg/g VSS, respectively [Fig. 3.22(a)]. An increasing trends in PS, PN, and EPS contents in aerobic sludge biomass have been observed during recovery phase operation. On 78th d of operation, PS, PN, and EPS contents for reactor $R_{\text{amx}500}$ biomass has marginally increased to 15.32, 40.03, and 55.32 mg/g VSS, respectively, from 14.23, 39.43, and 53.66 mg/g VSS of stressed phase operation (on 43rd d) [Fig. 3.22(b)]. Similarly, for reactor $R_{\text{amx}1000}$ biomass, PS, PN, and EPS contents on 78th d has slightly increased to 16.43 mg/g VSS; 41.43 mg/g VSS; 57.86 mg/g VSS, respectively, from 15.23, 40.20, and 55.43 mg/g VSS of stressed phase operation (on 43rd d) [Fig. 3.22(c)]. PS, PN, and EPS contents for reactor $R_{\text{amx}2000}$ biomass has been obtained as 16.99, 43.33, and 60.32 mg/g VSS, respectively [Fig. 3.22(d)], which has increased from 16.53, 41.23, and 57.76 mg/g VSS of stressed phase operation (on 43rd d). However, these little increase in PS, PN, and EPS contents obtained during recovery phase is not able to shed much light on the changes in biomass characteristics after removal of stress caused by AMX.

Enzyme activity

E_{Catalase} for all the reactor biomasses on the 78th d of operation has been shown in Fig. 3.23(a) and summary of data has been presented in Table 3.22. When AMX has been discontinued in the feed, E_{Catalase} for reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$ and $R_{\text{amx}2000}$ biomasses have been observed as 0.41, 0.43, 0.44, and 0.43 mg/g MLSS h, respectively, towards the end of recovery phase. During stressed phase operation, E_{Catalase} for reactor $R_{\text{amx}0}$, $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses have been observed as 0.37, 0.18, 0.14, and 0.05 mg/g MLSS h, respectively, which is much lower than the values obtained during recovery phase. These

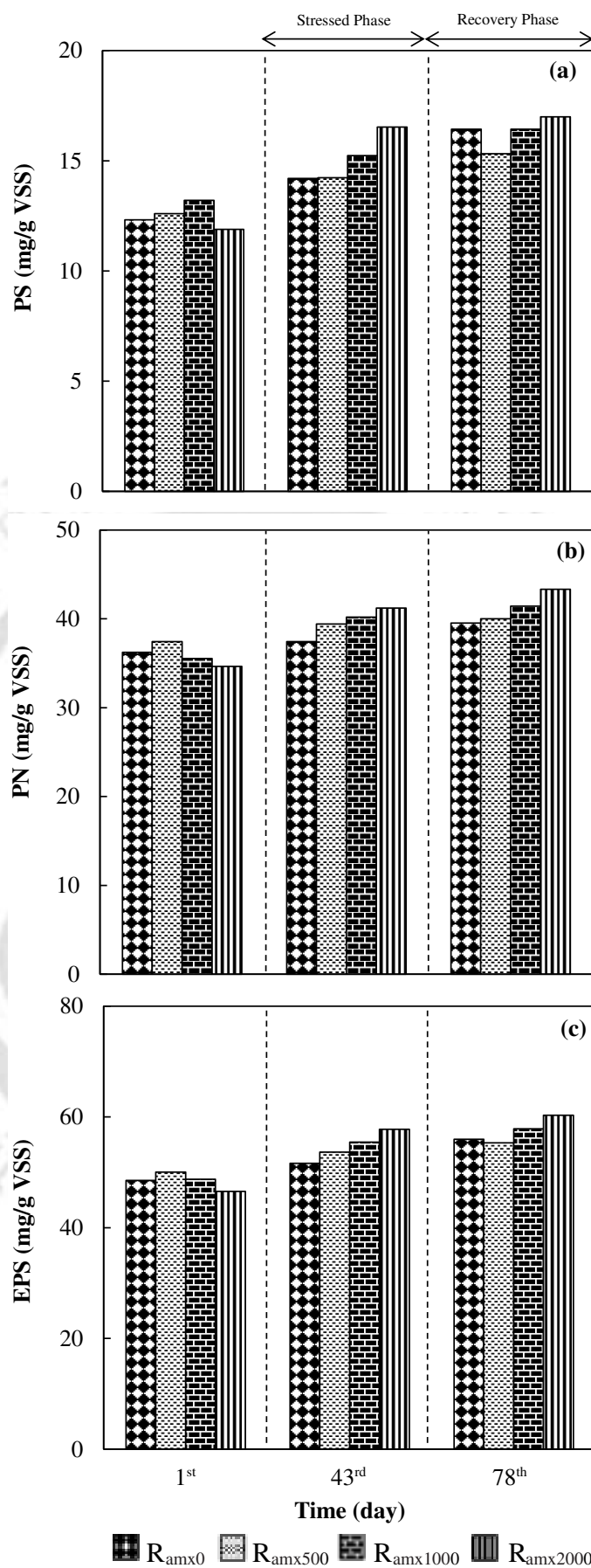


Fig. 3.22 (a) PS, (b) PN, and (c) EPS contents in aerobic sludge biomass during stressed and recovery phase operations.

Table 3.21 PS, PN, and EPS contents in aerobic sludge biomass estimated during stressed and recovery phase operations.

Biomass withdrawn on	Reactor biomass	PS	PN	EPS
mg/g VSS				
Stressed phase operation				
1st d	R_{amx0}	12.32 ±0.62	36.23±0.70	48.55±0.66
	R_{amx500}	12.60 ±1.03	37.43±2.10	50.03±1.56
	R_{amx1000}	13.20±0.92	35.53±2.00	48.73±1.46
	R_{amx2000}	11.89±0.63	34.65±2.20	46.54±1.41
43rd d	R_{amx0}	14.20±1.07	37.43±0.90	51.63±0.98
	R_{amx500}	14.23±0.46	39.43±1.90	53.66±1.18
	R_{amx1000}	15.23±0.72	40.20±1.40	55.43±1.06
	R_{amx2000}	16.53±0.83	41.23±1.10	57.76±0.96
Recovery phase operation				
78th d	R_{amx0}	16.43±0.70	39.54±1.12	55.97±0.93
	R_{amx500}	15.32±1.33	40.00±1.30	55.32±1.29
	R_{amx1000}	16.43±0.73	41.43±2.00	57.86±1.36
	R_{amx2000}	16.99±0.82	43.33±1.91	60.32±1.32

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

results suggest that E_{Catalase} has reached to its normal level as compared to reactor R_{amx0} biomass towards the end of the recovery phase operation.

$E_{\text{Dehydrogenase}}$ for all the reactor biomasses has been shown in Fig. 3.23(b) and data has been summarized in Table 3.22. On 78th d of operation, $E_{\text{Dehydrogenase}}$ for reactor R_{amx0} , R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses have been observed as 28.54, 26.54, 26.43, and 25.34 $\mu\text{g/g MLSS h}$, respectively. Whereas, $E_{\text{Dehydrogenase}}$ for reactor R_{amx0} , R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses have been obtained as 27.34, 20.34, 15.23, and 13.43 $\mu\text{g/g MLSS h}$, respectively, during stressed phase operation. When compared with values obtained in stressed phase operation, it indicates that reactor R_{amx1000} and R_{amx2000} biomasses have shown significant improvement in $E_{\text{Dehydrogenase}}$. The results also suggest that $E_{\text{Dehydrogenase}}$ has completely recovered towards the end of the recovery phase operation for all the reactor biomasses.

E_{Protease} for all the reactor biomasses has been shown in Fig. 3.23(c) and Table 3.22 represent summary of data obtained during recovery phase operation. The reactors have shown improvement in E_{Protease} . On 78th d of operation, 1.70, 1.63, 1.53 and 1.54 mg/g MLSS h of E_{Protease} has been observed for reactor R_{amx0} , R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses, respectively, which has increased from 1.65, 1.32, 0.85, and 0.76 mg/g MLSS h , respectively, of the stressed phase operation (on 43rd d). Fig. 3.23(c) illustrates that E_{Protease} has been affected in the presence of AMX and it has recovered to its normal level in absence of AMX in the feed.

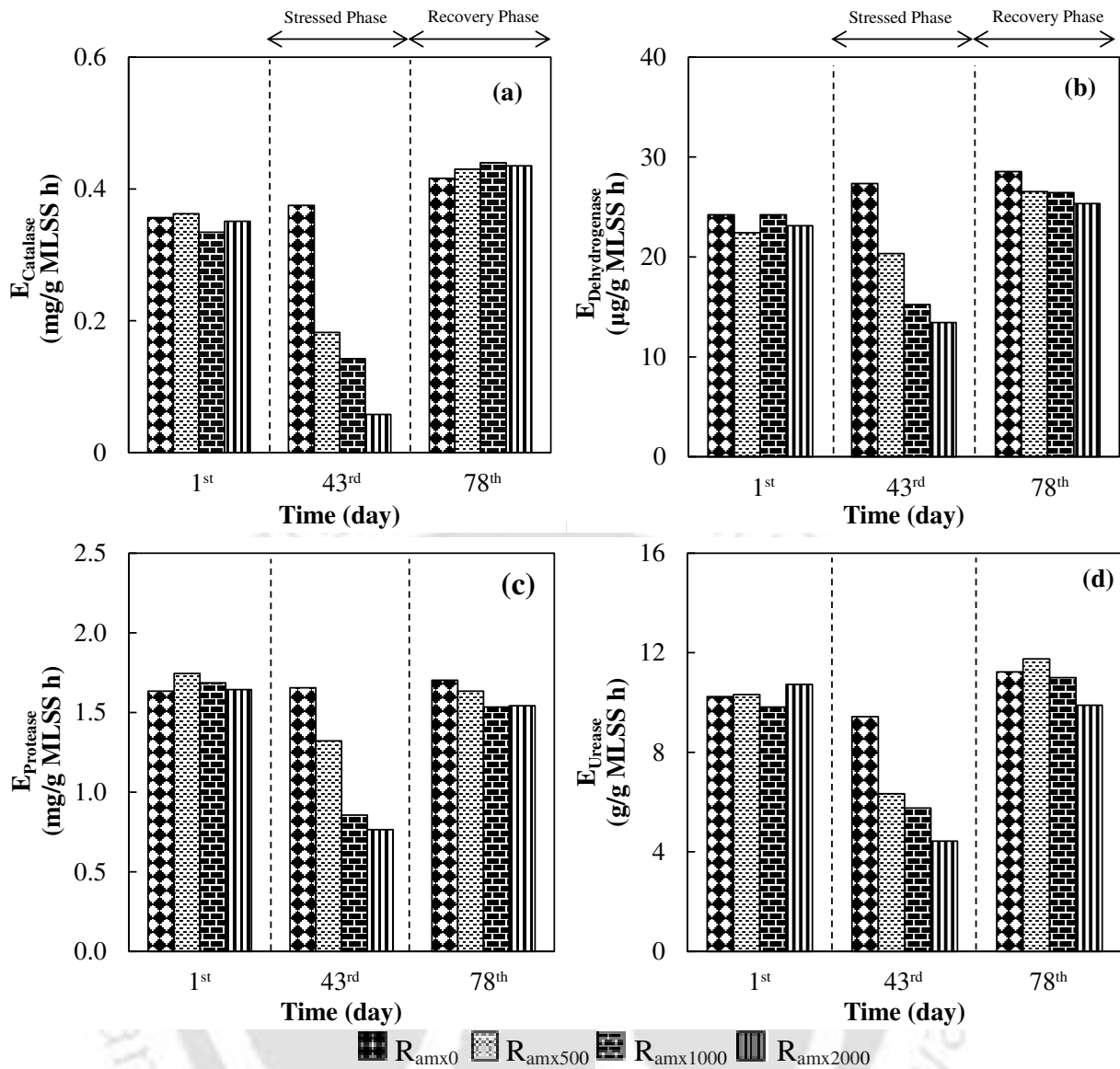


Fig. 3.23 Enzyme activities of aerobic sludge biomass in recovery phase operation (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} along with data for stressed phase operation.

E_{Urease} for all the reactor biomasses has also been affected in the presence of AMX in the feed and recovered during recovery phase operation as shown in Fig. 3.23(d) and Table 3.22. On 78th d of operation, E_{Urease} has been observed as 11.23, 11.75, 11.02, and 9.89 g/g MLSS h for reactor R_{амx0}, R_{амx500}, R_{амx1000}, and R_{амx2000} biomasses, respectively. E_{Urease} values have been 9.43, 6.34, 5.76, and 4.43 g/g MLSS h for reactor R_{амx0}, R_{амx500}, R_{амx1000}, and R_{амx2000} biomasses, respectively, on 43rd d of stressed phase operation. Similar to all other enzymes, E_{Urease} has also recovered when AMX addition in the feed has been discontinued during recovery phase operation.

It can be observed from Fig. 3.23 that aerobic sludge biomass has regained its enzyme activities at the end of the recovery phase operation. The possible reason could be that microorganisms must have started to repair their cellular machinery when AMX addition has been stopped in the feed to reactors. There is no literature available which can correlate the

Table 3.22 Summary of enzyme activities of aerobic sludge biomass during recovery phase operation along with data for stressed phase operation.

Biomass withdrawn on	Reactor biomass	E _{Catalase} mg/g MLSS h	E _{Dehydrogenase} μg/g MLSS h	E _{Protease} mg/g MLSS h	E _{Urease} g/g MLSS h
Stressed phase operation					
1st d	R_{amx0}	0.35±0.03	24.23±2.00	1.63±0.08	10.24±1.12
	R_{amx500}	0.36±0.03	22.42±2.50	1.74±0.05	10.32±1.10
	R_{amx1000}	0.33±0.02	24.23±1.80	1.68±0.15	9.82±1.40
	R_{amx2000}	0.35±0.03	23.13±2.20	1.64±0.07	10.73±0.50
43rd d	R_{amx0}	0.37±0.02	27.34±2.98	1.65±0.09	9.43±2.32
	R_{amx500}	0.18±0.02	20.34±1.50	1.32±0.06	6.34±0.50
	R_{amx1000}	0.14±0.02	15.23±1.70	0.85±0.13	5.76±0.60
	R_{amx2000}	0.05±0.02	13.43±1.10	0.76±0.17	4.43±1.10
Recovery phase operation					
78th d	R_{amx0}	0.41±0.03	28.54±0.82	1.70±0.11	11.23±0.82
	R_{amx500}	0.43±0.04	26.54±1.71	1.63±0.09	11.75±0.30
	R_{amx1000}	0.44±0.03	26.43±2.23	1.53±0.09	11.02±1.20
	R_{amx2000}	0.43±0.02	25.34±1.95	1.54±0.05	9.89±0.70

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

AMX concentration in feed with enzyme activity, hence mode of action on enzyme activity is still missing.

Amplified ribosomal DNA restriction analysis (ARDRA)

The aerobic sludge biomass samples have been withdrawn on 78th d of the operation and examined through ARDRA to determine the changes in microbial communities. Fig. 3.24 shows the gel run after the treatment of three different restriction enzymes (*AluI*, *HhaI*, and *MspI*) and dendrogram has been prepared based on the similarity matrix presented in Table 3.23. The restriction enzyme *AluI* has shown 91.2%, 74.3%, and 68.0% similarity with the reactor R_{amx0} biomass towards the end of the recovery phase operation for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively, which has improved from 78.6%, 68.7%, and 57.1%, respectively, of stressed phase operation. Similarly, *HhaI* has exhibited 94.3%, 84.5%, and 84.5% similarity for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses, respectively, which has also improved from 80.0%, 75.0%, and 74.1%, respectively, when compared to stressed phase operation. Similarity of 80.0%, 73.3%, and 83.9% has been observed for reactor R_{amx500}, R_{amx1000}, and R_{amx2000} biomasses withdrawn towards the end of the recovery phase operation, respectively, when digested with *MspI*, which shows improvement from 72.2%, 59.3%, and 51.9%, respectively, of stressed phase operation. When AMX addition has been discontinued in the feed to reactors during recovery phase operation, aerobic sludge biomass have tried to regain its microbial community structure. The reactor R_{amx500} and R_{amx1000} biomasses have shown higher similarity to reactor R_{amx0} biomass as compared to reactor R_{amx1000} and R_{amx2000} biomasses.

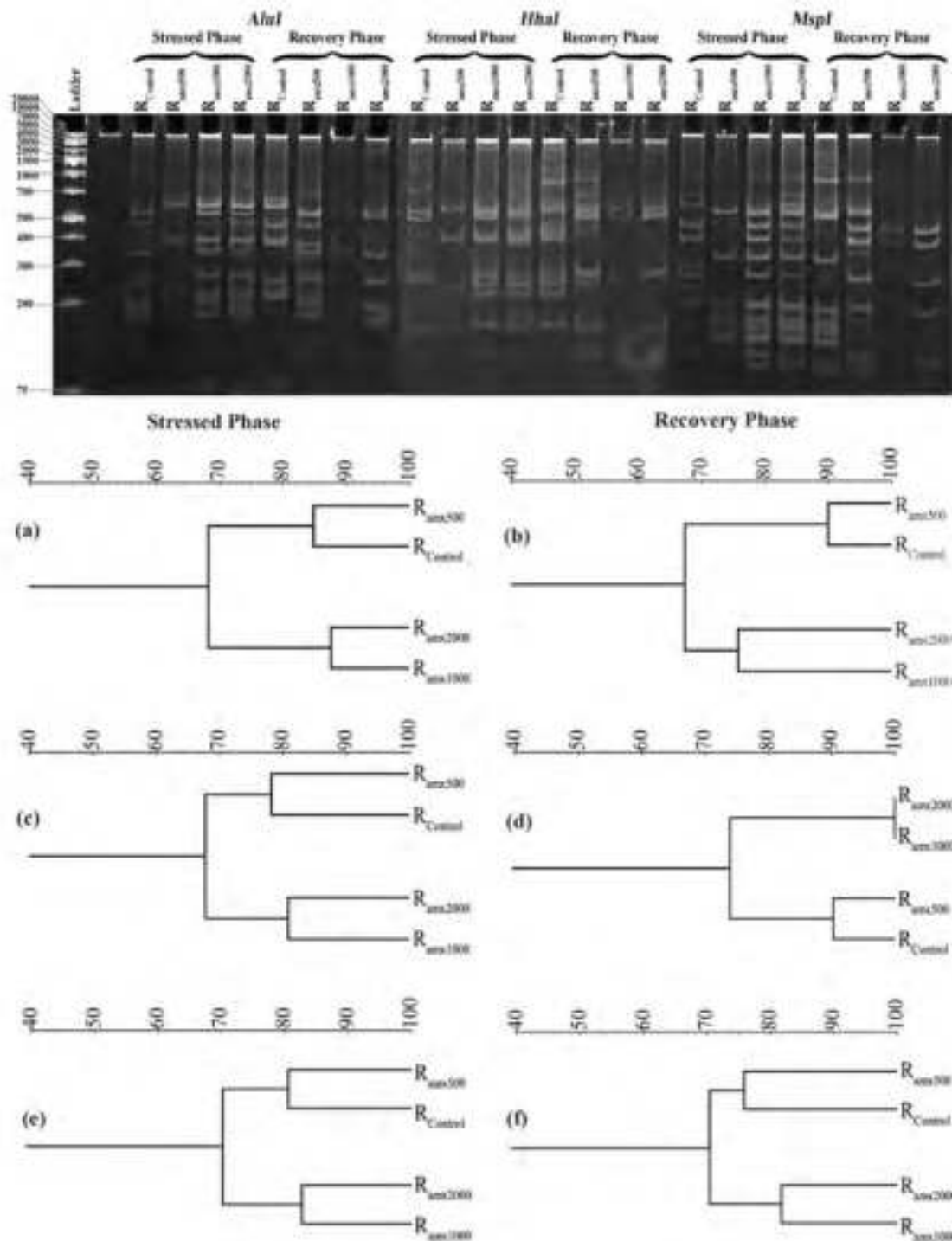


Fig. 3.24 Digestion pattern and dendrogram of aerobic sludge biomass under stressed and recovery phase operation treated with *AluI*: (a) stressed phase (b) recovery phase; *HhaI*: (c) stressed phase (d) recovery phase; and *MspI*: (e) stressed phase (f) recovery phase restriction enzymes.

Table 3.23 Similarity matrix resulting from digestion of aerobic sludge biomass samples collected during recovery phase operation by restriction enzymes along with data for stressed phase operation.

Stressed phase operation												
Reactor	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
R_{amx0}	1	0.7857	0.6875	0.5714	1	0.8000	0.7500	0.7407	1	0.7223	0.5934	0.5192
R_{amx500}	0.7857	1	0.5217	0.4444	0.8000	1	0.6897	0.6452	0.7223	1	0.5798	0.6234
$R_{amx1000}$	0.6875	0.5217	1	0.8235	0.7500	0.6897	1	0.8235	0.5934	0.5798	1	0.7534
$R_{amx2000}$	0.5714	0.4444	0.8235	1	0.7407	0.6452	0.8235	1	0.5192	0.6234	0.7534	1
Recovery phase operation												
Reactor	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$	R_{amx0}	R_{amx500}	$R_{amx1000}$	$R_{amx2000}$
R_{amx0}	1	0.9123	0.7429	0.6800	1	0.9429	0.8452	0.8452	1	0.8000	0.7333	0.8387
R_{amx500}	0.9123	1	0.7833	0.6926	0.7429	1	0.7500	0.7500	0.8000	1	0.7407	0.7143
$R_{amx1000}$	0.7429	0.7833	1	0.7957	0.6452	0.7500	1	1	0.7333	0.7407	1	0.8485
$R_{amx2000}$	0.6800	0.6926	0.7957	1	0.6452	0.7500	1	1	0.8387	0.7143	0.8485	1

As AMX has been discontinued from the feed of the reactors, the biomass flocs grew in size and form. Uncontrolled filamentous growth has been observed for all reactors during recovery phase. Images of flocs during the stressed and recovery phases have revealed minimal changes in their shape. The thick and denser floc structures have been observed towards the end of the recovery phase for all reactor biomasses within the range of 2.0-20.0 mm. Photographic observation has easily differentiated between stressed and recovery phases aerobic sludge biomass morphology. Aerobic sludge biomass in the recovery phase appears to be thicker and more denser than in the stressed phase. FI values maintained at 5 during the recovery phase operation, indicating that filamentous biomass has been in plenty in all reactors throughout the operation. Biomasses from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ have 98.1%, 88.2%, 81.3%, and 64.2% live cell concentrations during recovery phase operation, up from 97.7%, 87.2%, 39.2%, and 17.3% during stressed phase operation. Similarly, dead cells concentration have decreased during recovery phase operation to 0.8%, 7.4%, 11.0%, and 10.7% from 0.4%, 30.8%, 58.9%, and 76.4% of stressed phase operation for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively. During the recovery phase operation, all four enzymes recovered and showed pretty much identical values between AMX fed reactors and control reactor. $E_{Catalase}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 0.43-0.44 mg/g MLSS h, while 0.41 mg/g MLSS h for reactor R_{amx0} biomass. $E_{Dehydrogenase}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 25.34-26-54 μ g/g MLSS h, while 28.54 μ g/g MLSS h for reactor R_{amx0} biomass. $E_{Protease}$ for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged 1.54-1.63 mg/g MLSS h, while 1.70 mg/g MLSS h for reactor R_{amx0} biomass. Similarly, the E_{Urease} for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged from 9.89-11.75 g/g MLSS h, while 11.23 g/g MLSS h for reactor R_{amx0} biomass. ARDRA has revealed that the aerobic sludge biomass has regained its microbial community structure during recovery phase operation. *AluI* has shown 91.2%, 72.3%, and 68.0% of similarity; *HhaI* has shown 94.3%, 84.5%, and 84.5% of similarity; and *MspI* has shown 80.0%, 73.3%, and 93.9% of similarity for reactor biomasses fed with AMX concentration of 500, 1000, and 2000 mg/L, respectively, during stressed phase operation as compared to reactor R_{amx0} biomass.

3.4 Summary

The imposition of AMX in feed has adversely affected the organic removal efficiency, which has decreased with increase in AMX doses. The reactor fed with higher doses of AMX has been badly affected in terms of COD removal due to AMX stress. The COD removal for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ has been 74%, 38%, and 23%, respectively, whereas reactor R_{amx0} has shown 93% of COD removal during the steady-state condition of stressed phase operation. AMX has encouraged biomass growth, and morphological studies have indicated that AMX has transformed the biomass from denser flocs to weaker filaments than the control reactor biomass. AMX addition in feed has slightly lowered SVI for reactor $R_{amx1000}$ (121 mL/g) and $R_{amx2000}$ (126 mL/g) biomasses, while reactor R_{amx0} and R_{amx500} biomasses have shown SVI in the range of 140-145 mL/g. ISV have been observed in the range of 1.49-1.93

m/h during stressed phase operation for all reactor biomasses. The organic degradation capability is represented by ASBA which has been observed as 7.65, 3.84, and 2.22 mg COD removed/mg VSS d for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, which has been on a lower side compared to 9.43 mg COD removed/mg VSS d for control reactor R_{amx0} biomass. The imposition of AMX has no significant impact on filamentous biomass growth. The microscopic examination has revealed presence of abundance of filaments during stressed phase operation. The photographic observation has indicated flocs size between 1.0-7.0 mm for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses. A slight change in FI values has been observed for reactor $R_{amx2000}$ (FI value 4) biomass due to AMX to filamentous biomass, while FI values for other reactor biomasses (R_{amx0} , R_{amx500} , $R_{amx1000}$) have remained as 5 towards the end of the stressed phase operation. AMX has decreased the active portion of biomass, which has further reduced with increased AMX concentration in the feed. The fluorescence microscopic results have shown 97.7%, 87.2%, 39.2%, and 17.3% of live cells and 0.4%, 30.8%, 58.9%, and 76.4% of dead cells for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, at steady-state condition of stressed phase operation. $E_{Catalase}$, $E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease} values have confirmed the adverse impacts of AMX on organic removal capability of aerobic sludge biomass during stressed phase operation. PS, PN, and EPS secretion by aerobic sludge biomass have increased due to AMX. ARDRA has established the relation between biomass of control and AMX fed reactors and confirmed the microbial community changes (52-80% similarity with control reactor) during stressed phase operation.

During the recovery phase operation, all the reactors have shown significant COD removal (almost 93% at the steady-state condition). Also, reactors have maintained biomass growth with increased SVI. ISV has deteriorated due to the abundance of filaments in all reactors during the recovery phase operation. The average SVI for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged between 130-188 mL/g, whereas ISV have been observed in the range of 0.89-1.84 m/h during recovery phase operation. The discontinuation of AMX in the feed has allowed the aerobic sludge biomass to regain its organic degradation capability and ASBA values has improved from 9.43, 7.65, 3.84, and 2.22 mg COD removed/mg VSS d in the stressed phase to 11.58, 9.22, 8.96, and 7.96 mg COD removed/mg VSS d in recovery phase for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively. The discontinuation of AMX in the feed has also enhanced the filamentous growth; hence, thick and denser flocs (size 2.0-20.0 mm) structure have been confirmed by photographic observation for all the reactors. The FI values have remained at 5 during the recovery phase operation. Biomasses from reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ have showed 88.2%, 81.3% and 64.2% live cell concentrations during recovery phase operation, up from 87.2%, 39.2%, and 17.3% during stressed phase operation. All four enzyme activities have also shown improvement for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses during the recovery phase operation and indicated almost similar enzyme activity values compared to reactor R_{amx0} biomass. ARDRA has revealed that reactor R_{amx500} , $R_{amx1000}$,

and $R_{amx2000}$ biomasses have regained its microbial community structure and has shown 68-94% similarity compared to reactor R_{amx0} biomass. The investigation has indicated unhindered increase in filamentous biomasses during stressed phase operation in AMX fed reactors even-though COD reductions have been impacted severely along with reduction in ASBA, live cell fraction and microbial community structure. In addition, during recovery phase operation, COD removal, ASBA, live cell fraction and microbial community structure have improved significantly with the filamentous biomass in reactors. This requires further in-depth investigation.

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Effects of Pesticide (Chlorpyrifos) Stress on Aerobic Sludge Biomass

Chlorpyrifos [$(C_9H_{11}Cl_3NO_3PS)$ or *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinol) phosphorothioate], a member of phosphorothioate class of chlorinated organo-phosphorous compounds, is a toxic broad-spectrum pesticide (Maya et al., 2012; Racke, 1993). It is widely applied in agriculture, animal houses, and for the control of household pests and subterranean termites. The trends of kitchen gardening and terrace gardening are also adding small pesticide content in sewage. The shopkeepers usually dump expired pesticides in toilets/nearby drains. The dumped pesticide finds its way to the sewage treatment plant (STP). This chapter discusses the effect of pesticide on aerobic sludge biomass when added in influent and the recovery potential of the pesticide exposed aerobic sludge biomass when the pesticide is removed from the influent.

4.1 Background

Chlorpyrifos (CPS) is used widely in direct soil application to control mosquitoes, flies, various crop pests in soil and foliage, household pests, and aquatic larvae. It is also used on sheep and cattle for the control of ectoparasites. Nowadays, trends in kitchen gardening, home farming, and terrace gardening are the major source of CPS in domestic wastewater. In addition, through careless handling and illegal discharge of CPS in sewer lines by agrochemical industries, stockholders, and shopkeepers, it reaches STP. CPS is toxic to the plant's microorganisms, resulting in decreased plant efficiency (Mohan et al., 2004; Zhang et al., 2016). CPS in wastewater is likely to cause morphological and structural changes in aerobic sludge biomass (Maya et al., 2012). The literature survey suggests that a considerable amount of published works have focused on bio-degradation of CPS (Affam et al., 2014; Maya et al., 2012; Mohan et al., 2004; Zhang et al., 2016). Most of the studies have emphasized on bio-degradation kinetics of CPS and its products (Affam et al., 2014; Maya et al., 2011;

Sasikala et al., 2012). Researchers haven't focused wastewater treatment plant microbes and their activity in the presence of CPS in the available literature, despite the fact that biomass plays an important role in the biological process. There is limited literature on the effects of CPS on aerobic sludge biomass as most of the studies have focused on performance and monitoring of treatment plant (Affam et al., 2014; Maya et al., 2012; Mohan et al., 2004; Zhang et al., 2016). These studies reveal the impact of CPS on the performance of the activated sludge process; however, there is a lack of correlation between performance and sludge morphology. The studies have been carried out to describe the toxicity of CPS in terms of plant performance without specifying flocs morphology, aerobic sludge biomass, and enzyme activity, as well as the recovery potential of aerobic sludge biomass after stoppage of exposure to CPS. Enzymes play an essential role in the breakdown of organic matter present in wastewater and flocs morphology significantly plays an important role in biosolid-liquid separation. It is strongly believed that the enzymatic approach will substantially contribute to the knowledge of the biochemical factors that control the capability of wastewater treatment, and it will also help in the characterization of aerobic sludge biomass. There is no literature available to correlate the aerobic sludge biomass activity and enzyme activity with respect to flocs morphology. Therefore, the objective of this research is to assess the impact of CPS on aerobic sludge biomass morphology along with treatment performance. In this research, the aim is to study the effects of different concentration of CPS on the activities of catalase, dehydrogenase, protease, and urease of aerobic sludge biomass and correlate with the performance along with the morphology of aerobic sludge biomass. The study also offers some important insights into the recovery potential of aerobic sludge biomass exposed to CPS in terms of COD removal, aerobic sludge biomass activity, and flocs morphology.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

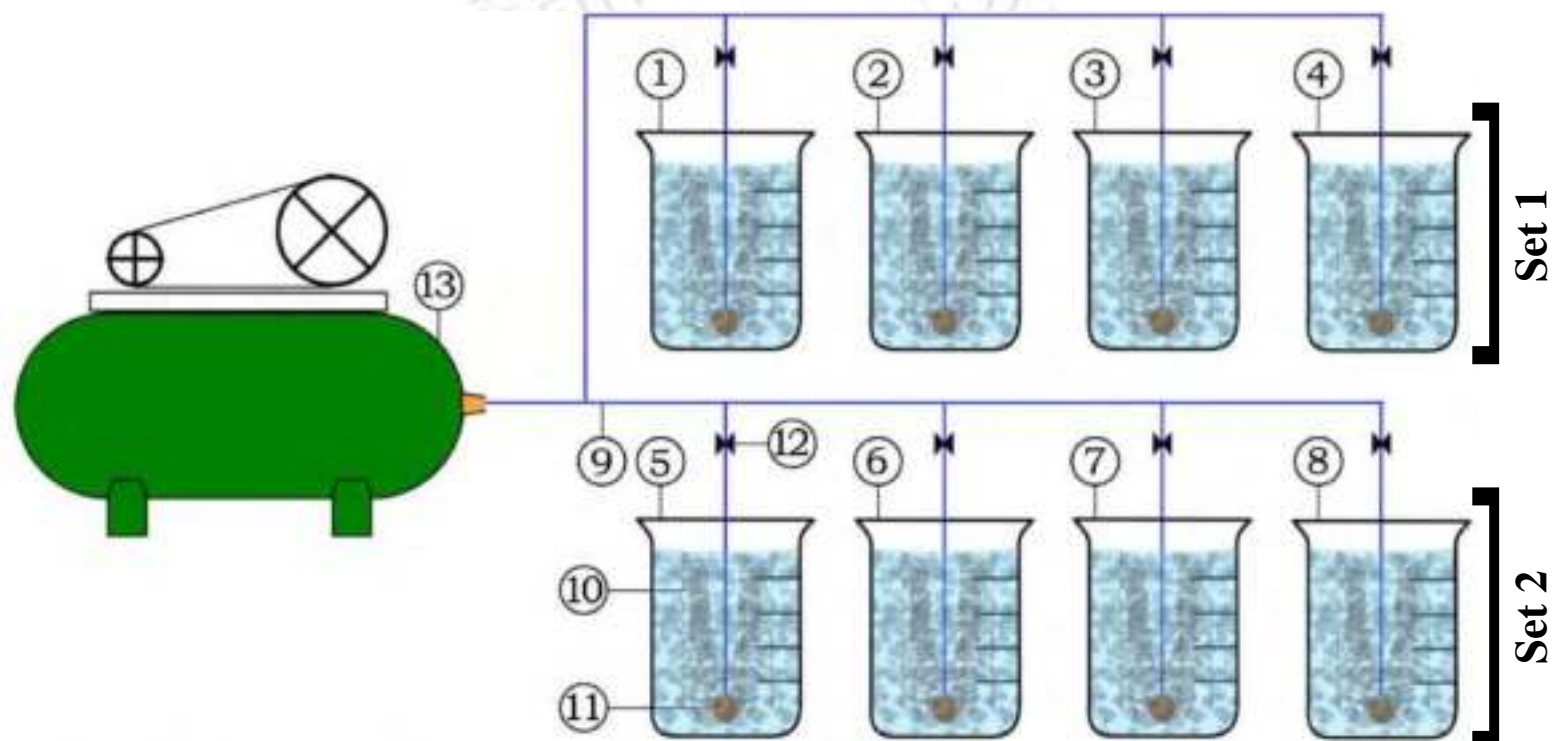
All chemicals and reagents used in this study are analytical or HPLC grade, manufactured by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals is presented in *Appendix-I* (Table A-I.1). The selection of CPS concentration for experiment has been briefly described in *Appendix-IV*. Tricel (chlorpyrifos 20% EC) has been purchased from a local agriculture shop in Guwahati, manufactured by Excel Crop Care Limited, Mumbai, India.

4.2.2 Source of aerobic sludge biomass

The aerobic sludge biomass, withdrawn from the mother reactor (MR), has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in detail in *Appendix-II*.

4.2.3 Reactor setup and operation

The experimental set-up consists of two sets of four identical reactors (height 14.5 cm × diameter 12.5 cm) with a working volume of 1 L (Fig. 4.1). Reactors have been designated as R_{cps0_1} and R_{cps0_2} (control reactors with no CPS in the feed), R_{cps50_1} and R_{cps50_2} (50 mg/L CPS in the feed), R_{cps100_1} and R_{cps100_2} (100 mg/L CPS in the feed), R_{cps200_1} and R_{cps200_2}



1. R_{cps0_1} 2. R_{cps50_1} 3. R_{cps100_1} 4. R_{cps200_1} 5. R_{cps0_2} 6. R_{cps50_2} 7. R_{cps100_2} 8. R_{cps200_2}
 9. Flexible PVC pipe 10. Aerobic sludge biomass 11. Diffused aeration 12. Air valve 13. Air compressor

Fig. 4.1 Schematic diagram of reactor setup used for experiment.

(200 mg/L CPS in the feed). The reactors have been fed with CPS along with Lactogen 3 as a source of carbon and nutrients. The composition of Lactogen 3 powder is presented in Table A-II.2. The reactors have been operated at ambient conditions without temperature and pH control for two cycles per day with a fill time of 10 min, a react time of 665 min, a settling time of 10 min, a decant time of 15 min, and a wash time (two times) of 20 min (as shown in Fig. 4.2).

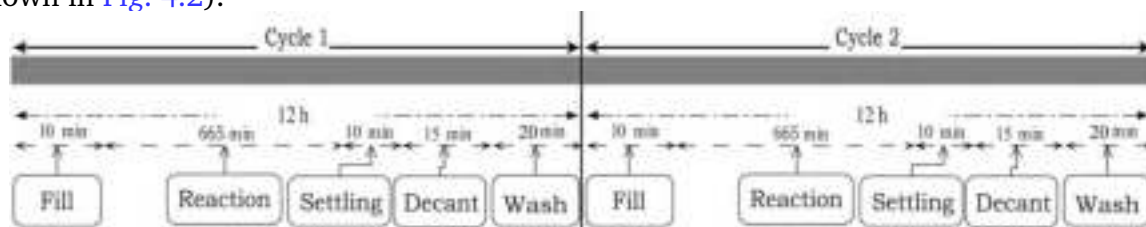


Fig. 4.2 Schematic representation of cycle time for reactor operation.

To prevent the progressive build-up of increasing CPS concentrations (residuals from previous feedings and fresh addition in the subsequent feeding) and subsequent exposure of biomass to more than the planned CPS load, the retained settled mixed liquor in the reactor after decantation is washed twice with tap water before starting the next feeding. The washing is carried out by refilling the reactors with tap water up to 1 L volume and followed by gentle mixing the content for 1-2 min. After that, the content is allowed to settle for 5 min before the supernatant is decanted. This process has been repeated two times. After washing, the reactor is again filled with tap water up to a 1 L volume. The clear liquid is sampled to estimate background (or residual) CPS concentration left after the washing— designated as 'BG_{CPS}'. The loss in the liquid volume due to the withdrawal of the sample has to be made up by the new feed. The settling time has been kept short (10 min) in order to decant dispersed and slow-settling flocs. Each reactor has been initially filled with ~450 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~3000 mg/L] withdrawn from the mother reactor (MR). The synthetic feed has been prepared using Lactogen 3 (2.70 g for reactor R_{CPS0}, 2.14 g for reactor R_{CPS50}, 1.58 g for reactor R_{CPS100}, and 0.50 g for reactor R_{CPS200}) and lukewarm water (~100 mL) followed by proper mixing using magnetic stirrer. Tricel has been used as the source of CPS in the feed, which contains 21.5 g/L of CPS. The reactors R_{CPS0}, R_{CPS50}, R_{CPS100}, and R_{CPS200} have been fed with 0, 2.32 mL (2 mL+320 µL), 4.65 mL (4 mL+650 µL), and 9.30 mL (9 mL+300 µL) of CPS solution in each cycle (using 0.5-5.0 mL variable volume pipette, Thermo Scientific™ Finnpiptette™ F2, Finland). After the addition of CPS solution, the final volume of the reactors has been made to 1 L with tap water. The COD provided by CPS has been estimated in this study by diluting 232 µL (using 100-1000 µL variable volume pipette, Thermo Scientific™ Finnpiptette™ F2, Finland) of CPS solution (Tricel) into 500 mL of milli-Q water. The COD of the prepared solution has been determined by Standard Methods (APHA et al., 2005). The result suggests that 1 mg/L of CPS gives COD of 11.13±0.82 mg/L. Hence, feed (different amount of Lactogen 3 and CPS) is giving a COD of ~2700 mg/L in each reactor. For keeping the biomass in suspension and imparting mixing, the air has been supplied using an air compressor at a flow rate of 2.5 L/min through an air diffuser.

The 1st cycle has been started around 9.30 am in the morning, and initial room temperature has been recorded at that time, while room temperature during decanting has been recorded at 8.45 pm in the night and then 2nd cycle started around 9.30 pm. Since the reactors have been fed with CPS solution, which is likely to cause stress on aerobic sludge biomass; hence the operation of these reactors has been termed as *stressed phase operation*. The stressed phase operation has lasted for 34 d in which the steady-state condition with respect to COD removal (%) has been achieved after 24th d from the start and steady-state condition data obtained till 34th d of operation. Thereafter, the reactors have been operated without CPS in the feed for the next 42 d. This phase of the operation has been termed as *recovery phase operation*. The steady-state condition has been achieved after 25-30 d from the start of the recovery phase operation with respect to COD removal (%).

The initial liquid samples have been withdrawn after the fill stage (proper mixing of the feed and CPS solution ensured), and effluent samples have been collected during the decantation step at the end of a cycle. During a settling time of 10 min, the settled mixed liquor (sludge biomass + liquid) has occupied up to 600 mL for poorly settling biomass and 230 mL for rapidly settling biomass. The volume exchange ratio for all reactors has been in the range of 40-75% throughout the study. The aerobic sludge biomass has been washed thrice with tap water at the end of the stressed phase operation but before starting the recovery phase operation.

4.2.4 Analytical methods

The frequencies of initial and effluent sample analysis are presented in [Table 4.1](#). The analytical parameters have been grouped into four different categories, as explained earlier. The analytical techniques for estimation of temperature, pH, alkalinity, COD, MLSS, MLVSS, interface settling velocity (ISV), sludge volume index (SVI), photographic observation, microscopic analysis, aerobic sludge biomass activity (ASBA) test, enzymes activity, and ARDRA, used in this study, have already been explained in *Chapter 2*. The cell viability test, estimation of polysaccharides, proteins, and extracellular polymeric substance contents have been explained in *Chapter 3*.

4.2.4.1 Determination of CPS

The initial and effluent samples have been centrifuged at 12,000 rpm and supernatant has been collected for CPS estimation. Then samples have been filtered through a 0.22 μ m nylon membrane. Samples have been stored at 4°C until analyzed. CPS concentration has been determined by using high-performance liquid chromatography (HPLC) (Prostar 210, Varian, USA) equipped with a micro-vacuum degasser, a dual pump, UV detector (UV) at the working wavelength of 230 nm, and with an Agilent 5 TC-C18 analytical column (250×4.5 mm, particle size 5 μ m). The samples have been eluted with a linear gradient of acetonitrile solutions (10%-100%, 0-10 min), 100% acetonitrile (10-12 min), and a linear gradient of acetonitrile (100%-10%, 12-15 min) at a flow rate of 1.0 mL/min ([Liu et al., 2016](#)). The standard curve is given in *Appendix-I* ([Fig. A-I.8](#)).

Table 4.1 Analysis frequency of initial and effluent sample parameters.

Category	Parameters	Frequency
Environmental related parameters	Room temperature	Daily, during stressed phase and alternate day, during recovery phase
	Initial pH	
	Initial CPS concentration	
Reactor related performance parameters	Effluent pH	Towards end of the stressed and recovery phase operations
	Effluent CPS concentration	
	Chemical oxygen demand (COD)	
	Alkalinity	
Biomass related general parameters	Mixed liquor suspended solids (MLSS)	Every 7 th day
	Mixed liquor volatile suspended solids (MLVSS)	
	Sludge volume index (SVI)	
	Interface settling velocity (ISV)	
Biomass related microbiological parameters	Aerobic sludge biomass activity (ASBA)	Towards end of the stressed and recovery phase operations
	Microscopic observation after Gram's staining	
	Photographic observation	
	Filament index (FI)	
	Cell viability test	
	Polysaccharides (PS), proteins (PN) and extracellular-polymeric substances (EPS)	
	Catalase activity	
	Dehydrogenase activity	
	Protease activity	
	Urease activity	
	Amplified ribosomal DNA restriction analysis (ARDRA)	

4.3 Results and Discussion

The reactors have been operated during 0-34 d with CPS in the feed — termed as stressed phase operation and afterward for 35-76 d without CPS in the feed — termed as recovery phase operation. The results obtained are discussed in subsequent subsections. The average values of duplicate reactors (average of R_{cps0_1} and R_{cps0_2} have been represented as R_{cps0} and similarly for other reactors as R_{cps50} , R_{cps100} , and R_{cps200}) along with the standard deviation in the respective tables.

4.3.1 Stressed phase operation

4.3.1.1 Environmental and performance parameters

The reactors have been operated for 34 d under stressed phase operation. Variation in environmental and performance-related parameters is shown in Fig. 4.3 to 4.6 and summary of data are presented in Table 4.2. The reactors have been operated under ambient conditions throughout the operation; hence, pH and reactor temperature have not been adjusted. The room temperature variation has been mainly observed due to weather changes. Temperature variation has been shown in Fig. 4.3 and data has been summarized in Table 4.2. Since all the reactors have been operated at room temperature, variation in room temperature recorded at 9.30 am and 8.45 pm have been presented in Fig. 4.3.

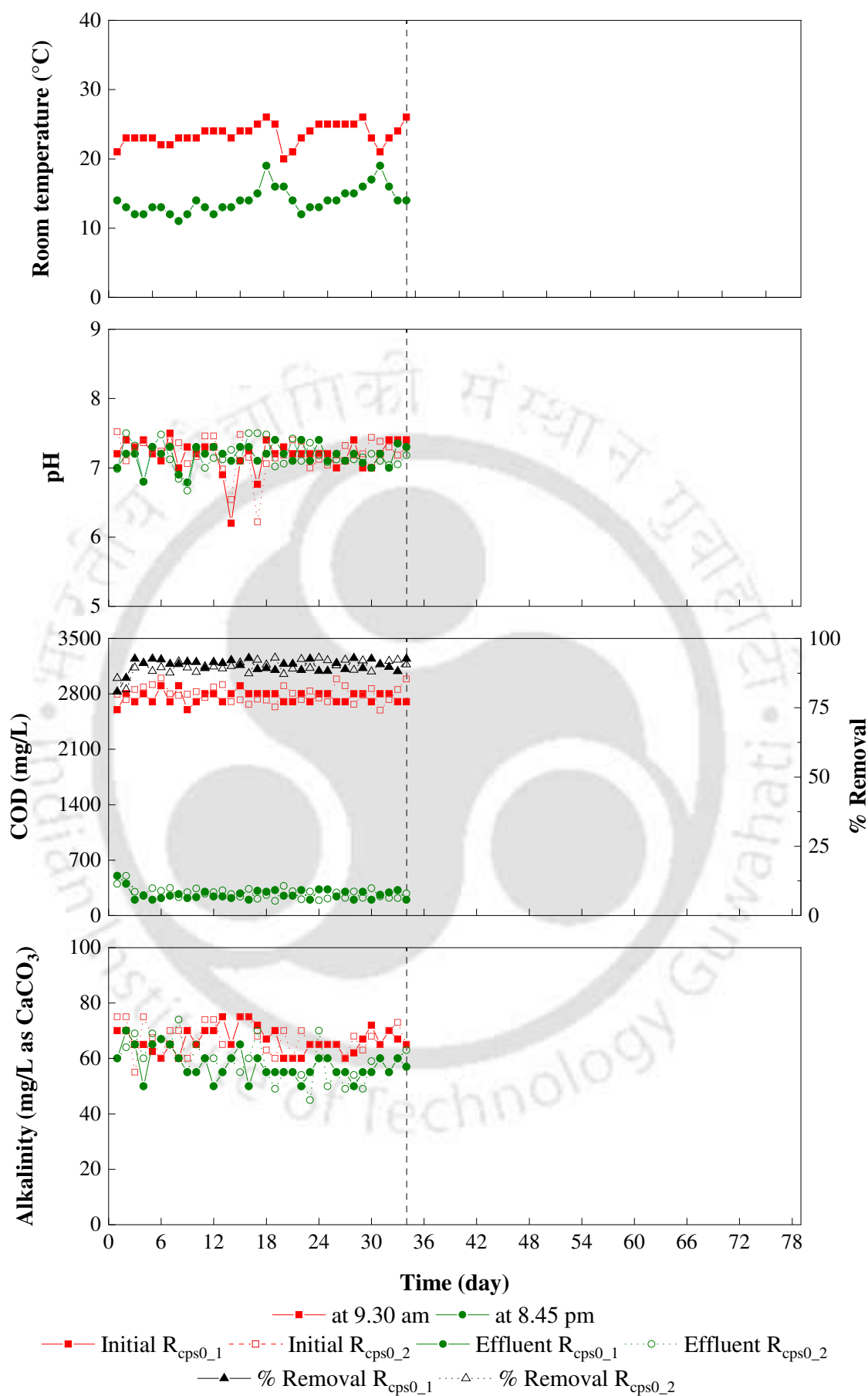


Fig. 4.3 Variation in environmental and performance related parameters for reactor R_{cps0} during 0-34 d of operation.

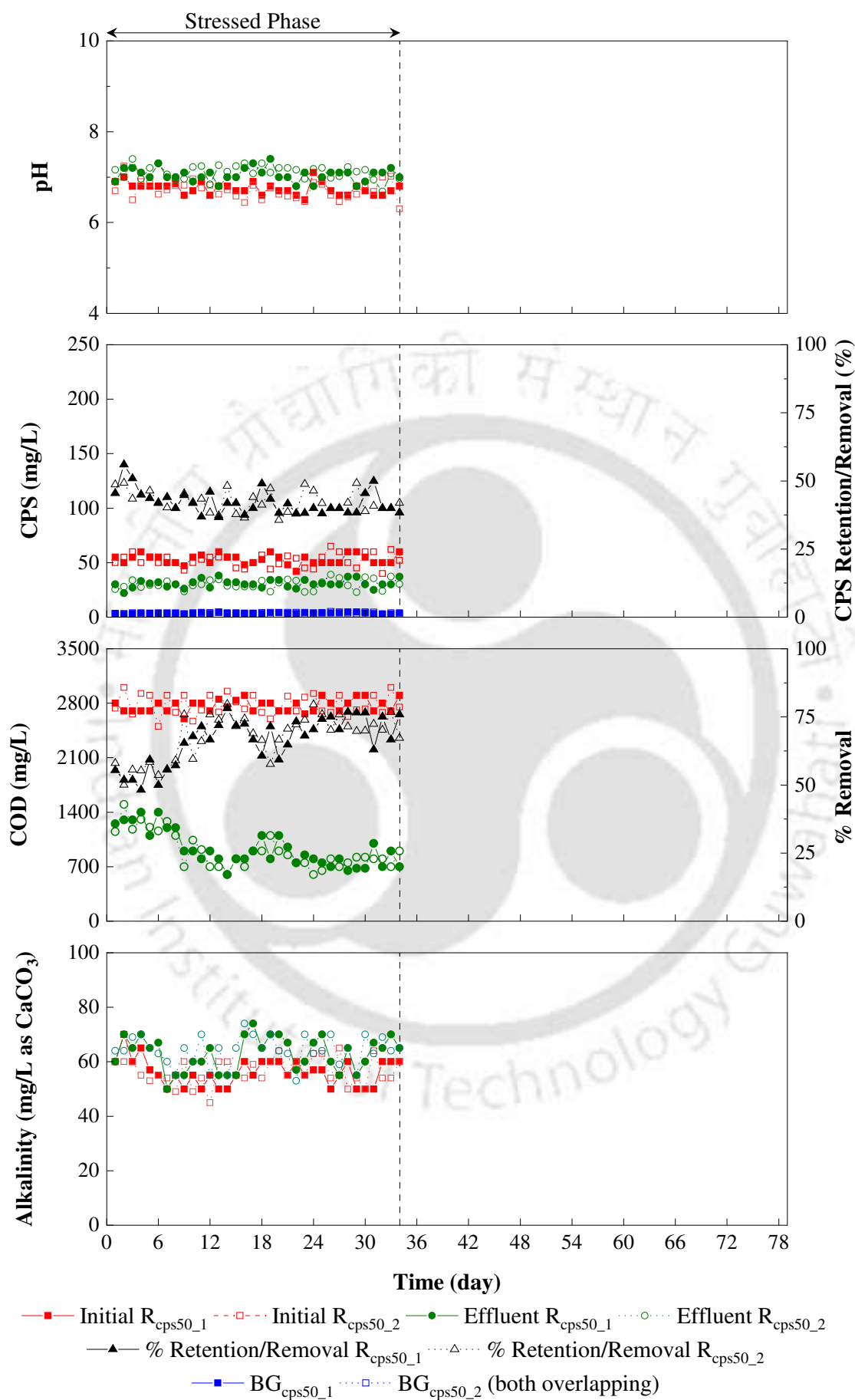


Fig. 4.4 Variation in environmental and performance related parameters for reactor R_{cps50} during 0-34 d of operation.

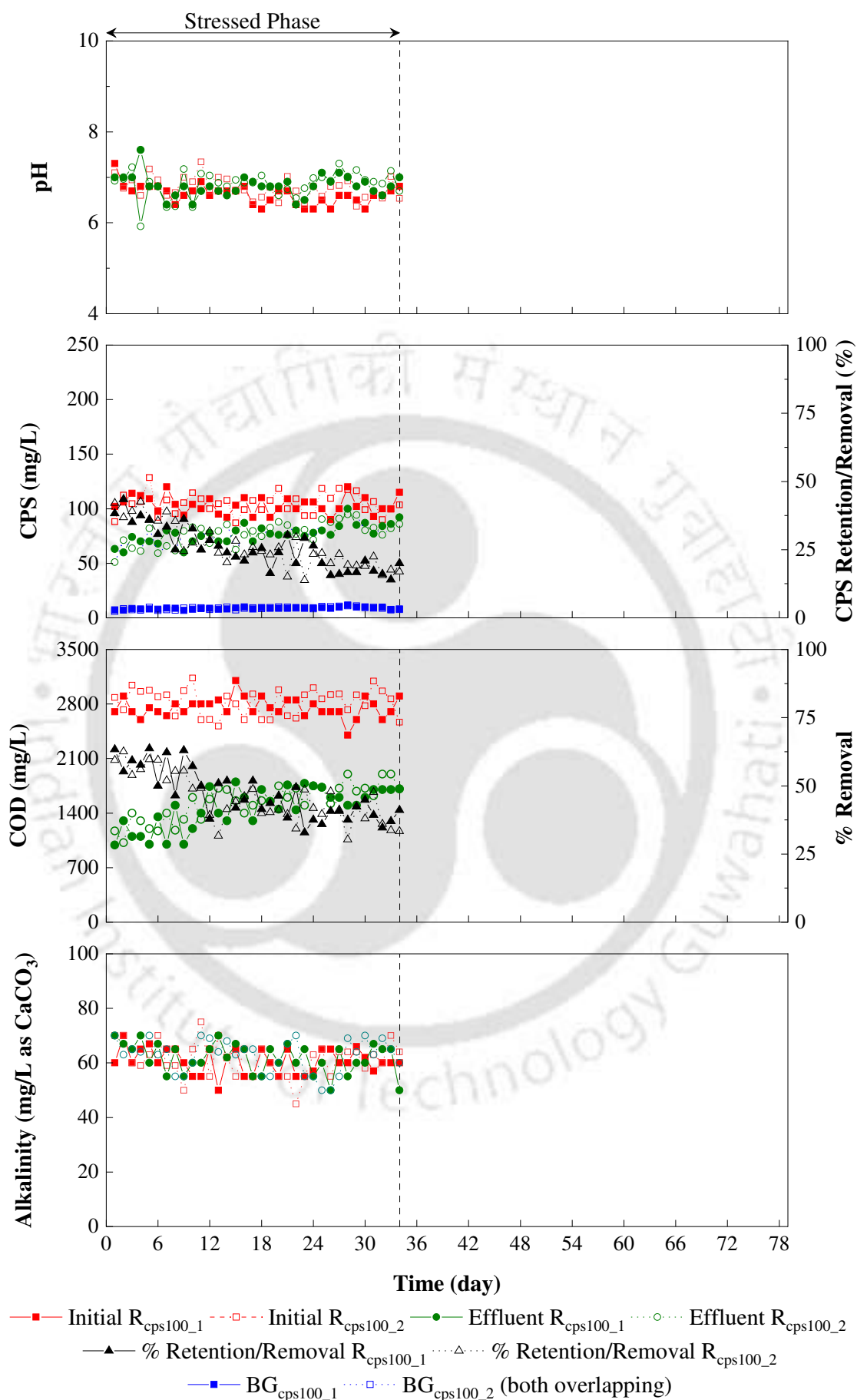


Fig. 4.5 Variation in environmental and performance related parameters for reactor R_{cps100} during 0-34 d of operation.

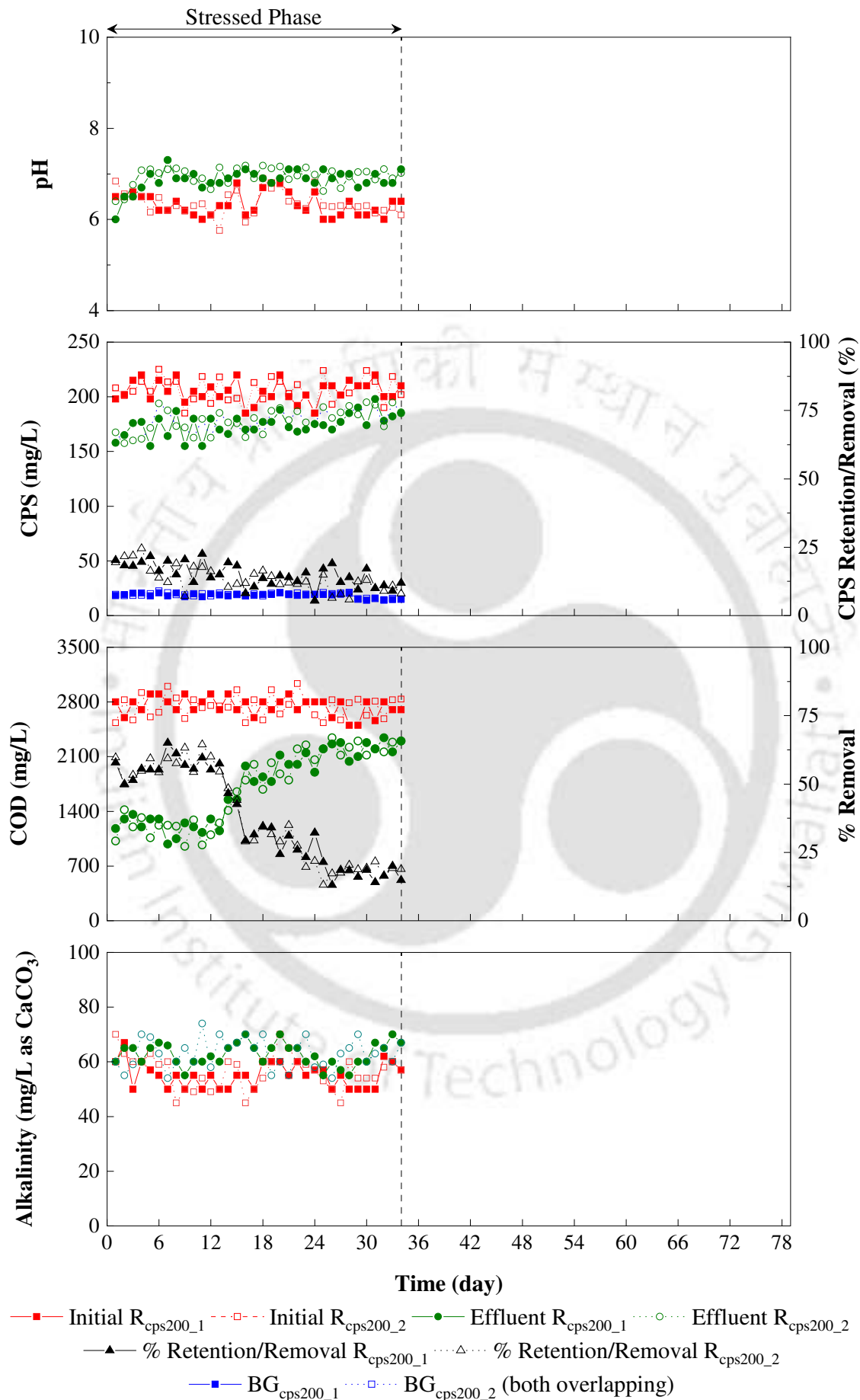


Fig. 4.6 Variation in environmental and performance related parameters for reactor R_{cps200} during 0-34 d of operation.

Table 4.2 Summary of environmental and performance related parameters for reactors operated during 0-34 d of stressed phase operation.

Parameters	Day-wise	0-34			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.14	1.58	0.47
	CPS (mg/L)	0	50	100	200
Stressed phase	Environmental				
	Room temperature (°C) at 9.30 am	23.5±1.5 (34)	23.5±1.5 (34)	23.5±1.5 (34)	23.5±1.5 (34)
	Room temperature (°C) at 8.45 pm	14.0±1.9 (34)	14.0±1.9 (34)	14.0±1.9 (34)	14.0±1.9 (34)
	Initial pH	7.19±0.25 (68)	6.73±0.16 (68)	6.69±0.22 (68)	6.34±0.23 (68)
	Initial CPS (mg/L)	0	52.62±5.30 (68)	104.22±8.86 (68)	205.54±10.58 (68)
	Effluent pH	7.17±0.18 (68)	7.08±0.15 (68)	6.83±0.26 (68)	6.91±0.22 (68)
	Effluent CPS (mg/L)	0	30.45±4.11 (68)	77.26±9.63 (68)	176.34±10.66 (68)
	CPS retention/removal (%)	0	42.10±4.21 (68)	26.18±6.78 (68)	14.62±3.75 (68)
	Background CPS (mg/L)	0	3.64±0.56 (68)	8.70±1.12 (68)	18.57±1.97 (68)
	Performance				
	Initial soluble COD (mg/L)	2779.26±88.57 (68)	2773.98±105.61 (68)	2790.20±148.18 (68)	2746.66±127.87 (68)
	Effluent soluble COD (mg/L)	276.17±65.82 (68)	911.69±226.92 (68)	1503.24±244.04 (68)	1716.68±467.93 (68)
	COD removal (%)	90.07±2.39 (68)	67.02±8.45 (68)	46.04±8.98 (68)	37.35±17.45 (68)
	Initial alkalinity (mg CaCO ₃ /L)	66.81±4.96 (68)	58.35±4.89 (68)	61.00 ±5.51 (68)	55.71±5.49 (68)
	Effluent alkalinity (mg CaCO ₃ /L)	58.29 ±6.09 (68)	63.53 ±5.72 (68)	62.18±5.81 (68)	62.91±4.85 (68)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

Variations in the initial and effluent pH for reactors have been shown in Fig. 4.3 to 4.6. The average initial and effluent pH for reactor R_{cps0} during 0-34 d of operation has been observed as 7.19 ± 0.25 and 7.17 ± 0.18 , respectively. However, pH for other reactors R_{cps50} , R_{cps100} , and R_{cps200} have slightly decreased as compare to the reactor R_{cps0} during stressed phase operation due to addition of CPS in the feed. The average initial pH for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 6.73 ± 0.16 , 6.69 ± 0.22 , and 6.34 ± 0.23 , respectively, while the average effluent pH have been obtained as 7.08 ± 0.15 , 6.83 ± 0.26 , and 6.91 ± 0.22 , respectively.

The variation in CPS retention/removal has been shown in Fig. 4.4 to 4.6 and data has been summarized in Table 4.2. On the 1st d, 17th d, and 34th d of operation, the CPS retention/removal for reactor R_{cps50} has been observed as 47.1%, 42.3%, and 40.0% (Fig. 4.4), respectively, while reactor R_{cps100} has shown 40.2%, 20.2%, and 20.0% of CPS retention/removal (Fig. 4.5), respectively. Similarly, reactor R_{cps200} has shown 25.3%, 13.0%, and 10.2% of CPS retention/removal on 1st d, 17th d, and 34th d of operation, respectively (Fig. 4.6). The average CPS retention/removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 42.1%, 26.2%, and 14.6%, respectively. The reactors R_{cps50} , R_{cps100} , and R_{cps200} have been fed with a different concentrations of CPS in the feed; therefore, to prevent the accumulation of CPS in reactors, aerobic sludge biomass has been washed at the end of the cycle before subsequent feedings in the next cycle. The background CPS (BG_{cps}) concentration has also been estimated (Fig. 4.4 to 4.6). The reactor R_{cps50} has retained 3.17 mg/L and 3.41 mg/L of CPS on 1st and 34th d of operation, respectively. Similarly, reactor R_{cps100} has retained 6.58 mg/L and 7.79 mg/L of CPS, while 18.80 mg/L and 14.92 mg/L of CPS has been retained by reactor R_{cps200} on 1st and 34th d of operation, respectively. The average variation in BG_{cps} concentration for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 3.64, 8.70, and 18.57 mg/L, respectively (Table 4.2). It can be observed that increased CPS concentration has reduced the CPS retention/removal (Fig. 4.4 to Fig. 4.6). CPS has been removed in a controlled environment by pure cultured microorganism in the majority of the literature, however, the studies are lacking to show the impact of CPS on mixed culture of aerobic sludge biomass.

The variations in COD removal during the stressed phase operation for all reactors have been shown in Fig. 4.3 to 4.6 and data has been summarized in Table 4.2. Initially, a lower COD removal has been observed for reactor R_{cps0} due to the transfer of aerobic sludge biomass from MR to reactors and a sudden increase in organic content in the feed. MR has been fed with 500-550 mg/L of COD (Appendix-II), while the reactors have been fed with a COD of ~ 2700 mg/L in this study. The aerobic sludge biomass has taken 4 d to adapt to the new level of COD in the feed and thereafter started to degrade the organic matter significantly. The performance of reactor R_{cps0} has been observed uniform throughout the experiment. On the 1st d, 17th d, and 34th d of operation, 83.3%, 90.6%, and 91.6% of COD removal has been observed for reactor R_{cps0} , respectively. The average COD removal for reactor R_{cps0} has been observed as 90.0% during stressed phase operation. The addition of CPS (50, 100, and

200 mg/L) to the reactors has negatively impacted COD removal. The reactor $R_{\text{cps}50}$ fed with 50 mg/L of CPS has shown a gradual increase in COD removal (till 15 d of operation), and thereafter COD removal has been stabilized (Fig. 4.4). On the 1st d, 17th d, and 34th d of operation, 56.6%, 67.9%, and 71.7% of COD removal has been observed for reactor $R_{\text{cps}50}$, respectively. The increased COD removal pattern may be attributed to the time taken by aerobic sludge biomass to respond to the presence of CPS in the feed. At this time, aerobic sludge biomass adapts to the new conditions and synthesizes the enzymes needed to degrade CPS. CPS is a complex pesticide that is not easily degradable, which may also attribute to decreased COD removal by CPS-fed reactors. As the CPS concentration increased in reactor $R_{\text{cps}100}$, the COD removal decreased significantly as compared to reactor $R_{\text{cps}50}$. On the 1st d, 17th d, and 34th d of operation, the COD removal for reactor $R_{\text{cps}100}$ has been observed as 61.3%, 50.2%, and 37.4%, respectively as shown in Fig. 4.5. The addition of CPS has caused a decrease in COD removal due to its stress. The CPS is toxic to microorganisms, and its adverse impact has increased with increased CPS concentration in the feed. Initial variation in COD removal could be due to addition of increased CPS concentration in the reactor. Microbes of aerobic sludge biomass could not adapt themselves to the new condition. The COD removal for reactor fed with 200 mg/L of CPS has been severely affected due to the presence of CPS in the feed. On the 1st d, 17th d, and 34th d of operation, 58.7%, 30.4%, and 16.9% COD removal has been observed for reactor $R_{\text{cps}200}$, respectively as shown in Fig. 4.6. The results reveal that increased CPS concentration under stressed phase operation significantly affected the COD removal. The average COD removal for reactors $R_{\text{cps}50}$, $R_{\text{cps}100}$, and $R_{\text{cps}200}$ has been observed as 67.0%, 46.0%, and 37.3%, respectively, under-stressed phase operation. The available literature suggests that the bio-degradation product of CPS is 3, 5, 6-trichloropyridinol (TCP), which is more toxic to aerobic sludge biomass than CPS due to its antimicrobial property. The literature also suggests that microbes are sensitive to the change of CPS concentration, and the performance of the system has been affected mainly by TCP accumulation. The degradation product TCP is more toxic than CPS and has excellent solubility in water (81 mg/L), and it also has antimicrobial properties, which prevents the proliferation of CPS degrading microorganisms (Hou et al., 2018; Wang et al., 2017).

Fig. 4.4 to 4.6 illustrates that the presence of CPS has a negative effect on the COD removal process, and decreased COD removal has been observed with increase in CPS concentration. Very limited literature is available related to reactor performance in the presence of CPS. John and Shaik (2015) have reported that the impact of a pesticide depends on environmental factors, concentration, and the frequency at which they are being applied. Lower concentrations have detrimental effects on microbes and their enzymes. However, concentrations greater than recommended levels exert a deleterious effect on the total microbial population and their biological activity. The stressed phase results suggest that CPS addition to the reactor has inhibited COD degradation and leads to microbial inhibition. García-Mancha et al. (2017) have reported that biological activity has decreased with increasing pollution and toxic load. Pant and Rai (2020) have reported that CPS affects

the metabolism of microorganisms. Microbes release inhibitory stress by converting CPS into less inhibitory by-products as long as their concentrations are below an inhibitory threshold level. Most of the research on CPS removal has been carried out with either pure cultured bacteria (Feng et al., 2017; Khalid and Hashmi, 2016; Niu et al., 2011; Sasikala et al., 2012; Shabbir et al., 2018; Zhang et al., 2018b) or oxidation with Fenton's agent followed by biological oxidation process (Sanchis et al., 2013; Stasinakis et al., 2009; Zhang et al., 2018a).

The addition of CPS has no significant effects on alkalinity. However slight variation in initial and effluent alkalinity has been observed. The average initial alkalinity for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 66.81, 58.35, 61.00, and 55.71 mg CaCO_3/L , respectively, while average effluent alkalinity have been observed as 58.29, 63.53, 62.18, and 62.91 mg CaCO_3/L , respectively.

The COD removal appears to be stabilized after 24th d of operation during stressed phase operation for CPS fed reactors and thereafter yielding steady performance. Summary of environmental and performance related parameters for reactors operated during steady-state of stressed phase operation during 25-34 d are presented in Table 4.3. The data suggests minimum variation in reactors performance. During the steady-state condition, the average CPS retention/removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 41.2%, 18.8%, and 11.5%, respectively. The average BG_{cps} for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 3.83, 9.42, and 17.00 mg/L, respectively, during steady-state of stressed phase operation. The average COD removal for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 90.7%, 72.5%, 39.2%, and 17.7%, respectively, during steady-state condition of stressed phase operation. The variation in alkalinity for all the reactors have been observed in the range of 54.30–66.00 mg CaCO_3/L (Table 4.3).

4.3.1.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The impact of CPS on aerobic sludge biomass characteristics has been assessed grossly through MLSS and MLVSS, ISV, SVI, and ASBA tests. The mixed liquor concentration has been maintained at ~3000 mg/L in each reactor, and excess sludge has been withdrawn every week throughout the operation for characterization. The continuous growth of aerobic sludge biomass and steadily increase in SVI has been observed in reactor R_{cps0} biomass as illustrated in Fig. 4.7(a). The reactor R_{cps0} has been fed with ~2700 mg/L of COD without CPS, which has been easily biodegraded and utilized by the aerobic sludge biomass for their growth. The steadily increase in SVI has been due to uncontrolled growth of filamentous microorganism in reactor R_{cps0} biomass. However, addition of CPS has slowed down biomass growth in reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses as shown in Fig. 4.7(b-d) compared to reactor R_{cps0} biomass [Fig. 4.7(a)]. The MLSS concentration in reactor R_{cps0} has increased from 3098 mg/L (on 1st d) to 3978 mg/L (on 7th d) in a week and has yielded almost similar growth pattern throughout the operation. Similarly, reactor R_{cps50} biomass has shown significant

Table 4.3 Summary of environmental and performance related parameters for reactors operated during 25-34 d of steady-state condition of stressed phase operation.

Parameters	Day-wise	25-34			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.14	1.58	0.47
	CPS (mg/L)	0	50	100	200
Stressed phase	Environmental				
	Room temperature (°C) at 9.30 am	24.3±1.6 (10)	24.3±1.6 (10)	24.3±1.6 (10)	24.3±1.6 (10)
	Room temperature (°C) at 8.45 pm	15.40±1.6 (10)	15.40±1.6 (10)	15.40±1.6 (10)	15.40±1.6 (10)
	Initial pH	7.23±0.15 (20)	6.70±0.17 (20)	6.62±0.18 (20)	6.21±0.12 (20)
	Initial CPS (mg/L)	0	54.20±6.40 (20)	105.45±9.64 (20)	208.35±9.28 (20)
	Effluent pH	7.13±0.09 (20)	7.04±0.14 (20)	6.94±0.17 (20)	6.92±0.15 (20)
	Effluent CPS (mg/L)	0	31.97±4.82 (20)	85.78±6.65 (20)	184.31±7.72 (20)
	CPS retention/removal (%)	0	41.15±3.40 (20)	18.50±2.49 (20)	11.46±3.27 (20)
	Background CPS (mg/L)	0	3.83±0.73 (20)	9.42±1.23 (20)	17.00±2.70 (20)
	Performance				
	Initial soluble COD (mg/L)	2778.08±93.60 (20)	2789.48±104.62 (20)	2781.27±146.74 (20)	2700.00±127.08 (20)
	Effluent soluble COD (mg/L)	259.83±48.11 (20)	765.00±93.29 (20)	1687.00±106.75 (20)	2220.20±86.59 (20)
	COD removal (%)	90.65±1.68 (20)	72.48±3.97 (20)	39.21±4.53 (20)	17.70±2.53 (20)
	Initial alkalinity (mg CaCO ₃ /L)	66.00±3.58 (20)	56.00±5.22 (20)	62.10±3.53 (20)	54.30±4.66 (20)
	Effluent alkalinity (mg CaCO ₃ /L)	55.80±4.09 (20)	63.80±5.13 (20)	60.60±6.89 (20)	62.10±4.94 (20)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

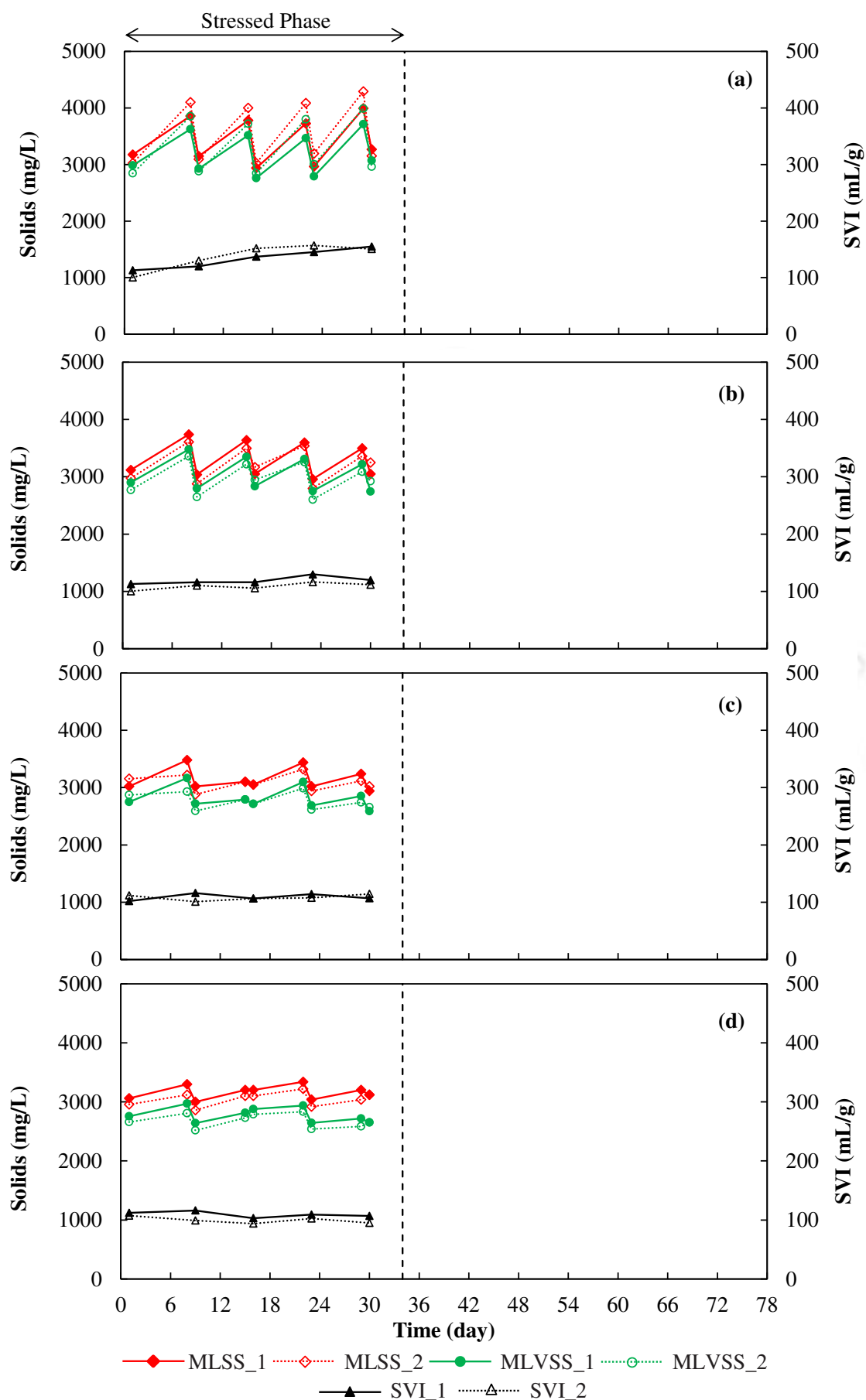


Fig. 4.7 Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} biomasses during 0-34 d under stressed phase operation.

Table 4.4 Summary of solids characteristics estimated during stressed phase operation.

Gross parameters	Stressed phase operation				
	Day-wise	0-34			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
MLSS (mg/L)		3097.20±108.38 (10)	3030.00±123.59 (10)	3009.60±73.54 (10)	3037.60±95.97 (10)
MIVSS (mg/L)		2905.13±101.71 (10)	2793.08±110.31 (10)	2690.84±86.72 (10)	2673.04±106.68 (10)
MIVSS/MLSS ratio		0.94±0.01 (10)	0.92±0.01 (10)	0.90±0.01 (10)	0.89±0.01 (10)
SVI (mL/g)		135.94±20.37 (10)	113.96±6.40 (10)	108.67±5.48 (10)	104.46±5.25 (10)
ISV (m/h)		2.13±0.28 (10)	2.42±0.05 (10)	2.49±0.03 (10)	2.52±0.05 (10)

[The data is presented in $xx \pm yy$ (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

growth in a week (from 3050 mg/L to 3676 mg/L) [Fig. 4.7(b)]. On the other hand, the reactors fed with higher doses of CPS (reactor R_{cps100} and R_{cps200} biomasses) have shown slower biomass growth as compared to reactor R_{cps0} and R_{cps50} biomasses. The reactor R_{cps200} biomass has shown little growth (from 3088 mg/L to 3350 mg/L in a week) [Fig. 4.7(d)] compared to reactor R_{cps0} biomass. The zig-zag pattern in solids concentrations are due to withdrawal of biomasses for analysis as well as to maintain its level around 3000 mg/L. The MIVSS/MLSS ratio has ranged between 0.89-0.94 for all the reactors during stressed phase operation (Table 4.4). The SRT for reactors R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} has been observed in the range of 29-56 d during stressed phase operation. CPS is toxic to aerobic sludge biomass and causes inhibition to microbial growth, resulting in slower biomass growth. As the concentration of CPS in the reactors rises, the biomass growth in the reactors has slowed down depending on the level of CPS concentration.

The SVI is the indicator of sludge settling and compactness. Grady et al. (2011) have characterized the aerobic sludge biomass for settling as excellent, moderate, and poor if the SVI are 80, 80-150, and >150 mL/g, respectively. The SVI of aerobic sludge biomass for all the reactors have been shown in Fig. 4.7 and data has been summarized in Table 4.4. The SVI for reactor R_{cps0} biomass has been observed as 107, 144, and 153 mL/g on 1st, 16th, and 30th d of operation, respectively (Fig. 4.7). While SVI has been obtained as 107, 110, and 116 mL/g for reactor R_{cps50} biomass; 107, 106, and 111 mL/g for reactor R_{cps100} biomass; 110, 98, and 101 mL/g for reactor R_{cps200} biomass on 1st, 16th, and 30th d of operation, respectively (Fig. 4.7). The reactor R_{cps0} biomass has shown continuous increasing trend in SVI under stressed phase operation due to abundance of filamentous microorganisms. Whereas CPS fed reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses have shown decrease in SVI. The SVI decreases with increase in CPS concentration in reactors (Fig. 4.7). The average SVI for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been observed as 135.94, 113.96, 108.67, and 104.46 mL/g, respectively, under stressed phase operation (Table 4.4).

The ISV tests for all the reactors have been carried out on every 7th d throughout the operation, as shown in Fig. 4.8 and ISV has been estimated, as shown in Fig. 4.9 and summarized data is presented in Table 4.4. Fig. 4.8(a) shows the settling behavior for

reactor R_{cps0} biomass, which has deteriorated as the operation progressed due to growth of filamentous biomass. However, the settling behavior for reactor R_{cps200} biomass has remained the same throughout the stressed phase operation as shown in Fig. 4.8(b).

The ISV has also been affected due to addition of CPS as shown in Fig. 4.9. The ISV for reactor R_{cps0} biomass have been observed as 2.48, 2.11, and 1.79 m/h, while 2.49, 2.42, and 2.37 m/h have been observed for reactor R_{cps50} biomass on 1st, 16th, and 30th d of operation. Similarly, ISV for reactor R_{cps100} biomass have been observed as 2.49, 2.50, and 2.49 m/h, respectively, while 2.49, 2.54, and 2.53 m/h of ISV have been observed for reactor R_{cps200} biomass on 1st, 16th, and 30th d of operation during stressed phase. The ISV has increased with increase in CPS concentration (for CPS fed reactor biomasses) compared to reactor R_{cps0} biomass. The decrease in ISV for reactor R_{cps0} biomass may be attributable to filamentous microorganism growth, but CPS fed reactor aerobic sludge biomass has transitioned from filamentous to non-filamentous form during stressed phase operation with insignificant variation in ISV. The average ISV for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have been observed as 2.13, 2.42, 2.49, and 2.52 m/h, respectively, during stressed phase operation (Table 4.4).

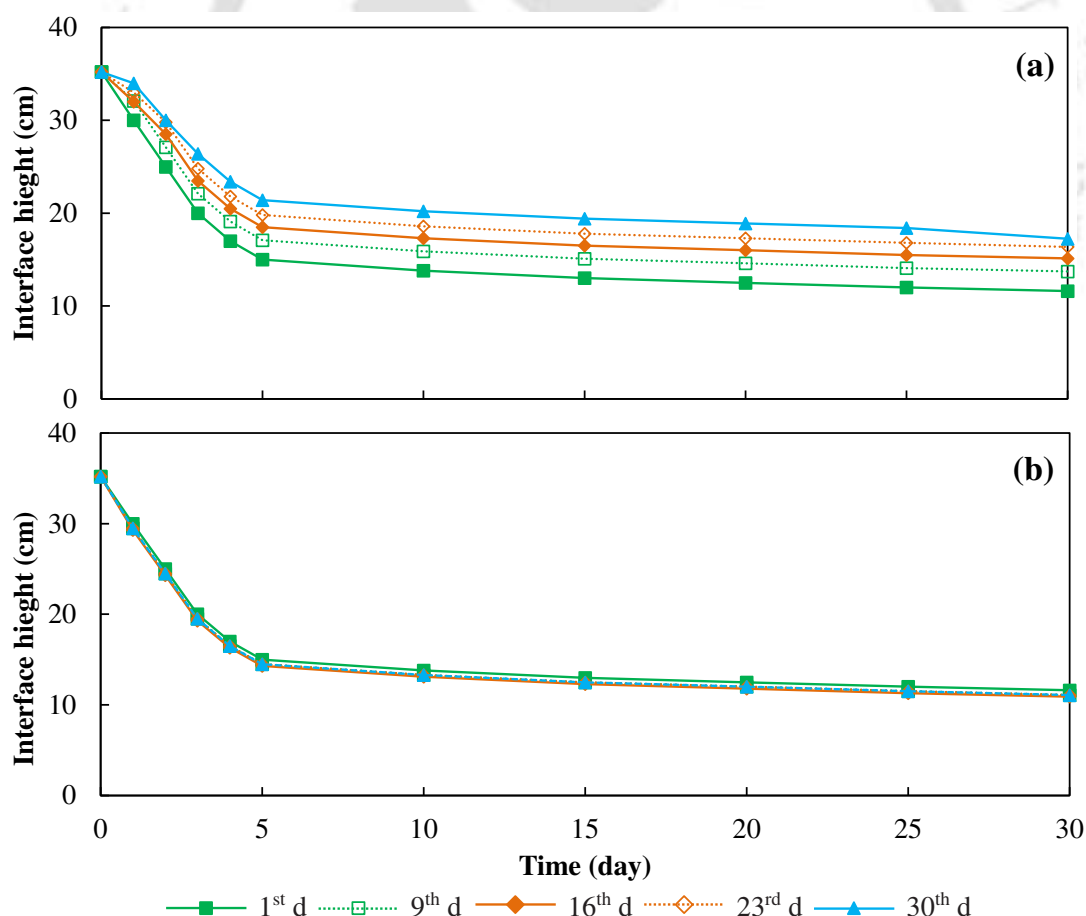


Fig. 4.8 Typical settling test results for reactor (a) R_{cps0} and (b) R_{cps200} biomasses during 0-34 d under stressed phase operation.

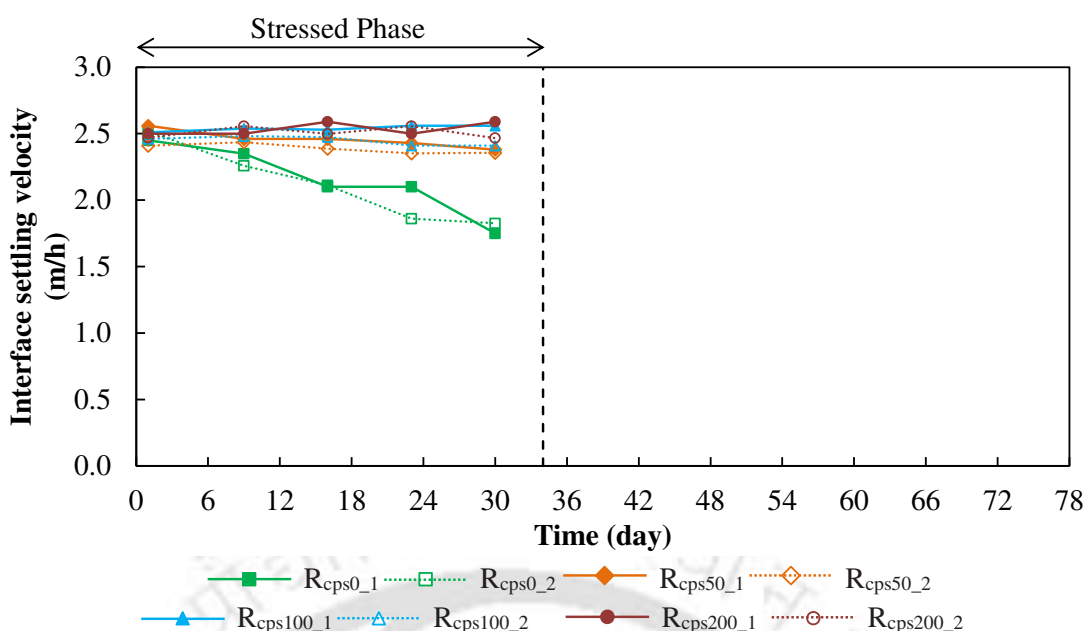


Fig. 4.9 Variation in interface settling velocities of reactor biomasses during 0-34 d under stressed phase operation.

Aerobic sludge biomass activity test

ASBA test has been carried out towards the end of the stressed phase operation (on 34th d) when reactors have been operated under the steady-state condition. The results have been shown in Fig. 4.10 and data has been presented in Table 4.5. The results obtained from the ASBA test suggest that aerobic sludge biomasses have been severely affected by CPS stress. The ASBA for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have been observed as 7.42, 6.57, 4.30, and 2.04 mg COD removed/mg VSS d, respectively, and it has decreased with increase in CPS concentration (Table 4.5). The results suggest that increased concentration of CPS has adversely impacted the microbial activity due to its stress. It has been reported that CPS could severely inhibit microbial activity and influence the degradation of COD (Wang et al., 2017). This may also be the reason for lower COD removal in CPS-fed reactors during stressed phase operation.

Biomass related general parameters such as MLSS and MLVSS have not reflected significant changes due to presence of CPS during stressed phase operation. Similarly, a minor variation in MLVSS/MLSS ratio has been observed in the range of 0.89-0.94 for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses. However, SVI measurements have demonstrated significant variation between control reactor biomass and CPS fed reactor biomasses. The reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have shown SVI of 136, 114, 109, and 104 mL/g, respectively. ISV for all reactor biomasses have revealed minor changes. The ISV for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses are 2.42, 2.49, and 2.52 m/h – on lower side compared to control reactor (2.13 m/h). The minor changes in SVI and ISV reflect the minor changes in aerobic sludge biomass characteristics. In addition, ASBA has been significantly affected due to CPS stress. ASBA for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses are 6.57, 4.30, and 2.04 mg COD removed/mg VSS d – on lower side compared to control reactor

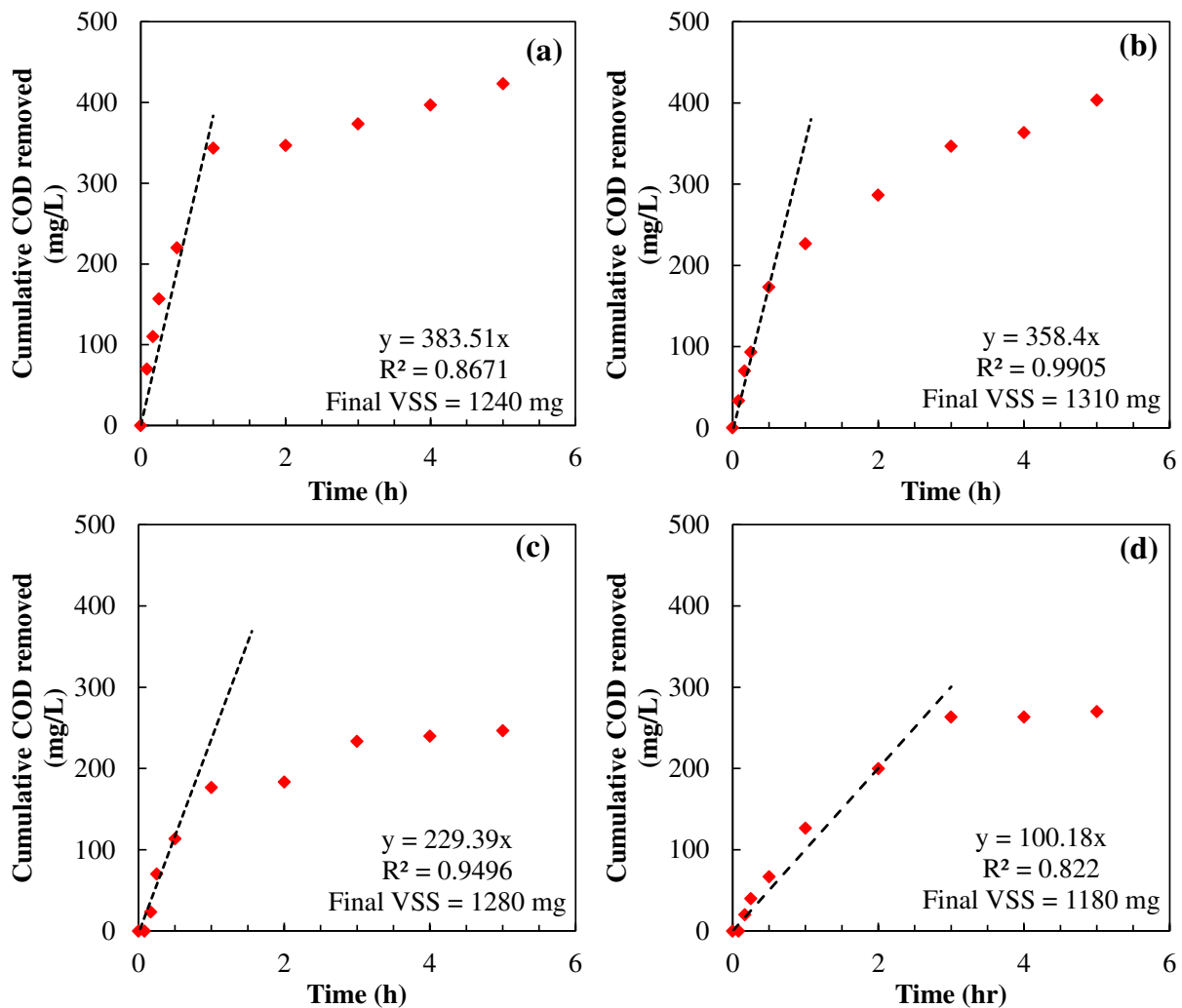


Fig. 4.10 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} on 34th d of stressed phase operation.

(7.42 mg COD removed/mg VSS d). The ASBA values suggested that CPS stress has affected the organic degrading capability of aerobic sludge biomass. Adverse impact of CPS on ASBA has been directly correlated to COD removal. During steady-state condition of stressed phase operation, the COD removal for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} are 91%, 72%, 39%, and 18%, respectively, which has also shown a declining trend similar to ASBA. According to the results, SVI and ISV may contribute to biomass evaluation to a lesser extent, while the ASBA test contributes to a greater extent.

Table 4.5 Aerobic sludge biomass activity test results carried out towards the end of stressed phase operation.

Stressed phase operation				
Biomass withdrawn on	34 th d			
Reactor	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
ASBA (mg COD removed/mg VSS d)	7.42	6.57	4.30	2.04

4.3.1.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

The floc structure and morphology of the sludge have been examined through microscopic observation. The images of aerobic sludge biomass samples have been taken at $5\times$ and $100\times$ magnification using the digital camera attached to the microscope after being stained (Gram's staining), and the result has been presented in Table 4.6. The changes in biomass morphology during stressed phase operation has also been observed through the photographic observation as shown in Table 4.7. The aerobic sludge biomass has been stained and observed under a fluorescence microscope for cell viability, and results have been presented in Table 4.8. The filament index (FI) has also been estimated during stressed phase operation, and data are summarized in Table 4.9.

Table 4.6 suggest that floc morphology of reactor R_{cps0} biomass has continuously been increased in size, and filament abundance has been observed throughout. Whereas, aerobic sludge biomass morphology of CPS fed reactors R_{cps50} , R_{cps100} , and R_{cps200} have changed with increased CPS concentration. The microscopic observation suggests that the abundance of filamentous microorganisms has decreased marginally in the presence of CPS under the stressed phase operation. On the 1st d of operation, all reactor biomasses have contained filaments. The presence of lesser filaments has been observed for CPS-fed reactor biomasses from 8th d onward (at $5\times$ magnification). The images taken on 8th d (at $5\times$ magnification) show decreased filaments with increase in CPS concentration. The density of filaments in reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses appear to have gradually decreased in comparison with reactor R_{cps0} biomass as stressed phase progressed. On 15th and 29th d of operation, reactor R_{cps100} and R_{cps200} biomasses have shown reduced and weaker filaments as compared to 1st d of operation. Little aerobic sludge biomass growth at 200 mg/L of CPS has appeared and apparently loose flocs have been observed under the stressed phase operation.

The photographic observation of aerobic sludge biomass has been shown in Table 4.7. The aerobic sludge biomass of reactor R_{cps0} has continuously increased in shape and size (from 1st to 29th d). The thick and denser flocs have been observed in reactor R_{cps0} biomass. Floc morphology on the 1st d has been observed to be almost the same for all reactor biomasses. However, the addition of CPS to the reactors has led to the disintegration of flocs. The floc size in CPS fed reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have decreased gradually with increase in CPS concentration in the feed. In reactor R_{cps50} biomass, flocs have completely been disintegrated till 22nd d of operation and no thick flocs have been observed thereafter. For reactor R_{cps100} and R_{cps200} biomasses, the density of filaments have decreased significantly after 15th d of operation. Loose filament strands have been observed towards the end of stressed phase operation (on 29th d) for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses. The possible reason could be the toxicity of CPS for microorganisms of aerobic sludge biomass. The presence of CPS in reactors has led to inhibition of filamentous growth, and the level of inhibition on growth depends on CPS concentration. There is currently no literature available

Table 4.6 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operation.

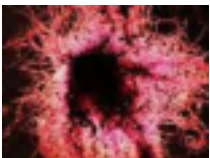
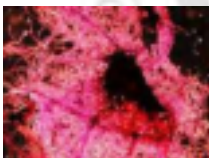
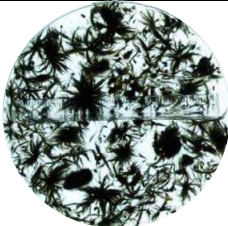
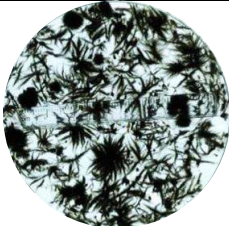
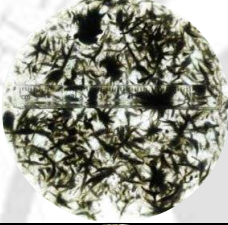
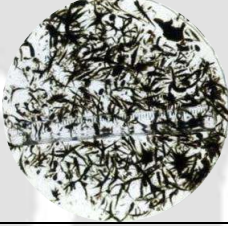
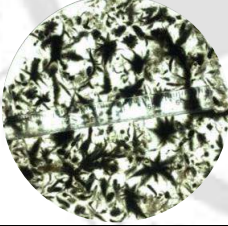
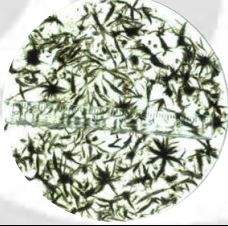
Reactor Mag.	Aerobic sludge biomass samples withdrawn on				
	Stressed phase operation				
	1 st d	8 th d	15 th d	22 nd d	29 th d
R_{cps0}	5× 	5× 	5× 	5× 	5× 
	100× 	100× 	100× 	100× 	100× 
R_{cps50}	5× 	5× 	5× 	5× 	5× 
	100× 	100× 	100× 	100× 	100× 
R_{cps100}	5× 	5× 	5× 	5× 	5× 
	100× 	100× 	100× 	100× 	100× 
R_{cps200}	5× 	5× 	5× 	5× 	5× 
	100× 	100× 	100× 	100× 	100× 

Table 4.7 Photographic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operations.

Sample withdrawn on	Aerobic sludge biomass withdrawn from reactors			
	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
1 st d				
8 th d				
15 th d				
22 nd d				
29 th d				

dealing with morphology of aerobic sludge biomass exposed to CPS-containing wastewater.

Fluorescence microscopic results distinguish the live and dead portion of aerobic sludge biomass as presented in Table 4.8. The bright-field images show the overall flocs structure; the red color represents the inactive/dead biomass portion, while the green color exhibits the active/live biomass portion of aerobic sludge biomass. On 1st d of operation, the live and dead portion of biomass have been observed as the same for all reactors. However, the addition of CPS has affected the biomass, and the dead/inactive portion has increased with the increase of CPS concentration in reactor feeds, as could be seen for biomasses withdrawn on 34th d of operation. The inactive portion in aerobic sludge biomass has gradually increased as the CPS concentration increased in the reactor (Table 4.8). According to the fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses, there were 98.2%, 48.3%,

33.7%, and 21.8% viable cells and 0.7%, 44.9%, 58.4, and 72.2% dead cells, respectively. Reactor R_{cps200} biomass has shown larger inactive portion than reactor R_{cps50} and R_{cps100} biomasses, indicating CPS might have toxic effect on aerobic sludge biomass.

FI values have been presented in Table 4.9, which demonstrates the abundance of filaments in reactors. Initially, all the reactors have contained more filamentous microorganisms, hence the FI values for all reactor have been observed as 5 on 1st d of the operation. On 8th d, FI value for reactor R_{cps0} , R_{cps50} , and R_{cps100} biomasses have been observed as 5, while R_{cps200} biomass has shown 4. On the other hand, FI values for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have been observed as 5, 5, 4, and 4, respectively, from 15th d of operation and no change has been observed till the end. While estimating FI,

Table 4.8 Fluorescence microscopic images of stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during stressed phase operations.

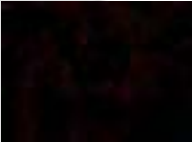
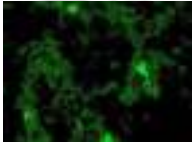
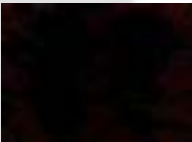
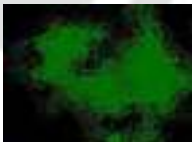
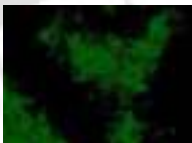
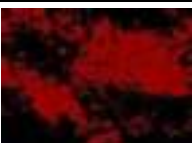
Sample withdrawn on	Reactor	Bright field (20×)	Dead Cells (20×)	Live Cells (20×)
1 st d	R_{cps0}			
	R_{cps50}			
	R_{cps100}			
	R_{cps200}			
34 th d	R_{cps0}			
	R_{cps50}			
	R_{cps100}			
	R_{cps200}			

Table 4.9 Filament index of aerobic sludge biomass estimated during stressed phase operation.

Biomass withdrawn on Day	Stressed phase operation			
	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
1 st d	5	5	5	5
8 th d	5	5	5	4
15 th d	5	5	4	4
22 nd d	5	5	4	4
29 th d	5	5	4	4

all the filaments have been counted without making any distinction between dead and live filaments. This might be probable cause for no change being observed in FI values during stress imposed by CPS.

Polysaccharide, protein and extracellular polymeric substance

Polysaccharide (PS), protein (PN), and extracellular polymeric substance (EPS) contents in aerobic sludge biomass have been estimated on 1st and 34th d of the operation. The results have been shown in Fig. 4.11 and data has been presented in Table 4.10. There has not been much change observed in PS, PN, and EPS contents in aerobic sludge biomass throughout the experiment. On 1st and 34th d of operation, PS content for reactor R_{cps0} biomass has been obtained as 10.23 and 13.23 mg/g VSS, respectively, PN content as 36.34 and 44.34 mg/g VSS, respectively, and EPS contents as 46.57 and 57.57 mg/g VSS, respectively. The CPS fed reactor biomasses have shown relatively higher PS, PN, and EPS contents. The PS content for CPS fed reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been observed as 14.21, 14.42, and 14.34 mg/g VSS, respectively, on 34th d of operation. Similarly, PN content for CPS fed reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been observed as 42.02, 43.07, and 42.06 mg/g VSS, respectively. While, 56.21, 57.42, and 56.34 mg/g VSS of EPS have been obtained for reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively, on 34th d of operation. The microorganism secretes and synthesizes EPS and protein under the stressed phase to protect themselves from the toxic effect of CPS. Hence, an enhanced level of PS, PN, and EPS has been observed. EPS also protects the flocs structure in the presence of toxic compounds in the feed (Kong et al., 2017). EPS also contain functional groups responsible for CPS removal and act as a permeability barrier to hinder intracellular penetration (Kong et al., 2017; Wang et al., 2018).

Enzyme activity

Microorganisms perform an indispensable role in CPS degradation in various environments (soil, liquid) due to their hydrolytic and oxidative enzymes (Dar et al., 2019). CPS inhibits bacterial enzymes, including hydrolases and oxidoreductases, which depend upon environmental factors, i.e., pH and temperature (Kollah et al., 2019). The impact of CPS on catalase, dehydrogenase, urease, and protease activities have been estimated on 1st and 34th d of operation. The results have been shown in Fig. 4.12, and a summary of enzyme activity values have been presented in Table 4.11.

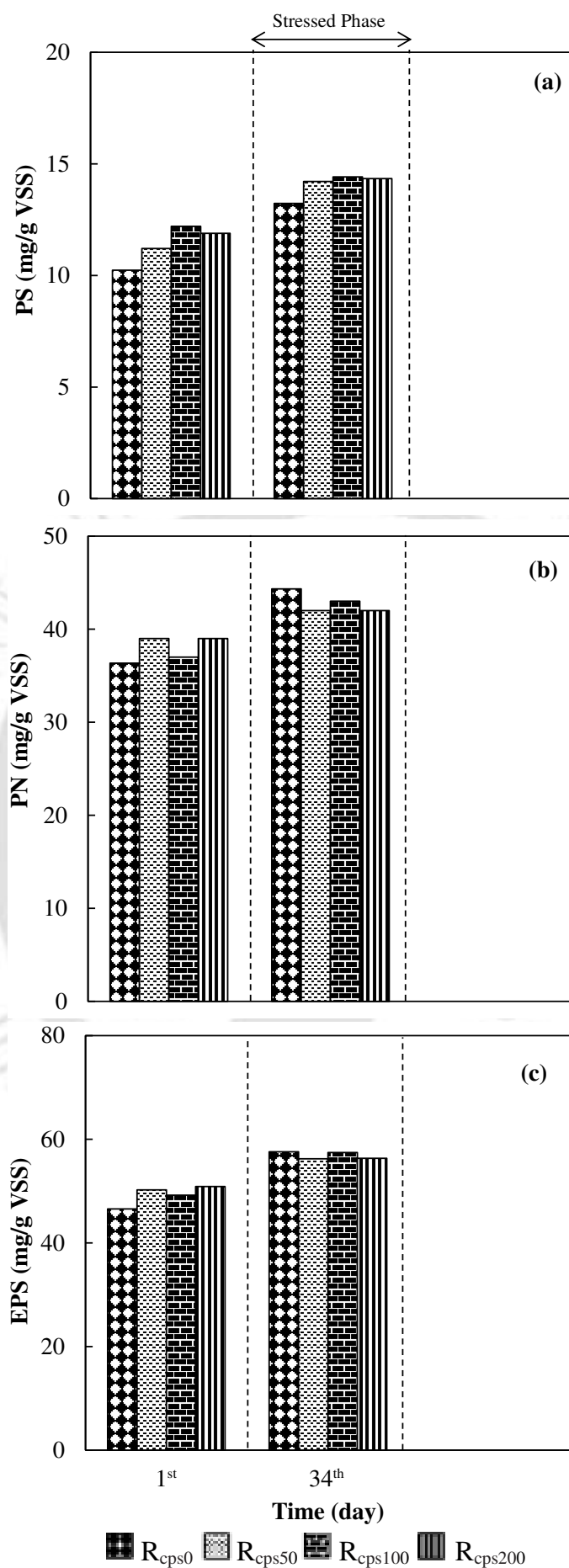


Fig. 4.11 Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase operation.

Table 4.10 PS, PN, and EPS contents in aerobic sludge biomass estimated under stressed phase operation.

Biomass withdrawn on	Reactor biomass	PS	PN	EPS
		mg/g VSS		
Stressed phase operation				
1 st d	R _{cps0}	10.23±0.86	36.34±0.78	46.57±0.82
	R _{cps50}	11.21±0.74	39.12±1.41	50.21±1.07
	R _{cps100}	12.21±1.21	37.02±1.22	49.21±1.20
	R _{cps200}	11.89±0.63	39.11±1.42	50.89±1.01
34 th d	R _{cps0}	13.23±1.23	44.34±0.98	57.57±1.10
	R _{cps50}	14.21±0.89	42.02±1.82	56.21±1.34
	R _{cps100}	14.42±0.92	43.08±1.03	57.42±0.96
	R _{cps200}	14.34±0.83	42.06±1.81	56.34±1.31

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

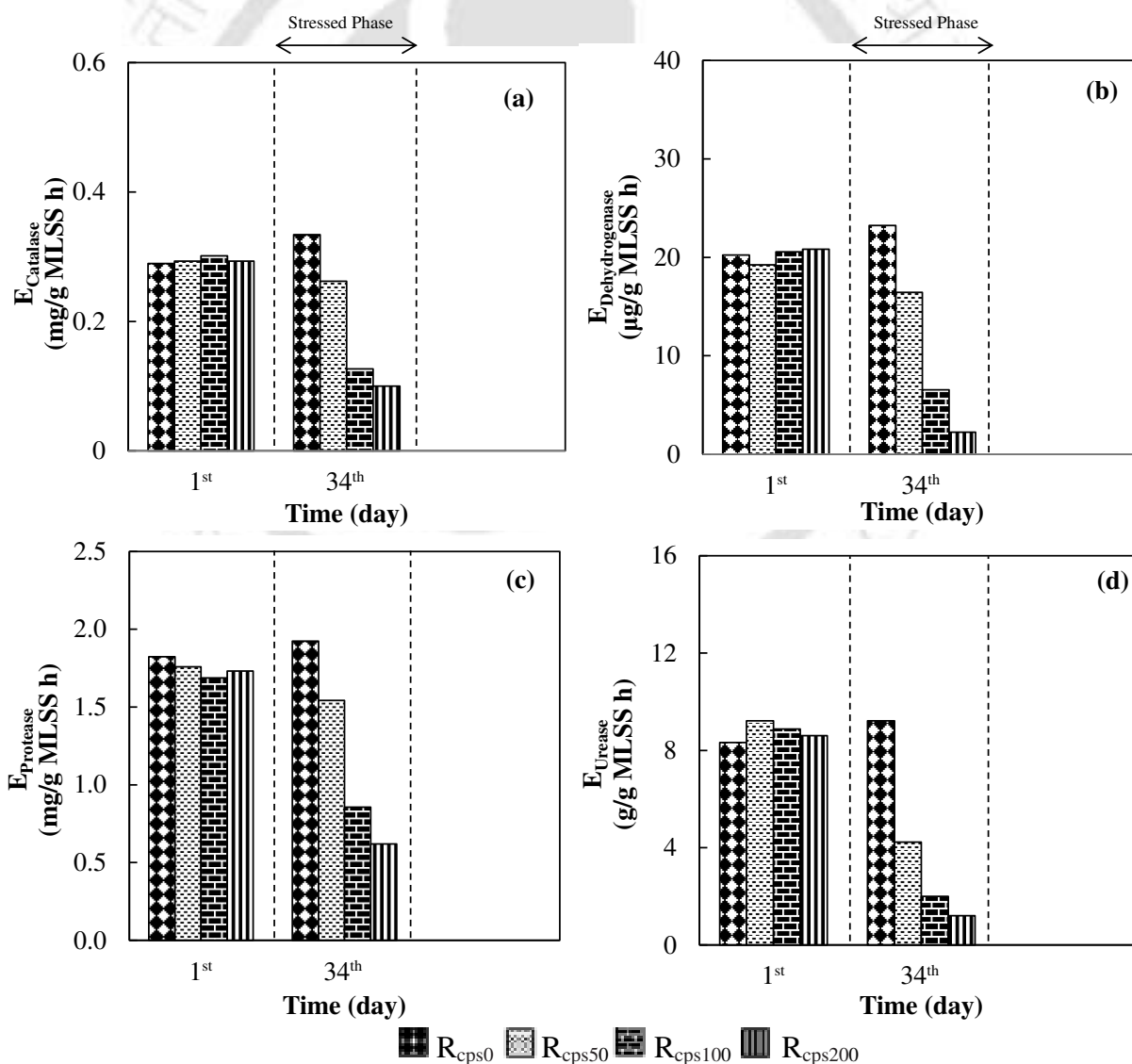
**Fig. 4.12** Enzyme activities of aerobic sludge biomass (a) E_{Catalase}, (b) E_{Dehydrogenase}, (c) E_{Protease}, and (d) E_{Urease} assessed during stressed phase operation.

Table 4.11 Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase operation.

Biomass withdrawn on	Reactor biomass	E_{Catalase} (mg/g MLSS h)	$E_{\text{Dehydrogenase}}$ ($\mu\text{g/g MLSS h}$)	E_{Protease} (mg/g MLSS h)	E_{Urease} (g/g MLSS h)
Stressed phase operation					
1 st d	R _{cps0}	0.29±0.03	20.23±1.32	1.82±0.09	8.32±0.80
	R _{cps50}	0.29±0.02	19.23±1.52	1.76±0.05	9.21±0.90
	R _{cps100}	0.30±0.01	20.55±0.92	1.69±0.15	8.88±1.20
	R _{cps200}	0.29±0.01	20.82±1.41	1.73±0.07	8.61±1.30
34 th d	R _{cps0}	0.33±0.02	23.23±1.64	1.92±0.14	9.21±1.60
	R _{cps50}	0.26±0.02	16.43±1.96	1.54±0.06	4.23±0.51
	R _{cps100}	0.12±0.02	6.54±2.01	0.85±0.13	2.02±0.61
	R _{cps200}	0.10±0.02	2.23±0.81	0.62±0.17	1.20±0.32

[The data is presented in $xx \pm yy$ format where xx is the average value, yy is the standard deviation.]

On 1st d of operation, catalase activity (E_{Catalase}) for all reactors has been observed between 0.29-0.30 mg/g MLSS h. Towards the end of the stressed phase operation (on 34th d), E_{Catalase} has been observed as 0.33, 0.26, 0.12, and 0.10 mg/g MLSS h for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively [Fig. 4.12(a)]. The results suggest that the addition of CPS has lowered E_{Catalase} of aerobic sludge biomasses. CPS concentration at 100 mg/L has reduced E_{Catalase} almost half as compared to reactor R_{cps0} biomass. Moreover, higher CPS concentration (200 mg/L) has severely affected E_{Catalase} . There is no literature currently available which could explain the effect of CPS on E_{Catalase} . The % inhibition of E_{Catalase} for CPS fed reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses has been obtained as 21.6%, 64.0%, and 70.0%, respectively.

The results for dehydrogenase activity ($E_{\text{Dehydrogenase}}$) has been shown in Fig. 4.12(b) and data has been summarized in Table 4.11. $E_{\text{Dehydrogenase}}$ for all reactor has ranged between 19.23-20.82 $\mu\text{g/g MLSS h}$ on 1st d of operation. Towards the end of the stressed phase operation (on 34th d), $E_{\text{Dehydrogenase}}$ for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses has been observed as 23.23, 16.43, 6.54, and 2.23 $\mu\text{g/g MLSS h}$, respectively [Fig. 4.12(b)], which suggest that CPS has negative impact on $E_{\text{Dehydrogenase}}$. The high dose of CPS could inhibit $E_{\text{Dehydrogenase}}$ almost by 10 times as compared to reactor R_{cps0} biomass. The % inhibition of $E_{\text{Dehydrogenase}}$ for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been observed 29.3%, 71.8%, and 90.4%, respectively, under stressed phase operation. The results indicate that CPS could completely inhibit $E_{\text{Dehydrogenase}}$ at higher concentration (200 mg/L of CPS).

The effect of CPS on protease activity (E_{Protease}) has been shown in Fig. 4.12(c) and data has been summarized in Table 4.11. Initially (1st d of operation), E_{Protease} has been observed between 1.69-1.82 mg/g MLSS h. E_{Protease} has been affected due to CPS exposure. On 34th d of operation, E_{Protease} has been observed as 1.92, 1.54, 0.86, and 0.13 mg/g MLSS h for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively. The lower dose (50 mg/L) of CPS has slightly affected E_{Protease} ; however, high dose (200 mg/L) has severely inhibited E_{Protease} . The impact of CPS on E_{Protease} has been calculated in terms of % inhibition, which has

been observed as 19.8%, 55.5%, and 67.8% for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively under stressed phase operation.

Urease activity (E_{Urease}) has also been affected due to presence of CPS in the feed as shown in Fig. 4.12(d) and Table 4.11. The addition of CPS has caused a negative impact on E_{Urease} . On the 1st d of operation, E_{Urease} has been observed in the range of 8.32-9.21 g/g MLSS h for all reactor biomasses. In contrast, on 34th d of operation, E_{Urease} for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses has been observed as 9.21, 4.23, 2.00, and 1.20 g/g MLSS h, respectively. The addition of CPS has caused 54.1%, 78.3%, and 86.9% of E_{Urease} inhibition, which has increased with increase in CPS concentration in the feed. Similar to other three enzymes, urease activity has also been affected by CPS and its concentration in the feed.

The results indicate that $E_{Dehydrogenase}$ and E_{Urease} have been severely affected as compared to $E_{Catalase}$ and $E_{Protease}$ at 200 mg/L of CPS in the feed. The % inhibition of enzyme activity has increased with an increase in CPS concentration. There is no literature available to relate aerobic sludge biomass enzyme activity with CPS concentration in the feed. The available literature is related to soil enzyme activity which suggests that CPS causes a negative effect on soil enzymatic activities and soil microorganisms diversity (Tejada et al., 2014). Pradeep and Subbaiah (2015) have reported that the enzymes involved in the degradation of CPS are membrane-bound. It has also been reported that CPS affects the enzymatic activity (dehydrogenase, urease, and protease) of soil (Jastrzbska, 2011).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA has been performed with the biomass samples withdrawn towards the end of the stressed phase operation (on 34th d) to assess the possible shift in microbial community structure of aerobic sludge biomass due to CPS exposure. Three different (*AluI*, *HhaI*, and *MspI*) tetra-cutter restriction enzymes have been used to digest the biomass samples as suggested by Das and Dash (2015). Fig. 4.13 shows the digestion pattern and dendrogram of stressed phase aerobic biomass samples digested with *AluI*, *HhaI*, and *MspI*. The dendrogram has been prepared using GelJ software, and a similarity table has been generated based on the gel image presented in Table 4.12. The results obtained after gel run (Fig. 4.13) implies that the reactor biomasses show different banding patterns for each restriction enzyme. The dendrogram has demonstrated the relationship among all the reactor biomasses. Fig. 4.13(a) exhibit that the biomass from reactor R_{cps50} is closest to the reactor R_{cps0} biomass, whereas, biomass from reactor R_{cps200} exist faraway to reactor R_{cps0} biomass. Similar observations have been made for biomass samples digested with *HhaI* [Fig. 4.13(b)] and *MspI* [Fig. 4.13(b)]. Table 4.12 shows the similarity among all reactor biomasses for each restriction enzymes obtained on the basis of dendrogram. The aerobic sludge biomass samples have shown 69.3%, 57.5%, and 42.3% similarity for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, compared with reactor R_{cps0} biomass, while 78.5%, 68.4%, and 54.6% similarity has been observed with *HhaI* for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, compared

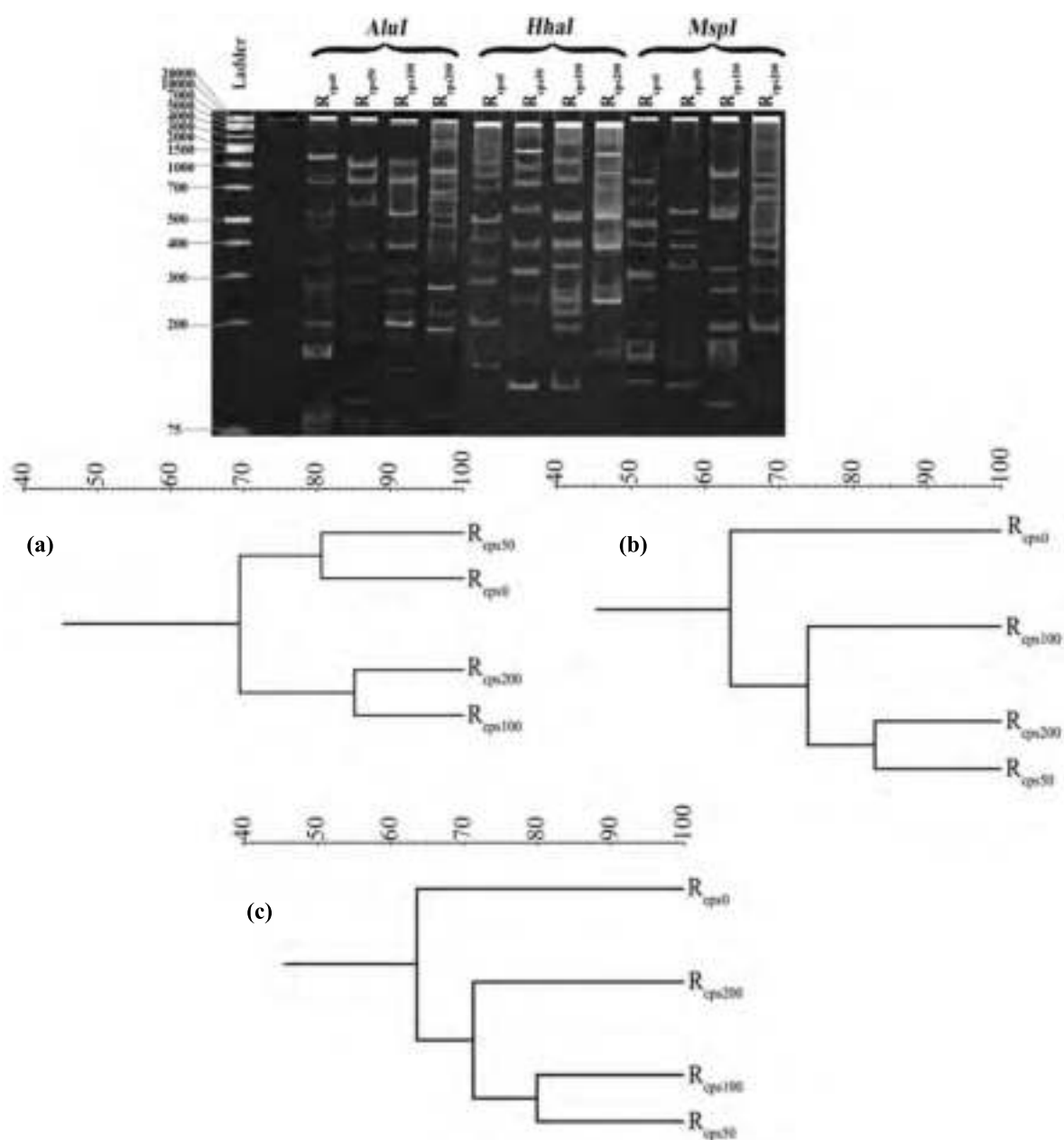


Fig. 4.13 Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase operation treated with (a) *AluI*, (b) *HhaI*, and (c) *MspI* restriction enzymes.

with reactor R_{cps0} biomass. The restriction enzyme *MspI* has shown 72.3%, 63.2%, and 51.9% similarity for reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively, compared with reactor R_{cps0} biomass, respectively.

These findings suggest that an increase in CPS concentration in reactors has decreased the similarity between the CPS fed reactor biomasses and the control reactor biomass. The reactor biomass fed with a lower CPS dose has shown highest similarity with the control reactor biomass as compared to the reactor biomasses fed with higher CPS doses. It can be assumed that an increase in CPS concentration may cause a significant change in the microbial community in aerobic sludge biomass, and only CPS tolerant microbes have been growing in the reactors. [Tejada et al. \(2014\)](#) have also reported that CPS addition to the soil may cause possible changes in the microbial community of soil. [Zhang et al. \(2016\)](#) have

Table 4.12 Similarity matrix resulting from digestion of aerobic sludge biomass samples withdrawn during stressed phase operation by restriction enzymes.

Stressed phase operation												
Reactor	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
R_{cps0}	1	0.6930	0.5753	0.4232	1	0.7850	0.6840	0.5455	1	0.7231	0.6323	0.5192
R_{cps50}	0.6930	1	0.5834	0.5034	0.7850	1	0.7230	0.8420	0.7231	1	0.7923	0.6434
R_{cps100}	0.5753	0.5834	1	0.7632	0.6840	0.7230	1	0.8023	0.6323	0.7923	1	0.7643
R_{cps200}	0.4232	0.5034	0.7632	1	0.5455	0.8420	0.8023	1	0.5192	0.6434	0.7643	1

reported that the main difference in the microbial community structure took place after adding CPS, compared to the original artificial sewage. Sasikala et al. (2012) have tried to isolate CPS degrading bacteria from soil. The number of isolates has been lesser in the soil taken for the study. They suggested that it might be due to the inhibitory effect of CPS on the soil microbial population. Fang et al. (2009) have reported that CPS concentration provokes inhibitory effects on microbes, though the duration of inhibiting effects on microbial functional diversity has been temporary. Therefore, CPS concentration may cause microbial community shift, which is concentration-dependent.

The findings of the microscopic analysis has indicated that the control reactor has an abundance of filaments, whereas CPS-fed reactors have marginally lesser filaments. With a rise in the CPS concentration in the reactor, filaments have become lesser in number and weaker towards the end of stressed phase operation. The thick and denser flocs with filaments have been observed in reactor R_{cps0} biomass, however, the addition of CPS to the reactors has led to disintegration of flocs to filaments. The floc size in CPS fed reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has decreased gradually with increase in CPS concentration in the feed. At steady-state condition of stressed phase operation, the proportion of live cells has reduced, and the dead cells proportion has risen at the same time. The fluorescence microscopic results have shown 98.2%, 48.3%, 33.7%, and 21.8% viable cells and 0.7%, 44.9%, 58.4, and 72.2% dead cells for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. CPS stress to the biomass has slightly affected the FI values. The FI value has been observed as 5 on 1st d for all reactors, which has remained same for reactor R_{cps0} and R_{cps50} biomasses towards end of the stressed phase operation. Whereas, FI value for reactor R_{cps100} , and R_{cps200} biomasses has been reduced to 4 from 5. PS, PN, and EPS contents have not shown much variation during stressed phase operation. The PS content for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 14.21-14.42 mg/g VSS, while 13.23 mg/g VSS for reactor R_{cps0} biomass. Similarly, PN content for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 42.02-43.08 mg/g VSS, while 44.34 mg/g VSS for reactor R_{cps0} biomass. EPS content for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 56.21-56.34 mg/g VSS, which is slightly lower than reactor R_{cps0} biomass (57.57 mg/g VSS). $E_{Dehydrogenase}$ and E_{Urease} activities have been the most severely affected among the four enzymes tested. $E_{Catalase}$ for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 0.10-0.26 mg/g MLSS h, while 0.33 mg/g MLSS h for reactor R_{cps0} biomass. $E_{Dehydrogenase}$ for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 2.23-16.43 μ g/g MLSS h, while 23.23 μ g/g MLSS h for reactor R_{cps0} biomass. $E_{Protease}$ for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 0.62-1.54 mg/g MLSS h, while 1.92 mg/g MLSS h for reactor R_{cps0} biomass. Similarly, E_{Urease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged from 1.20-4.23 g/g MLSS h, while 9.21 g/g MLSS h for reactor R_{cps0} biomass. It seems that $E_{Dehydrogenase}$ and E_{Urease} have been more effective than $E_{Catalase}$ and $E_{Protease}$ in measuring the stress imposed by CPS. ARDRA has revealed the changes in microbial community structure. *AluI* has shown 69.3%, 57.5%, and 42.3% similarity; *HhaI* has shown 78.5%, 68.4%, and 54.6% similarity; and *MspI* has shown 72.3%, 63.2%, and

51.9% similarity for reactors biomasses fed with CPS concentration of 50, 100, and 200 mg/L, respectively, as compared to reactor R_{cps0} biomass. Reconsidering the techniques used in evaluation of biomass, it seems that fluorescence microscopy, $E_{Dehydrogenase}$, E_{Urease} , and ARDRA appeared as effective techniques, which have ability to reflect changes in biomass during CPS stress. It has been evident from the foregoing discussion that CPS stress has little impact on FI values, PS, PN, and EPS contents as well as $E_{Catalase}$ and $E_{Protease}$ activities, while it has severe impact on cell viability, $E_{Dehydrogenase}$ and E_{Urease} and microbial community structure. It is indeed important to investigate the reasons for the same.

4.3.2 Recovery phase operation

After operating reactors under steady-state conditions for 25-34 d during the stressed phase operation, the addition of CPS in feed has been discontinued and thereafter operated for 35-76 d terming the operation as recovery phase operation. To highlight the differences between observations and results of recovery phase operation with the stressed phase operation, most of the figures and tables have been reproduced with the recovery phase operation results along with stressed phase operation.

4.3.2.1 Environmental and performance parameters

The reactors have been operated for 42 d during recovery phase operation. The results obtained during recovery phase operation have been shown in Fig. 4.14 to 4.17 and summary of data has been presented in Table 4.13. Similar to stressed phase operation, room temperature and pH have not been controlled during recovery phase operation. The variation in room temperature has been observed mainly due to weather changes. The average variation in room temperature recorded at 9.30 am and 8.45 pm has been observed as 26.0 ± 3.8 and $17.9 \pm 2.4^\circ\text{C}$, respectively (Table 4.13). The average initial and effluent pH for reactor R_{cps0} have been obtained as 7.13 ± 0.18 and 7.11 ± 0.16 during recovery phase operation (Table 4.13). The reactor R_{cps0} has shown uniform pH throughout the operation (Fig. 4.13). However, pH for reactors R_{cps50} , R_{cps100} , and R_{cps200} have reached to neutral during recovery phase operation. The average pH variation during recovery phase operation has slightly changed from stressed phase operation. During stressed phase operation, average initial pH for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 6.73 ± 0.16 , 6.69 ± 0.22 , and 6.34 ± 0.23 , respectively, while the average effluent pH of the reactors have been obtained as 7.08 ± 0.15 , 6.83 ± 0.26 , and 6.91 ± 0.22 , respectively (Table 4.13). During recovery phase operation, the average initial pH for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been obtained as 7.12 ± 0.18 , 6.82 ± 0.34 , and 7.00 ± 0.29 , respectively, whereas effluent pH has been observed as 7.13 ± 0.13 , 7.08 ± 0.16 , and 6.92 ± 0.26 , respectively (Table 4.13).

The residual CPS in effluent has been detected during initial days of recovery phase operation (Fig. 4.14 to 4.17). On 35th d operation, the effluent CPS concentration has been 3.66, 9.34, and 15.75 mg/L for reactors R_{cps50} , R_{cps100} , and R_{cps200} , respectively, which has further decreased to 0.10 mg/L with in six days (on 40th d) for reactors R_{cps50} , R_{cps100} , and R_{cps200} as shown in Fig. 4.14 to 4.17.

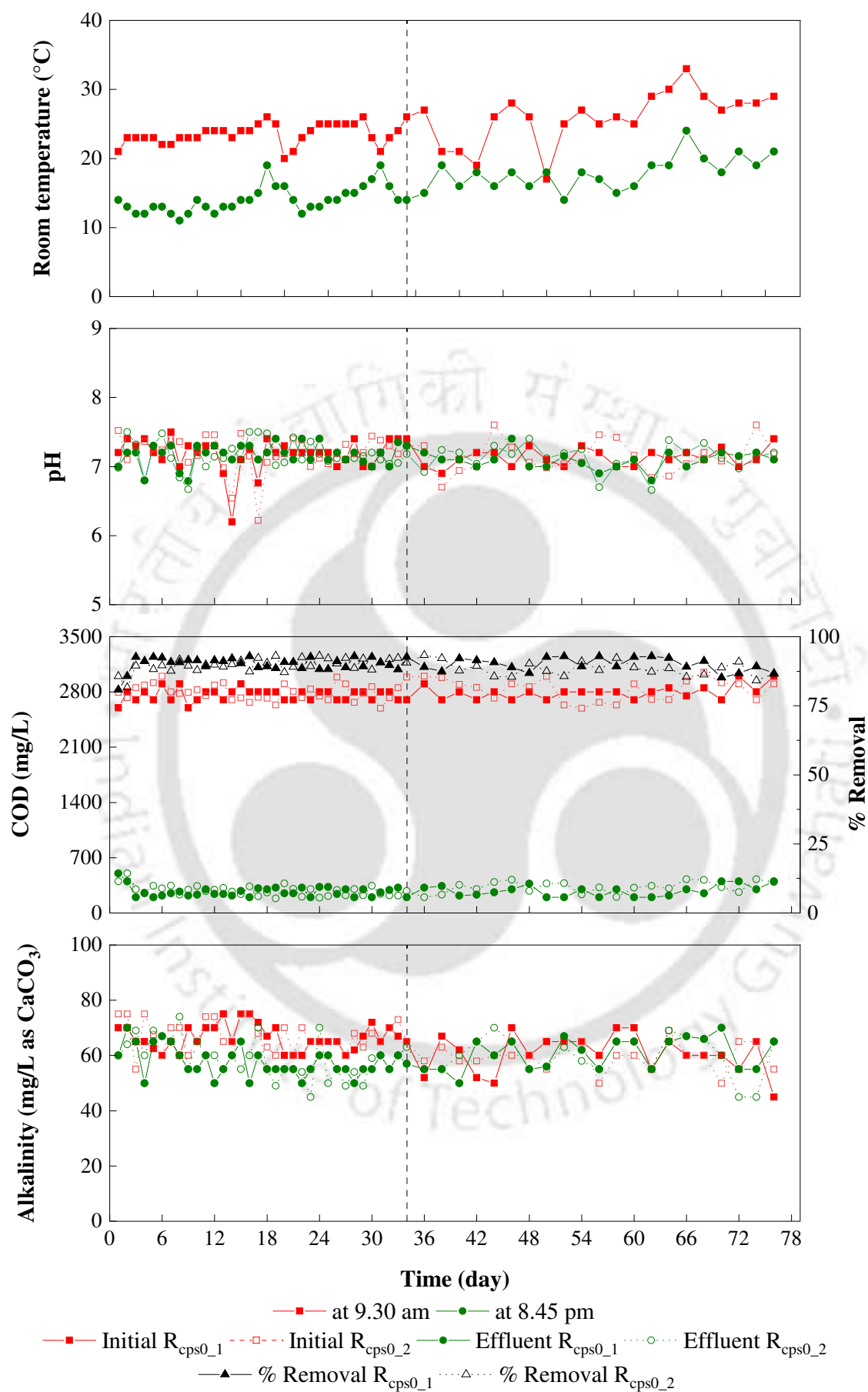


Fig. 4.14 Variation in environmental and performance related parameters for reactor R_{cps0} during 35-76 d of operation along with 0-34 d of operation (without CPS in the feed).

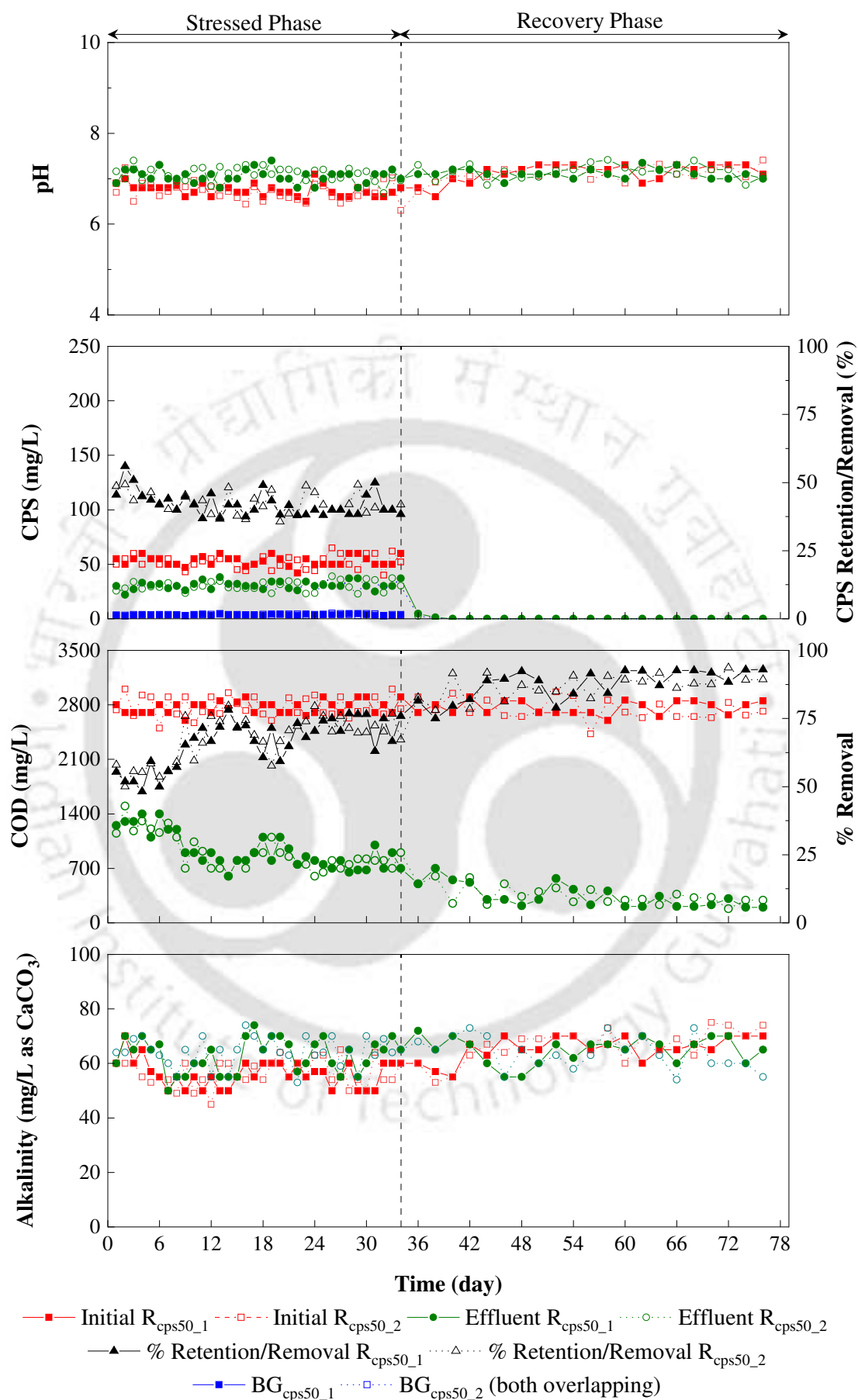


Fig. 4.15 Variation in environmental and performance related parameters for reactor R_{cps50} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.

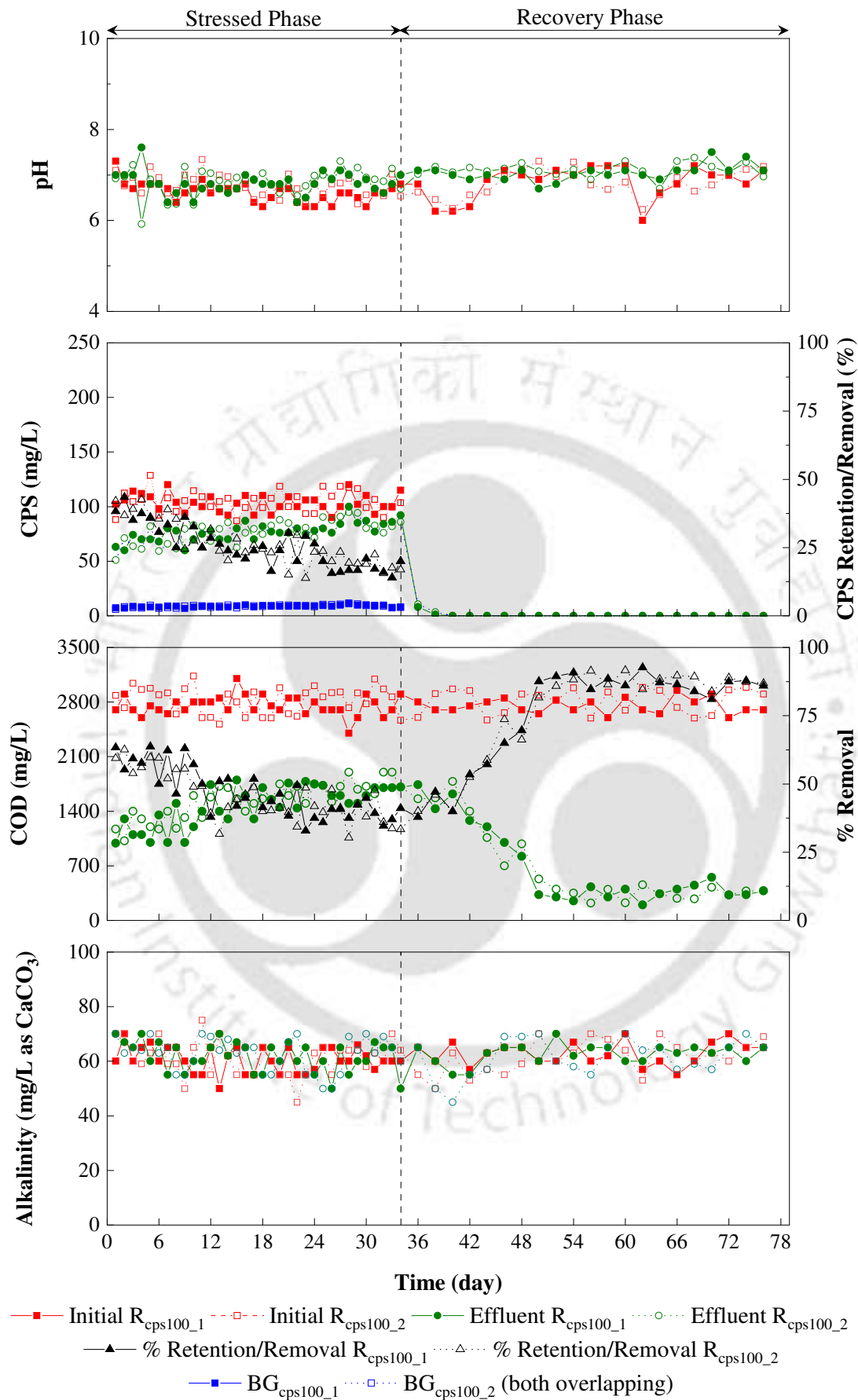


Fig. 4.16 Variation in environmental and performance related parameters for reactor R_{cps100} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.

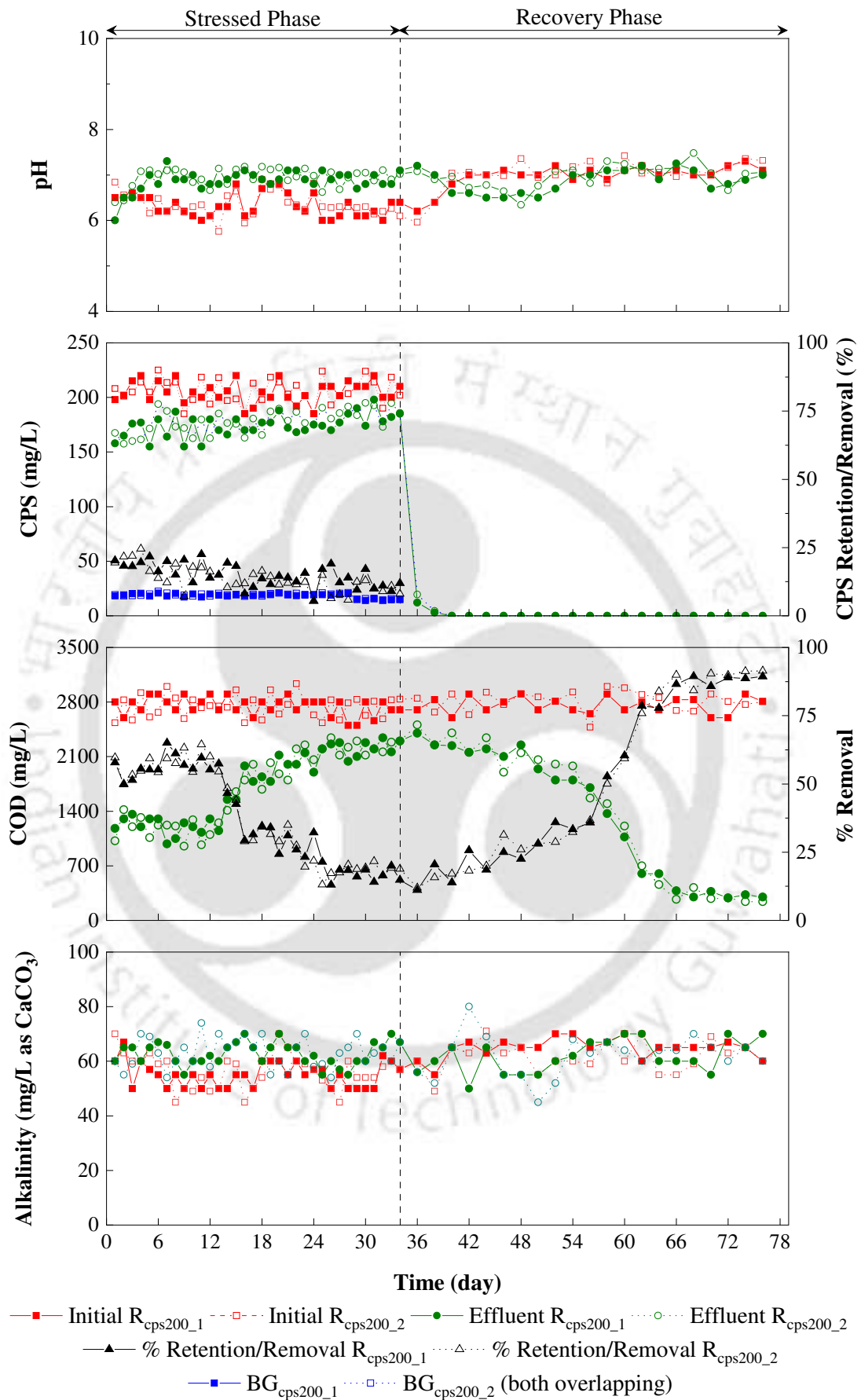


Fig. 4.17 Variation in environmental and performance related parameters for reactor R_{cps200} operated during 35-76 d under recovery phase operation along with data for stressed phase operation.

Table 4.13 Summary of environmental and performance related parameters for reactors operated during 35-76 d of recovery phase operation along with the data for stressed phase operation (0-34 d).

Parameters	Day-wise	0-34			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.14	1.58	0.47
	CPS (mg/L)	0	50	100	200
Stressed phase	Environmental	Room temperature (°C) at 9.30 am	23.5±1.5 (34)	23.5±1.5 (34)	23.5±1.5 (34)
		Room temperature (°C) at 8.45 pm	14.0±1.9 (34)	14.0±1.9 (34)	14.0±1.9 (34)
		Initial pH	7.19±0.25 (68)	6.73±0.16 (68)	6.69±0.22 (68)
		Initial CPS (mg/L)	0	52.62±5.30 (68)	104.22±8.86 (68)
	Performance	Effluent pH	7.17±0.18 (68)	7.08±0.15 (68)	6.83±0.26 (68)
		Effluent CPS (mg/L)	0	30.45±4.11 (68)	77.26±9.63 (68)
		CPS retention/removal (%)	0	42.10±4.21 (68)	26.18±6.78 (68)
		Background CPS (mg/L)	0	3.64±0.56 (68)	8.70±1.12 (68)
		Initial soluble COD (mg/L)	2779.26±88.57 (68)	2773.98±105.61 (68)	2790.20±148.18 (68)
		Effluent soluble COD (mg/L)	276.17±65.82 (68)	911.69±226.92 (68)	1503.24±244.04 (68)
		COD removal (%)	90.07±2.39 (68)	67.02±8.45 (68)	46.04±8.98 (68)
		Initial alkalinity (mg CaCO ₃ /L)	66.81±4.96 (68)	58.35±4.89 (68)	61.00 ±5.51 (68)
		Effluent alkalinity (mg CaCO ₃ /L)	58.29 ±6.09 (68)	63.53 ±5.72 (68)	62.18±5.81 (68)
Parameters	Day-wise	35-76			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.70	2.70	2.70
	CPS (mg/L)	0	0	0	0
Recovery phase	Environmental	Room temperature (°C) at 9.30 am	26.0±3.8 (21)	26.0±3.8 (21)	26.0±3.8 (21)
		Room temperature (°C) at 8.45 pm	17.9±2.4 (21)	17.9±2.4 (21)	17.9±2.4 (21)
		Initial pH	7.13±0.18 (42)	7.12±0.18 (42)	6.82±0.34 (42)
		Effluent pH	7.11±0.16 (42)	7.13±0.13 (42)	7.08±0.16 (42)
	Performance	Effluent CPS (mg/L)	0	0.31±0.79 (42)	0.64±2.05 (42)
		Initial soluble COD (mg/L)	2814.52±114.41 (42)	2753.93±109.56 (42)	2783.88±125.95 (42)
		Effluent soluble COD (mg/L)	305.37±72.30 (42)	347.00±133.13 (42)	669.40±499.47 (42)
		COD removal (%)	89.16±2.51 (42)	87.33±4.96 (42)	75.97±17.90 (42)
		Initial alkalinity (mg CaCO ₃ /L)	60.10±5.92 (42)	66.05 ±5.25 (42)	62.19±5.17 (42)
		Effluent alkalinity (mg CaCO ₃ /L)	60.29±6.42 (42)	64.38 ±5.48 (42)	62.05±5.27 (42)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

The variation in COD removal have been shown in Fig. 4.14 to 4.17 and summary of data has been presented in Table 4.13. The reactor R_{cps0} has exhibited uniform COD removal throughout the operation. While reactors R_{cps50} , R_{cps100} , and R_{cps200} have shown gross recovery in COD removal. The CPS fed reactors have tried to recovery under recovery phase operation. COD removal for reactor R_{cps50} has been observed as 82.1%, 87.2%, and 91.2% on 36th, 56th, and 76th d of operation, respectively. In addition, COD removal for reactor R_{cps100} has been observed as 38.9%, 87.9%, and 86.4% on 36th, 56th, and 76th d of operation, respectively. On the other hand, COD removal for reactor R_{cps200} has been observed as 11.5%, 36.2%, and 90.4% on 36th, 56th, and 76th d of operation, respectively. The reactor R_{cps50} has been fed with lower dose of CPS, hence fast recovery in terms of COD removal has been observed as compared to reactor R_{cps100} , and R_{cps200} . A slow recovery has been observed for the reactor fed with high dose (200 mg/L) of CPS. During recovery phase operation, COD removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have drastically improved as compared to stressed phase operation. During stressed phase operation, the average COD removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 67.0%, 46.0%, and 37.3%, respectively, which under recovery phase operation further increased to 87.3%, 76.0%, and 51.0%, respectively (Table 4.13). The recovery in COD removal is mainly attributed to discontinuation of CPS in the feed leading to termination of CPS toxicity to aerobic sludge biomass. The microbes of aerobic sludge biomass have recovered from the toxic effect of CPS and started consuming organics. The microbial cells adjust themselves and repair their cellular machinery and start growing. This is why aerobic sludge biomass has taken time to recovery from CPS effect.

The average initial alkalinity for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 60.10 ± 4.86 , 60.29 ± 5.90 , 66.05 ± 4.69 , and 64.38 ± 4.64 mg CaCO_3/L , respectively, while effluent alkalinity have been observed as 62.19 ± 3.88 , 62.05 ± 4.43 , 63.19 ± 3.57 , and 61.81 ± 5.04 mg CaCO_3/L , respectively, under recovery phase operation.

The steady-state condition has been achieved after 35-67 d of recovery phase operation, and reactors have been further operated in the steady-state conditions during 68-76 d of operation. The summary of steady-state performance data during recovery phase operation has been presented in Table 4.14. The steady-state operation has shown stabilization in the performance-related parameters. Both initial and effluent pH for all reactors have been observed in the range of 6.21-7.23 during the steady-state condition of the recovery phase operation; whereas, the initial and effluent pH values ranged 6.98-7.21 during the steady-state condition of the stressed phase operation (Table 4.14). The steady-state COD removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been obtained as 90.6%, 86.2%, and 88.9%, respectively, during recovery phase, which has significantly improved from 72.5%, 39.2%, and 17.7%, respectively, of the stressed phase steady-state COD removal (Table 4.14). The COD removal profile suggests that reactors have recovered from CPS impact and have shown significant organic removal. Minimum variation in alkalinity values have been obtained during the steady-state of the recovery phase operation, and it varied in the range of 58–69 mg CaCO_3/L .

Table 4.14 Summary of environmental and performance related parameters for reactors operated during steady-state condition of recovery phase operation (68-76 d) along with the data for stressed phase operation (25-34 d).

Parameters	Day-wise				
	25-34				
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.14	1.58	0.47
Stressed phase	CPS (mg/L)	0	50	100	200
	Environmental	Room temperature (°C) at 9.30 am	24.3±1.6 (10)	24.3±1.6 (10)	24.3±1.6 (10)
		Room temperature (°C) at 8.45 pm	15.40±1.6 (10)	15.40±1.6 (10)	15.40±1.6 (10)
		Initial pH	7.23±0.15 (20)	6.70±0.17 (20)	6.62±0.18 (20)
		Initial CPS (mg/L)	0	54.20±6.40 (20)	105.45±9.64 (20)
	Performance	Effluent pH	7.13±0.09 (20)	7.04±0.14 (20)	6.94±0.17 (20)
		Effluent CPS (mg/L)	0	31.97±4.82 (20)	85.78±6.65 (20)
		CPS retention/removal (%)	0	41.15±3.40 (20)	18.50±2.49 (20)
		Background CPS (mg/L)	0	3.83±0.73 (20)	9.42±1.23 (20)
		Initial soluble COD (mg/L)	2778.08±93.60 (20)	2789.48±104.62 (20)	2781.27±146.74 (20)
		Effluent soluble COD (mg/L)	259.83±48.11 (20)	765.00±93.29 (20)	1687.00±106.75 (20)
		COD removal (%)	90.65±1.68 (20)	72.48±3.97 (20)	39.21±4.53 (20)
		Initial alkalinity (mg CaCO ₃ /L)	66.00±3.58 (20)	56.00±5.22 (20)	62.10±3.53 (20)
		Effluent alkalinity (mg CaCO ₃ /L)	55.80±4.09 (20)	63.80±5.13 (20)	60.60±6.89 (20)
Recovery phase	Day-wise				
	68-76				
	Reactor	2.70	2.70	2.70	2.70
	CPS (mg/L)	0	0	0	0
	Environmental	Room temperature (°C) at 9.30 am	28.2±0.8 (5)	28.2±0.8 (5)	28.2±0.8 (5)
		Room temperature (°C) at 8.45 pm	19.8±1.3 (5)	19.8±1.3 (5)	19.8±1.3 (5)
		Initial pH	7.20±0.19 (10)	7.22±0.12 (10)	6.98±0.19 (10)
		Effluent pH	7.15±0.09 (10)	7.09±0.13 (10)	7.21±0.18 (10)
	Performance	Effluent CPS (mg/L)	0	0.10±0.03 (10)	0.10±0.03 (10)
		Initial soluble COD (mg/L)	2881.67±127.72 (10)	2746.67±76.56 (10)	2776.00±150.68 (10)
		Effluent soluble COD (mg/L)	357.40±69.94 (10)	256.00±53.01 (10)	382.00±75.23 (10)
		COD removal (%)	87.58±2.50 (10)	90.61±2.18 (10)	86.25±2.48 (10)
		Initial alkalinity (mg CaCO ₃ /L)	58.00±7.05 (10)	69.80±3.64 (10)	64.40±3.71 (10)
		Effluent alkalinity (mg CaCO ₃ /L)	59.20±8.65 (10)	64.00±5.44 (10)	63.40±3.70 (10)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

4.3.2.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The variation in solids characteristics and SVI has been shown in Fig. 4.18 and data has been summarized in Table 4.15. The sudden decrease in solids concentration (just after 34th d) in all reactors is due to biomass withdrawal for ASBA test during stressed phase operation. During stressed phase operation, CPS toxicity has limited biomass growth, whereas termination of CPS in the feed allowed reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses to recover in terms of their growth during recovery phase operation. The control reactor R_{cps0} biomass has shown continuous growth. From 37th to 43rd d, the MLSS concentration in reactor R_{cps0} has increased from 3022 mg/L to 4103 mg/L [Fig. 4.18(a)]. Similarly, reactor R_{cps50} has also shown increase in MLSS from 2170 mg/L to 2890 mg/L from 37th to 43rd d, which indicates that the reactor R_{cps50} biomass has shown accelerated growth [Fig. 4.18(b)]. The MLSS concentration in R_{cps100} has been observed as 2084 mg/L on 37th d which has increased to 2822 mg/L on 43rd d. On the other hand, reactor R_{cps200} has shown increase in MLSS 2090 mg/L on 37th d to 2900 mg/L on 43rd d, which indicates that the reactor R_{cps200} biomass has shown similar growth compared to reactor R_{cps100} biomass. The SVI been observed as 169 mL/g for reactor R_{cps50} biomass [Fig. 4.18(b)], 168 mg/L for reactor R_{cps100} biomass [Fig. 4.18(c)], and 151 mL/g for reactor R_{cps200} biomass [Fig. 4.18(d)] on 72nd d of operation. The MLVSS/MLSS ratio has remained similar to stressed phase operation and has ranged between 0.89-0.94 for all reactors during recovery phase operation (Table 4.15). The SRT for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} has been observed in the range of 28-44 d during recovery phase operation.

During recovery phase operation, all reactor biomasses have exhibited an increase in SVI [Fig. 4.18]. The SVI for reactor R_{cps0} biomass has been observed as 187, 185, and 194 mL/g on 44th, 58th, and 72nd d, respectively, while SVI for reactor R_{cps50} biomass has been observed as 137, 158, and 169 mL/g, respectively. On the other hand, SVI for reactor R_{cps100} biomass has been observed as 130, 152, and 168 mL/g, respectively, while SVI for reactor R_{cps200} biomass has been observed as 121, 147, and 151 mL/g on 44th, 58th, and 72nd d of operation during recovery phase. The increase in SVI could be due to growth of filamentous microorganisms. When CPS addition has been discontinued, thereby cutting off CPS stress to reactors, the growth of filamentous biomass has increased under recovery phase operation.

The ISV test has also been performed during recovery phase operation as shown in Fig. 4.19. The results suggest that settling behavior of reactor R_{cps0} biomass has been hindered due to abundance and overgrowth of filamentous microorganisms [Fig. 4.19(a)]; however, reactor R_{cps200} biomass has shown uniform settling behavior throughout the recovery phase operation [Fig. 4.19(b)]. The ISV results have also been shown in Fig. 4.20 and data has been summarized in Table 4.15. During recovery phase operation, ISV of control reactor R_{cps0} biomass has been observed as 1.50, 1.47, and 1.30 m/h on 44th, 58th, and 72nd d of operation, respectively, while ISV for reactor R_{cps50} biomass has been observed as 2.11, 1.95, and 1.85

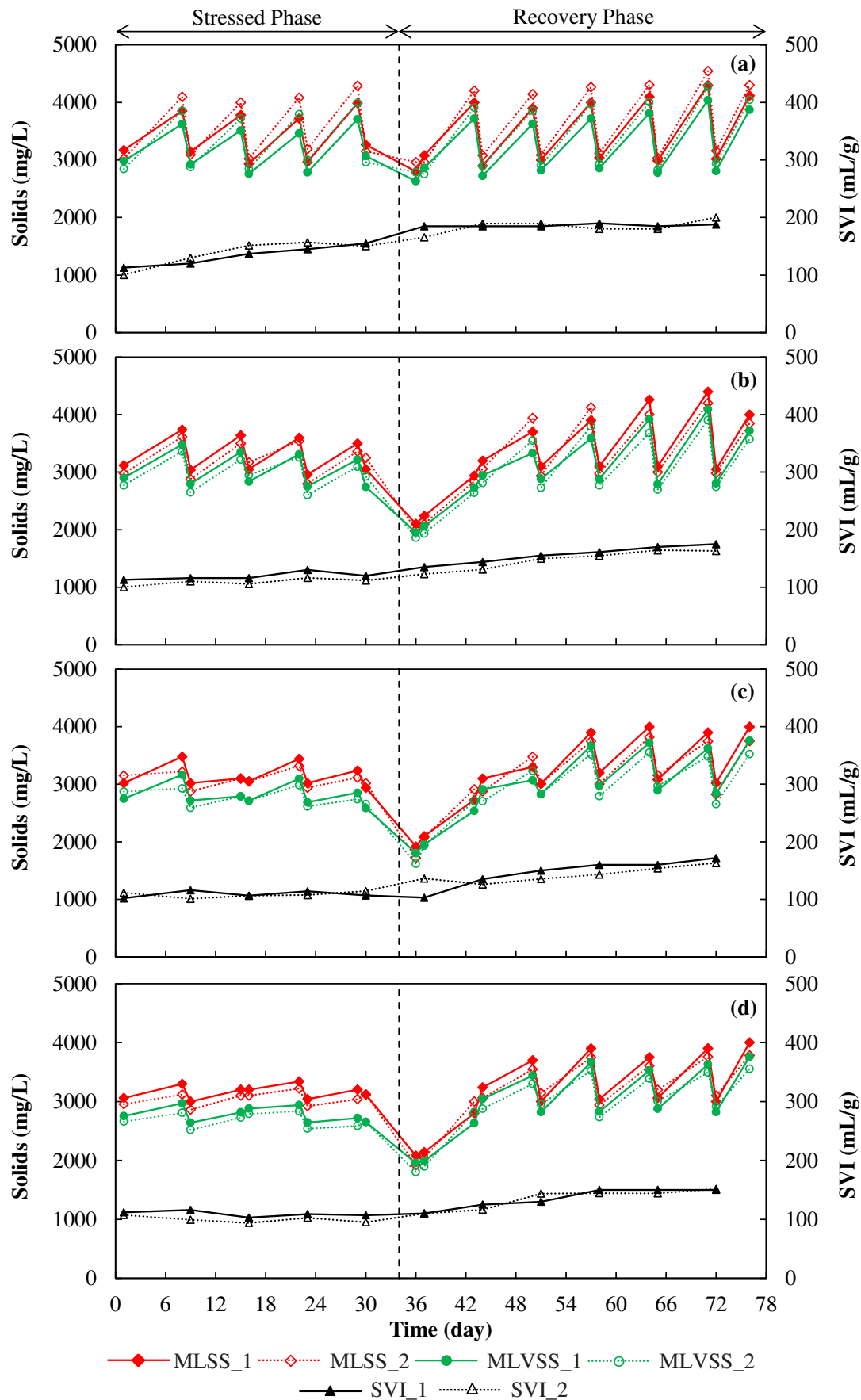


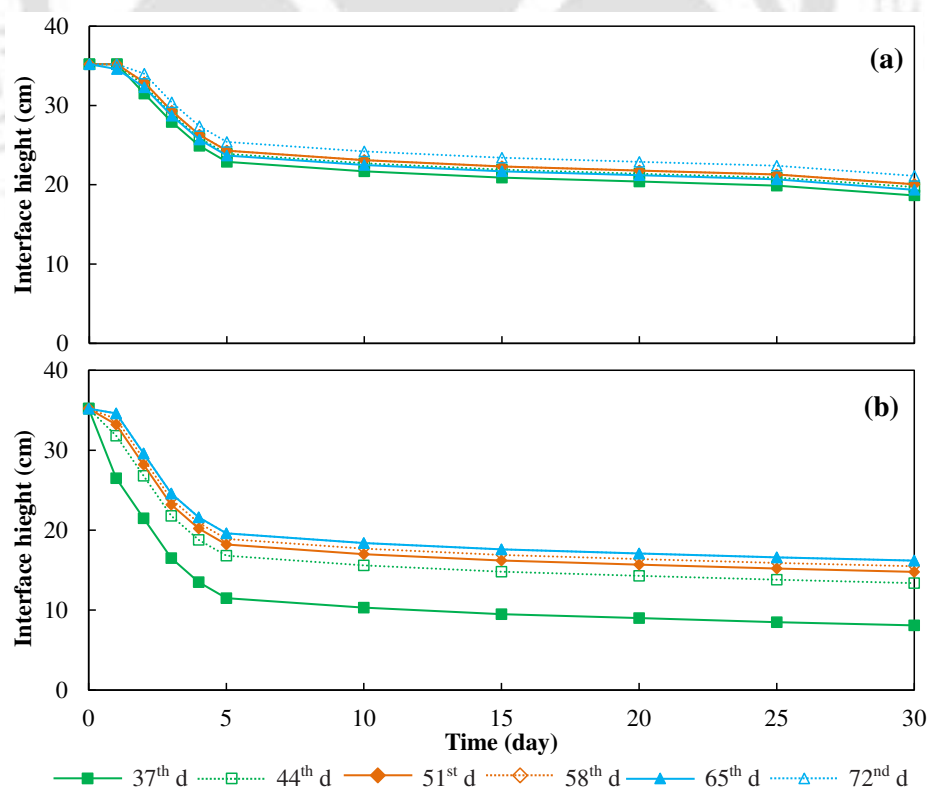
Fig. 4.18 Variation in MLSS, MLVSS, and SVI values for reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} biomasses during 35-76 d of recovery phase operation along with stressed phase operation.

Table 4.15 Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase operation.

Gross parameters	Stressed phase operation				
	Day-wise	0-34			
	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
MLSS (mg/L)		3097.20±108.38 (10)	3030.00±123.59 (10)	3009.60±73.54 (10)	3037.60±95.97 (10)
MLVSS (mg/L)		2905.13±101.71 (10)	2793.08±110.31 (10)	2690.84±86.72 (10)	2673.04±106.68 (10)
MLVSS/MLSS ratio		0.94±0.01 (10)	0.92±0.01 (10)	0.90±0.01 (10)	0.89±0.01 (10)
SVI (mL/g)		135.94±20.37 (10)	113.96±6.40 (10)	108.67±5.48 (10)	104.46±5.25 (10)
ISV (m/h)		2.13±0.28 (10)	2.42±0.05 (10)	2.49±0.03 (10)	2.52±0.05 (10)
Gross parameters	Recovery phase operation				
	Day-wise	35-76			
MLSS (mg/L)		3039.83±64.51 (12)	2904.00±362.37 (12)	2872.33±395.62 (12)	2913.00±412.66 (12)
MLVSS (mg/L)		2842.24±61.46 (12)	2671.61±334.34 (12)	2691.34±376.77 (12)	2729.75±395.22 (12)
MLVSS/MLSS ratio		0.94±0.01 (12)	0.94±0.01 (12)	0.90±0.03 (12)	0.89±0.04 (12)
SVI (mL/g)		185.28±6.95 (12)	152.17±16.20 (12)	144.86±19.15 (12)	135.42±17.07 (12)
ISV (m/h)		1.48±0.11 (12)	2.06±0.29 (12)	2.08±0.40 (12)	2.28±0.27 (12)

[The data is presented in $xx \pm yy$ (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

m/h, respectively. In addition, ISV for reactor R_{cps100} biomass has been observed as 2.31, 1.94, and 1.71 m/h on 44th, 58th, and 72nd d of operation, respectively, while ISV for reactor R_{cps200} biomass has been observed as 2.33, 2.15, and 2.09 m/h, respectively. The decrease in ISV may be due to increased growth of filamentous microorganisms in the reactor. However, ISV of all reactors have decreased in recovery phase due to uncontrolled growth of filamentous microorganisms in the reactors. The average ISV for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200}

**Fig. 4.19** Typical interface settling test results for reactor (a) R_{cps0} and (b) R_{cps200} biomasses during 35-76 d of recovery phase operation.

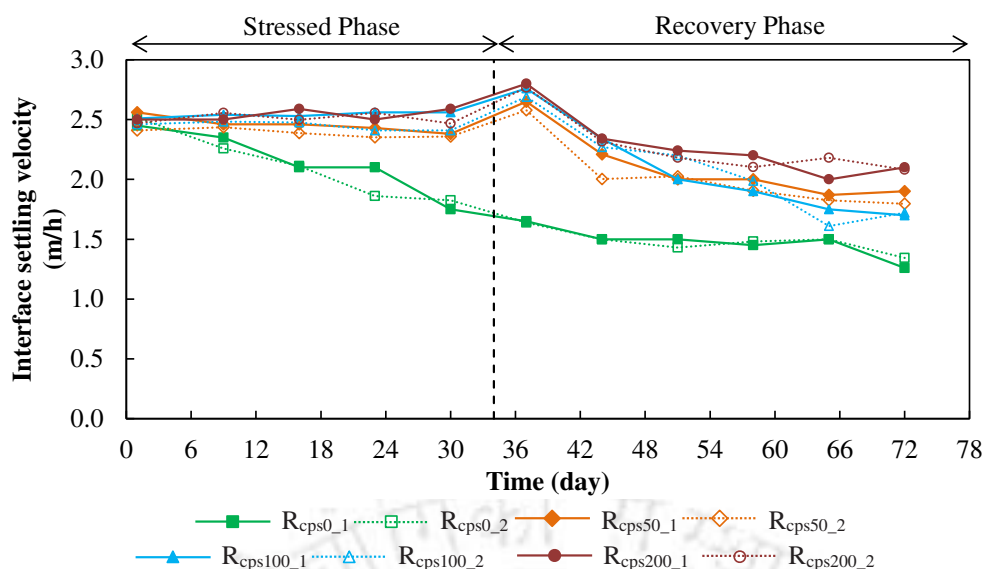


Fig. 4.20 Variation in interface settling velocities of reactor biomasses during 35-76 d of recovery phase operation along with data for stressed phase operation.

biomasses have been observed as 1.48 ± 0.11 , 2.06 ± 0.29 , 2.08 ± 0.40 , and 2.28 ± 0.27 m/h, respectively, under recovery phase operation.

Aerobic sludge biomass activity test

When the reactors have achieved steady-state condition in the recovery phase operation, biomass has been withdrawn from all the reactors on 76th d of recovery phase operation and ASBA tests have been performed. The test results have been shown in Fig. 4.21, while data has been presented in Table 4.16. According to the ASBA test results, aerobic sludge biomasses of reactors R_{cps50}, R_{cps100}, and R_{cps200} have appeared to recover to a large degree since CPS addition has been stopped in the feed. The ASBA of reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been obtained as 8.13, 7.45, 7.13, and 7.10 mg COD removed/mg VSS d, respectively (Table 4.16), towards the end of recovery phase operation – means that the inhibition induced by CPS stress is reversible. During the stressed phase operation, ASBA of reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been obtained as 7.42, 6.57, 4.30, and 2.04 mg COD removed/mg VSS d, respectively – lower than the recovery phase ASBA especially for reactors fed with higher CPS concentrations (Table 4.16). There is no literature available to relate recovery of aerobic sludge biomass after exposure to CPS in the feed. When CPS addition has been stopped in the feed to the reactors, the ASBA have improved towards the end of recovery phase operation as compare to control reactor biomass. It suggests that aerobic sludge biomass repair their cellular machinery to metabolize the organic matter during recovery phase operation.

In recovery phase operation, general biomass characteristics such as MLSS, MLVSS, and MLVSS/MLSS ratio have been the same as in stressed phase operation. However, SVI and ISV have varied during recovery phase operation. Reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have shown SVI of 185, 152, 145, and 135 mL/g, respectively. ISV values for all reactor biomasses have shown minor variation. The ISV for reactor R_{cps50}, R_{cps100}, and

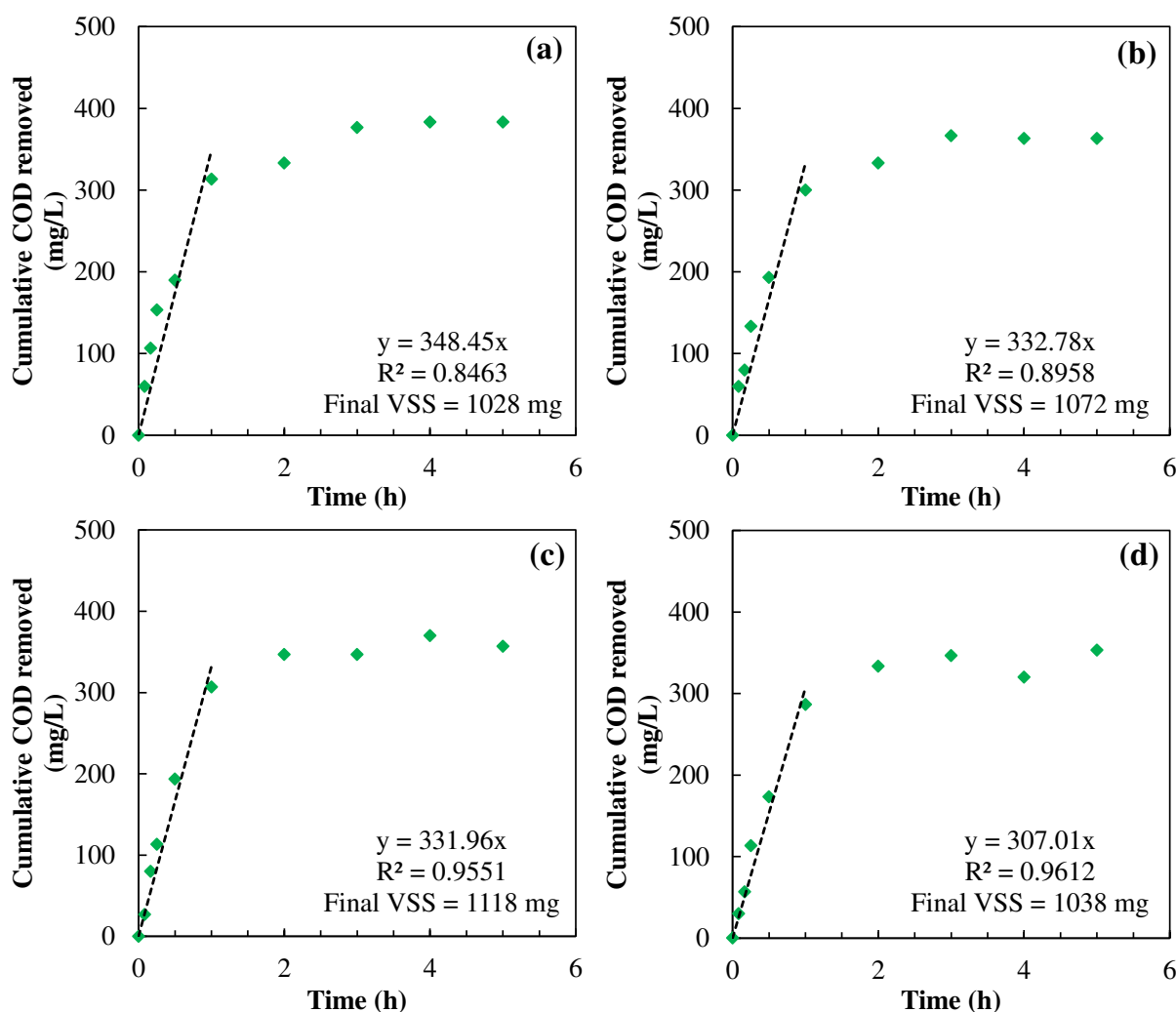


Fig. 4.21 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_{cps0} , (b) R_{cps50} , (c) R_{cps100} , and (d) R_{cps200} on 76th d of recovery phase operation.

Table 4.16 Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase operation.

Stressed phase operation				
Biomass withdrawn on 34 th d				
Reactor	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
ASBA (mg COD removed/mg VSS d)	7.42	6.57	4.30	2.04
Recovery phase operation				
Biomass withdrawn on 76 th d				
ASBA (mg COD removed/mg VSS d)	8.13	7.45	7.13	7.10

R_{cps200} biomasses are 2.06, 2.08, and 2.28 m/h – on lower side as compared to control reactor biomass (1.48 m/h). ASBA suggests significant recovery in organic degradation capability of biomass during recovery phase operation. The ASBA have improved from 7.42, 6.57, 4.30, and 2.04 mg COD removed/mg VSS d during stressed phase operation to 8.13, 7.45, 7.13, and 7.10 mg COD removed/mg VSS d during recovery phase operation for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. It's no coincidence that ASBA have improved along with COD removal in the reactors. The COD removal for reactors R_{cps0} , R_{cps50} ,

R_{cps100} , and R_{cps200} have ranged between 86-91% during steady-state condition of recovery phase operation. Among all biomass related parameters, ASBA has been found as a suitable tool to measure the recovery of biomass after CPS stress.

4.3.2.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

Microscopic examination after Gram's staining ($5\times$ and $100\times$ magnification), photographic observation, filament index, and cell viability test have been used to analyze the floc structure and morphology of aerobic sludge biomass. Microscopic images of aerobic biomass have been shown in Table 4.17, which has indicated presence of thick, denser, and abundance of filamentous microorganisms in all reactor biomasses towards the end of the recovery phase operation. The reactor R_{cps0} biomass has shown continuous growth of filamentous microorganisms, and fairly denser flocs structure has been observed throughout the experiment. On 36th d of operation, the microscopic images (at $5\times$ magnification) suggest that flocs structure has seen to be damaged with increased CPS concentration in reactor, whereas, on the same day (at $100\times$ magnification), reduction in filaments has been observed with the hike in CPS concentration (Table 4.17). The possible reason could be the toxicity of CPS for microorganisms of aerobic sludge biomass. The presence of CPS in the feed of reactors has inhibited the filamentous growth and this impact on microorganisms depend on CPS concentration. No literature is available to relate aerobic sludge biomass morphology exposed to CPS containing wastewater till now. As the recovery phase operation has progressed, the flocs morphology of aerobic biomass of reactors R_{cps50} , R_{cps100} , and R_{cps200} have started to regain its normal structure as compared to control reactor biomass (on 71st d). On 76th d, CPS fed reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have shown the similar morphology (visible at $5\times$ magnification) and abundance of filaments (visible at $100\times$ magnification) as compared to reactor R_{cps0} biomass (Table 4.17).

The photographic observations have also been carried out to see the changes in flocs morphology during recovery phase operations, and the images have been presented in Table 4.18. The reactor R_{cps0} biomass has shown continuous increase in floc size throughout the operation (36th to 71th d). The flocs size of biomass in reactor R_{cps0} has ranged between 2.0-12.0 mm on 71th d of operation. On the other hand, discontinuation of CPS in the feed has shown the increase of thick and denser flocs structure over time for reactor biomasses with size range of 2-12 mm for R_{cps50} , 1-10 mm for R_{cps100} , and 1-6 mm for R_{cps200} . On 36th d of operation, loose flocs with weak filaments have been observed for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses and slight increase in flocs size has been observed till 50th d of operation. From 57th to 71th d of operation, flocs size and density of filaments have continuously been increased. Moreover, the difference in flocs size and morphology between reactors fed with lower and higher doses of CPS during stressed phase operation can be observed from the images taken on 71th d of operation under recovery phase (Table 4.18). This indicates that the reactor fed with a lower CPS dose during stressed phase operation has shown larger and

Table 4.17 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with microscopic images of biomass withdrawn during stressed phase operation.

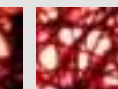
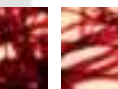
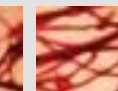
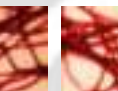
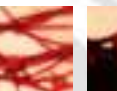
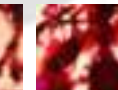
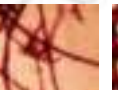
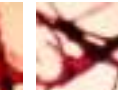
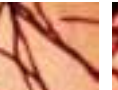
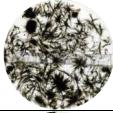
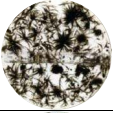
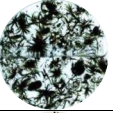
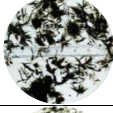
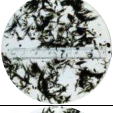
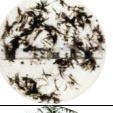
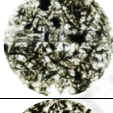
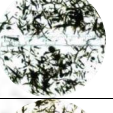
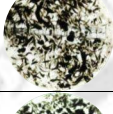
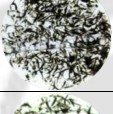
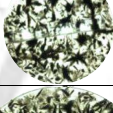
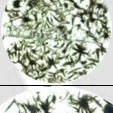
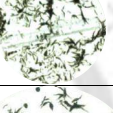
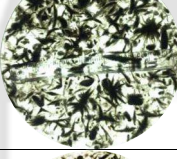
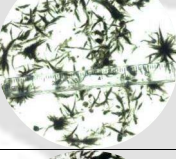
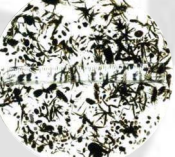
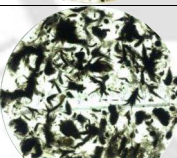
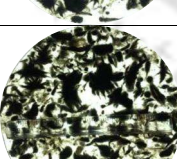
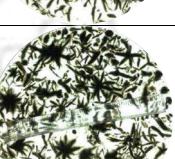
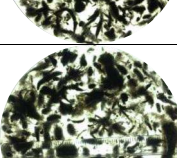
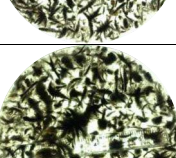
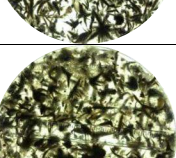
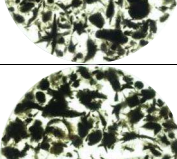
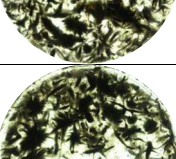
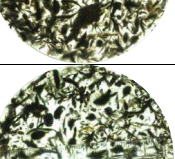
Reactor Mag.	Aerobic sludge biomass samples withdrawn on												
	Stressed phase operation					Recovery phase operation							
	1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d	43 rd d	50 th d	57 th d	64 th d	71 st d	76 th d	
R_{cps0}	5×												
	100×												
R_{cps50}	5×												
	100×												
R_{cps100}	5×												
	100×												
R_{cps200}	5×												
	100×												

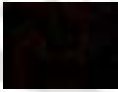
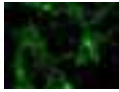
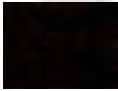
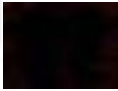
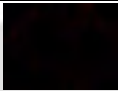
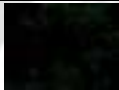
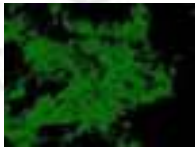
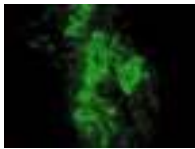
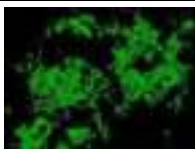
Table 4.18 Photographic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with images of biomasses withdrawn during stressed phase operation.

Sample withdrawn on	Aerobic sludge biomass withdrawn from reactors			
	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
Stressed phase operation	1 st d			
	8 th d			
	15 th d			
	22 nd d			
	29 th d			
Recovery phase operation	36 th d			
	43 rd d			
	50 th d			
	57 th d			
	64 th d			
	71 th d			

denser flocs during recovery phase compared to the reactor fed with higher doses during stressed phase operation.

The aerobic sludge biomass has been stained and observed under a fluorescence microscope at 20× magnification for cells viability, and results have been illustrated in Table 4.19. No change in the active portion of aerobic sludge biomass has been distinguished for control reactor R_{cps0} biomass. However, the discontinuation of CPS to reactors has

Table 4.19 Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} during recovery phase operation along with images of biomasses withdrawn during stressed phase operation.

Sample withdrawn on		Rector	Bright field (20×)	Dead Cells (20×)	Live Cells (20×)
Stressed phase operation	1 st d	R_{cps0}			
		R_{cps50}			
		R_{cps100}			
		R_{cps200}			
	34 th d	R_{cps0}			
		R_{cps50}			
		R_{cps100}			
		R_{cps200}			
Recovery phase operation	76 th d	R_{cps0}			
		R_{cps50}			
		R_{cps100}			
		R_{cps200}			

increased the active portion of biomass towards the end of the recovery phase operation. All the reactors have shown increased active biomass portion during the recovery phase operation along with a small part of inactive biomass observed for all the reactors (Table 4.19). According to the fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses, there were 94.8%, 85.7%, 67.6%, and 51.2% viable cells and 3.2%, 9.6%, 27.2, and 48.2% dead cells, respectively, towards the end of the recovery phase operation.

FI values have also been estimated during recovery phase operation, and data has been presented in Table 4.20, which shows abundance of filaments throughout the operation. FI value has suggested that filaments have never been reduced in any reactor biomasses throughout the operation. The reactor R_{cps0} biomass has shown FI value of 5 throughout the operation. On 36th d of operation, the FI for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have been observed as 4, 3, and 3 respectively, which remained same till 57th d of operation. The reactor R_{cps50} biomass has shown FI of 5 on 64th d of operation, which remained 5 till 76th d of operation. On the other hand, reactor R_{cps100} biomass has shown FI of 4 on 64th d of operation, which has further increased to 5 on 76th d of operation. Similarly, reactor R_{cps200} biomass has shown FI of 3 on 64th d of operation, which has further increased to 5 till 76th d of operation. The high FI has been observed for all reactors due to the continuous growth of filamentous biomass after termination of CPS in the feed during recovery phase operation.

Table 4.20 Filament index of aerobic sludge biomasses estimated during stressed and recovery phase operations.

Biomass withdrawn on	Stressed phase operation			
Day	R_{cps0}	R_{cps50}	R_{cps100}	R_{cps200}
1 st d	5	5	5	5
8 th d	5	5	5	4
15 th d	5	5	4	4
22 nd d	5	5	4	4
29 th d	5	5	4	4
Recovery phase operation				
36 th d	5	4	3	3
43 rd d	5	4	3	3
50 th d	5	4	3	3
57 th d	5	4	3	3
64 th d	5	5	4	3
71 st d	5	5	5	3
76 th d	5	5	5	5

Polysaccharide, protein and extracellular polymeric substance

The PS, PN, and EPS contents of aerobic sludge biomass during recovery phase operation have been shown in Fig. 4.22 and data has been summarized in Table 4.21. On 76th d of operation; PS, PN, and EPS contents for reactor R_{cps0} biomass has been obtained as 17.54, 55.02, and 72.54 mg/g VSS, respectively [Fig. 4.22(a)], which indicates significant increase over the respective values obtained previously. On 76th d of operation, PS, PN, and EPS contents for

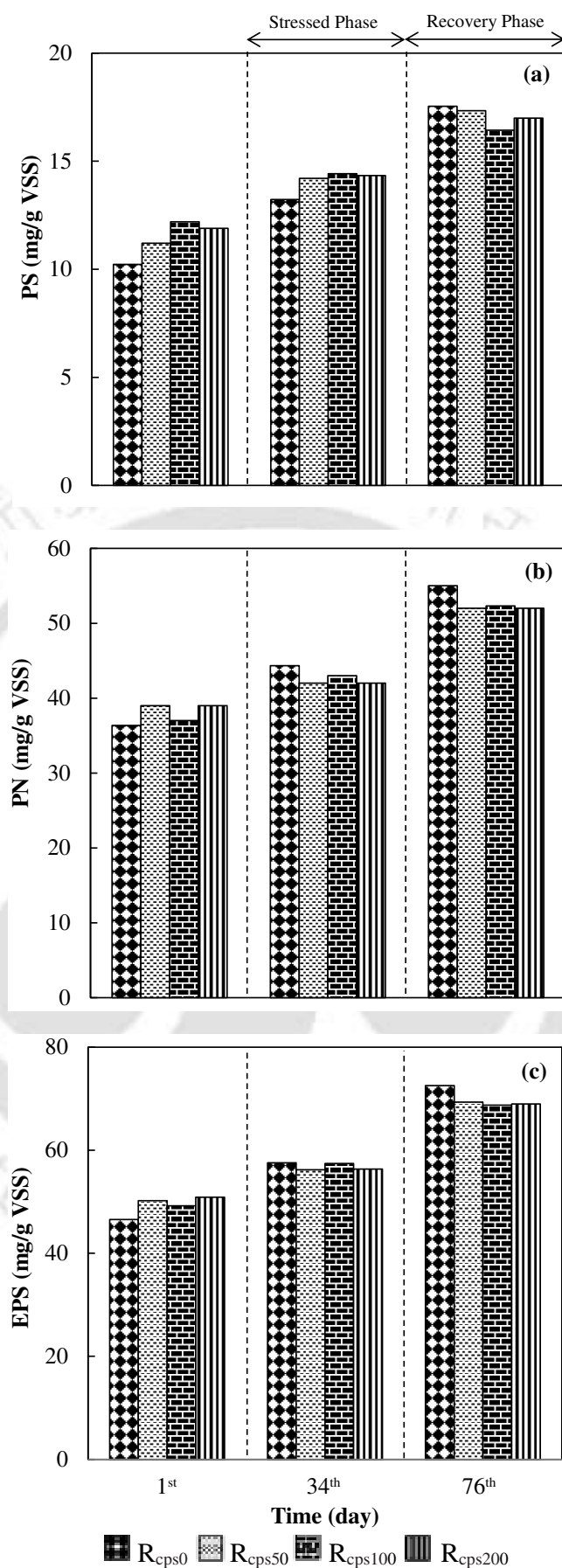


Fig. 4.22 Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during recovery phase operation along with data for stressed phase operation.

Table 4.21 PS, PN, and EPS contents in aerobic sludge biomass estimated under stressed and recovery phase operation.

Biomass withdrawn on	Reactor biomass	PS	PN	EPS
mg/g VSS				
Stressed phase operation				
1 st d	R _{cps0}	10.23±0.86	36.34±0.78	46.57±0.82
	R _{cps50}	11.21±0.74	39.12±1.41	50.21±1.07
	R _{cps100}	12.21±1.21	37.02±1.22	49.21±1.20
	R _{cps200}	11.89±0.63	39.11±1.42	50.89±1.01
34 th d	R _{cps0}	13.23±1.23	44.34±0.98	57.57±1.10
	R _{cps50}	14.21±0.89	42.02±1.82	56.21±1.34
	R _{cps100}	14.42±0.92	43.08±1.03	57.42±0.96
	R _{cps200}	14.34±0.83	42.06±1.81	56.34±1.31
Recovery phase operation				
76 th d	R _{cps0}	17.54±0.40	55.02±1.11	72.54±0.75
	R _{cps50}	17.34±0.80	52.03±1.04	69.34±0.90
	R _{cps100}	16.43±1.21	52.32±1.62	68.75±1.41
	R _{cps200}	16.99±0.82	52.02±1.03	68.99±0.91

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

reactor R_{cps50} biomass has increased to 17.34, 52.03, and 69.34 mg/g VSS, respectively, from 14.21, 42.02, and 56.21 mg/g VSS of stressed phase operation (on 34th d) [Fig. 4.22(b)].

Similarly, for reactor R_{cps100} biomass, PS, PN, and EPS contents on 76th d has increased to 16.43 mg/g VSS; 52.32 mg/g VSS; 68.75 mg/g VSS, respectively, from 14.42, 43.08, and 57.42 mg/g VSS of stressed phase operation (on 34th d) [Fig. 4.22(c)]. PS, PN, and EPS contents for reactor R_{cps200} biomass has been obtained as 16.99, 52.02, and 68.99 mg/g VSS, respectively [Fig. 4.22(d)], which has increased from 14.34, 42.01, and 56.34 mg/g VSS of stressed phase operation (on 34th d).

Enzyme activity

Four different enzyme catalase, dehydrogenase, protease, and urease activities have been estimated on the 76th d of operation under recovery phase operation as shown in Fig. 4.23 and summary is presented in Table 4.22. E_{Catalase} for all the reactor biomasses has been shown in Fig. 4.23(a) and summary of data has been presented in Table 4.22. When CPS has been discontinued in the feed, E_{Catalase} for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses has been observed as 0.38, 0.36, 0.37, and 0.37 mg/g MLSS h, respectively, towards the end of recovery phase operation. During stressed phase operation, E_{Catalase} for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses have been observed as 0.33, 0.26, 0.12, and 0.10 mg/g MLSS h, respectively, which is much lower than the values obtained during recovery phase. These results suggest recovery of aerobic biomass in terms of E_{Catalase}. E_{Catalase} of reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses have reached similar levels to reactor R_{cps0} biomass towards the end of the recovery phase operation.

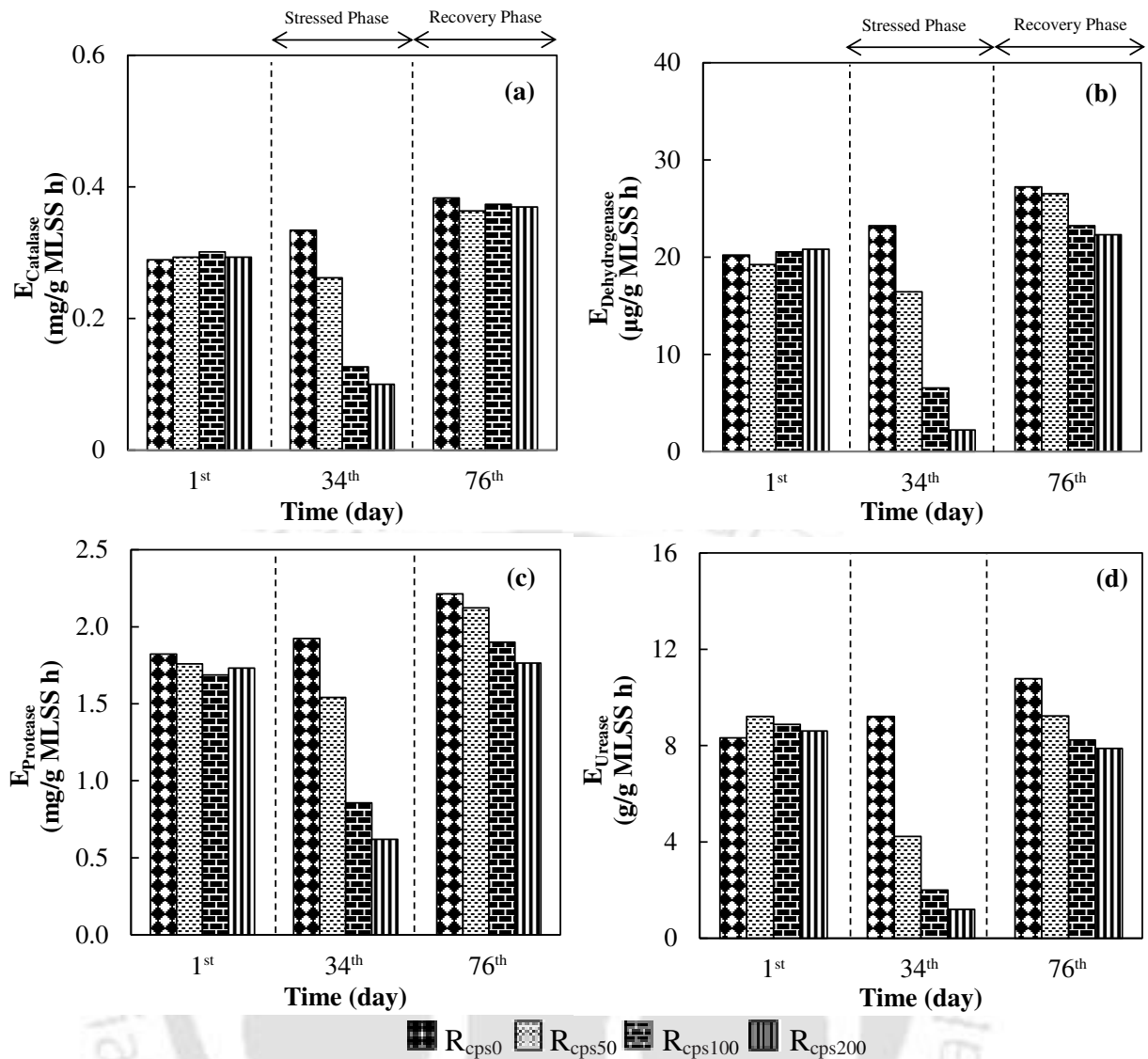


Fig. 4.23 Enzyme activities of aerobic sludge biomass (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} assessed during recovery phase operation along with data for stressed phase operation.

$E_{\text{Dehydrogenase}}$ for all reactor biomasses has been shown in Fig. 4.23(b) and data has been summarized in Table 4.22. On 76th d of operation, $E_{\text{Dehydrogenase}}$ for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses has been observed as 27.23, 26.52, 23.23, and 22.32 µg/g MLSS h, respectively. Whereas, during stressed phase operation, $E_{\text{Dehydrogenase}}$ for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses has been obtained as 23.23, 16.43, 6.54, and 2.23 µg/g MLSS h, respectively. Discontinuation of CPS in the feed has resulted in recovery of $E_{\text{Dehydrogenase}}$. The reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses have appeared to recover and has reached similar level to reactor R_{cps0} biomass.

E_{Protease} for all reactor biomasses has been shown in Fig. 4.23(c) and Table 4.22 represent summary of data obtained during recovery phase operation. The reactors have shown improvement in E_{Protease} . On 76th d of operation, 2.21, 2.12, 1.90, and 1.77 mg/g MLSS h of E_{Protease} has been observed for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively, which has increased from 1.92, 1.54, 0.86, and 0.13 mg/g MLSS h, respectively,

Table 4.22 Summary of enzyme activities of aerobic sludge biomass assessed during stressed and recovery phase operations.

Biomass withdrawn on	Reactor biomass	E _{Catalase} (mg/g MLSS h)	E _{Dehydrogenase} (μg/g MLSS h)	E _{Protease} (mg/g MLSS h)	E _{Urease} (g/g MLSS h)
Stressed phase operation					
1st d	R_{cps0}	0.29±0.03	20.23±1.32	1.82±0.09	8.32±0.80
	R_{cps50}	0.29±0.02	19.23±1.52	1.76±0.05	9.21±0.90
	R_{cps100}	0.30±0.01	20.55±0.92	1.69±0.15	8.88±1.20
	R_{cps200}	0.29±0.01	20.82±1.41	1.73±0.07	8.61±1.30
34th d	R_{cps0}	0.33±0.02	23.23±1.64	1.92±0.14	9.21±1.60
	R_{cps50}	0.26±0.02	16.43±1.96	1.54±0.06	4.23±0.51
	R_{cps100}	0.12±0.02	6.54±2.01	0.85±0.13	2.02±0.61
	R_{cps200}	0.10±0.02	2.23±0.81	0.62±0.17	1.20±0.32
Recovery phase operation					
76th d	R_{cps0}	0.38±0.03	27.23±0.83	2.21±0.22	10.78±1.20
	R_{cps50}	0.36±0.01	26.52±2.11	2.12±0.09	9.23±0.31
	R_{cps100}	0.37±0.02	23.23±1.30	1.90±0.09	8.23±1.21
	R_{cps200}	0.36±0.01	22.32±0.91	1.76±0.05	7.88±0.71

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

of the stressed phase operation (on 34th d). Fig. 4.23(c) illustrates that E_{Protease} has been affected in the presence of CPS and it has recovered to its normal level in absence of CPS.

E_{Urease} for all reactor biomasses has also been affected in the presence of CPS in the feed and has recovered during has recovery phase operation as shown in Fig. 4.23(d) and Table 4.22. On 76th d of operation, E_{Urease} has been observed as 10.78, 9.23, 8.23, and 7.88 g/g MLSS h for reactor R_{cps0}, R_{cps50}, R_{cps100} and R_{cps200} biomasses, respectively. E_{Urease} values have been 9.21, 4.23, 2.00, and 1.20 g/g MLSS h for reactor R_{cps0}, R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively, on 34th d of stressed phase operation. Similar to all other enzymes, E_{Urease} has also been recovered when CPS addition has been discontinued in the feed during recovery phase operation. The enzyme activity of aerobic sludge biomass has recovered when CPS addition has been stopped in the feed during recovery phase operation. Microbial cells of aerobic sludge biomass repair and synthesize enzymes and start metabolizing organic matters present in the feed in the absence of CPS.

Amplified ribosomal DNA restriction analysis (ARDRA)

The aerobic sludge biomass samples have been withdrawn on 76th d of the operation and examined through ARDRA to determine the changes in microbial communities. Fig. 4.24 shows the gel run after the treatment of three different restriction enzymes (*AluI*, *HhaI*, and *MspI*) and dendrogram has been prepared based on the similarity matrix presented in Table 4.23. For all reactors in both stressed and recovery phase operations, Fig. 4.24 and Table 4.23 have reflected DNA bands and similarity data. The restriction enzyme *AluI* has shown 93.4%, 81.2%, and 77.5% similarity during recovery phase operation for reactor R_{cps50}, R_{cps100}, and R_{cps200} biomasses, respectively, which has improved from 69.3%, 57.5%, and 42.3%, respectively, of stressed phase operation. Similarly, *HhaI* has exhibited 86.0%, 82.3%,

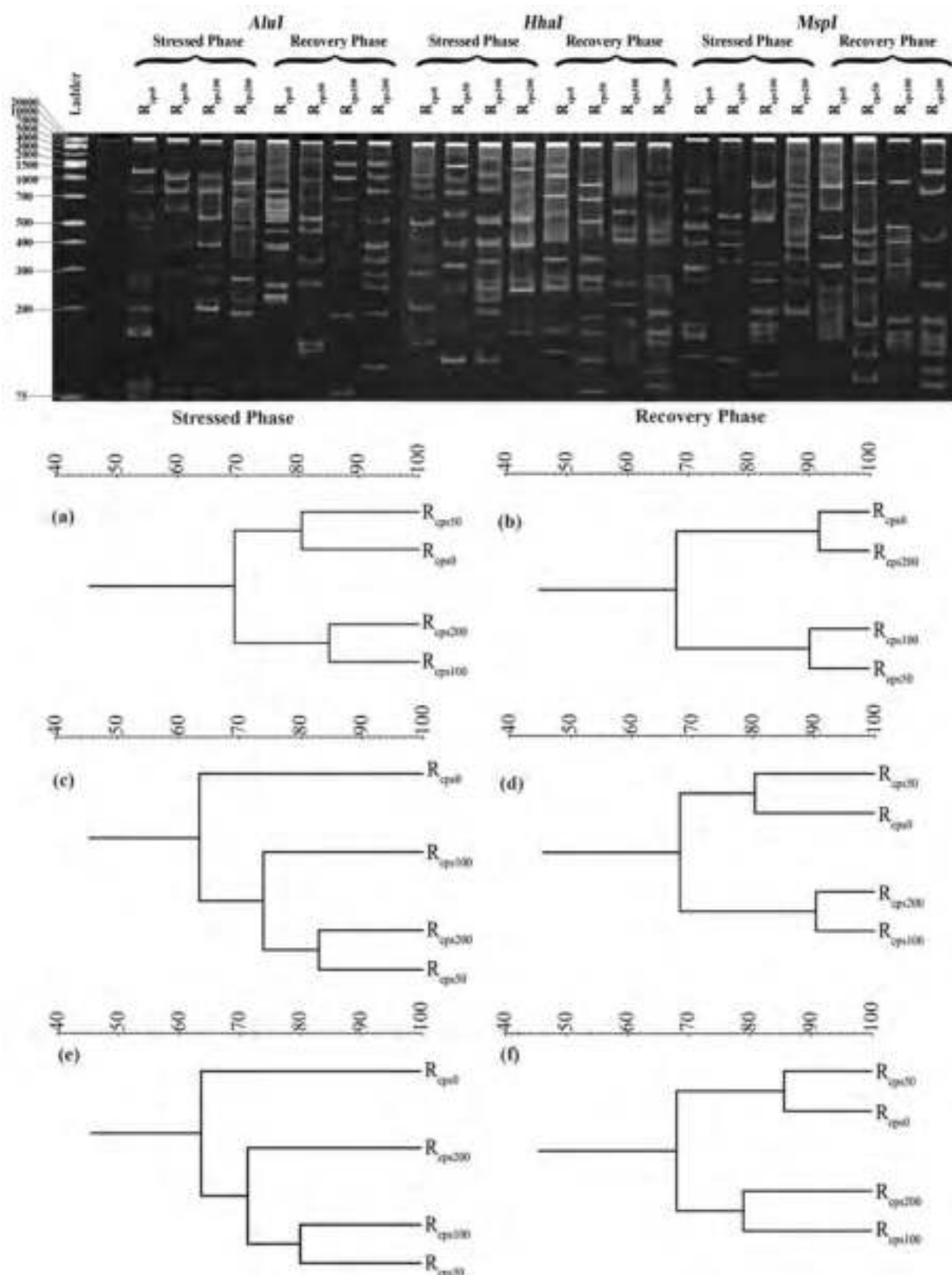


Fig. 4.24 Digestion pattern and dendrogram of aerobic sludge biomass treated with restriction enzyme *AluI*: (a) under stressed phase and (b) under recovery phase; *HhaI*: (c) under stressed phase (d) under recovery phase; and *MspI*: (e) under stressed phase (f) under recovery phase.

Table 4.23 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase operation.

Stressed phase operation												
Reactor	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
R _{cps0}	1	0.6930	0.5753	0.4232	1	0.7850	0.6840	0.5455	1	0.7231	0.6323	0.5192
R _{cps50}	0.6930	1	0.5834	0.5034	0.7850	1	0.7230	0.8420	0.7231	1	0.7923	0.6434
R _{cps100}	0.5753	0.5834	1	0.7632	0.6840	0.7230	1	0.8023	0.6323	0.7923	1	0.7643
R _{cps200}	0.4232	0.5034	0.7632	1	0.5455	0.8420	0.8023	1	0.5192	0.6434	0.7643	1
Recovery phase operation												
Reactor	<i>AluI</i>				<i>HhaI</i>				<i>MspI</i>			
	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
R _{cps0}	1	0.9342	0.8123	0.7754	1	0.8600	0.8230	0.8040	1	0.9010	0.8420	0.8120
R _{cps50}	0.9342	1	0.7323	0.7034	0.8600	1	0.7630	0.7043	0.9010	1	0.7507	0.7328
R _{cps100}	0.8123	0.7323	1	0.9223	0.8230	0.7630	1	0.9300	0.8420	0.7507	1	0.8554
R _{cps200}	0.7754	0.7034	0.9223	1	0.8040	0.7043	0.9300	1	0.8120	0.7328	0.8554	1

and 80.4% similarity for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, which has also improved from 78.5%, 68.4%, and 54.6%, respectively, when compared to stressed phase operation. Similarity of 90.1%, 84.2%, and 81.2% has been observed for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses withdrawn during recovery phase operation, respectively, when digested with *MspI*, which shows improvement from 72.3%, 63.2%, and 51.9%, respectively, of stressed phase operation. When CPS addition has been discontinued in the feed to the reactors during the recovery phase operation, aerobic sludge biomass has tried to regain its microbial community structure. The obtained result suggests recovery in the microbial community structure for reactor fed with a lower CPS concentration (50 mg/L) during stressed phase operation. The reactors fed with 100 and 200 mg/L of CPS during stressed phase operation have not been completely able to recover during recovery phase operation. It is clear from Table 4.23 that the similarity between CPS fed reactor biomass samples under the stressed phase has decreased with increased CPS concentration as compared to reactor R_{cps0} biomass. The literature is not available to relate microbial community shift in aerobic biomass due to CPS in the feed, while a few literature have suggested the adverse impact of CPS on soil microbial community.

Microscopic examination has indicated presence of thick, denser, and abundance of filamentous microorganisms in all reactor biomasses towards the end of the recovery phase operation. In reactor R_{cps50} biomass, the flocs size has ranged between 2.0-12.0 mm, whereas it has varied from 1.0 to 10.0 mm for reactor R_{cps100} biomass, and 1.0 to 6.0 mm for reactor R_{cps200} biomass towards the end of the recovery phase operation. According to the fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses, there have been 94.8%, 85.7%, 67.6%, and 51.2% viable cells and 3.2%, 9.6%, 27.2, and 48.2% dead cells, respectively. As recovery phase operation has progressed to a steady-state condition, the percentage of live cells has grown, which has indicated biomass recovery. A similar rise in filamentous biomass has been seen towards the end of the recovery phase operation, with a FI value of 5 for all reactor biomasses. As compared to reactor R_{cps0} biomass, PS, PN, and EPS contents for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have exhibited similar level. The PS content for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have ranged 16.43-17.54 mg/g VSS; PN content has ranged 52.02-55.02 mg/g VSS; and EPS content has ranged 68.75- 72.54 mg/g VSS. Similarly, enzyme activity values have also indicated the recovery as compared to stressed phase values. $E_{Catalase}$ and $E_{Dehydrogenase}$ for all reactor biomasses have ranged 0.36-0.38 mg/g VSS and 22.32-27.23 μ g/g VSS, respectively. $E_{Protease}$ for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have ranged 1.76-2.21 mg/g VSS. Similarly, the E_{Urease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have ranged from 7.88-9.23 g/g VSS, while 10.78 g/g VSS for reactor R_{cps0} biomass. The cellular machinery of aerobic sludge biomass microorganisms has been recovered with discontinuation of CPS in the feed, and enzyme activity levels have recovered. ARDRA has revealed the recovery in microbial community structure. *AluI* has shown 93.4%, 81.2%, and 77.5% similarity; *HhaI* has shown 86.0%, 82.3%, and 80.4% similarity; and *MspI* has shown 90.1%, 84.2%, and 81.2% similarity for

reactors biomasses fed with CPS concentration of 50, 100, and 200 mg/L, respectively, as compared to reactor R_{cps0} biomass.

4.4 Summary

The influence of CPS fed reactors have an adverse impact on the organic removal levels. With increased CPS concentration in feed, COD removal has deteriorated for CPS fed reactors during stressed phase operation. The COD removal for reactor R_{cps50} , R_{cps100} , and R_{cps200} have been 72%, 39%, and 18%, whereas reactor R_{cps0} has shown 91% of COD removal during the steady-state condition of stressed phase operation. The existence of CPS in feed seems to have a slight impact on pH and alkalinity. The biomass growth in control reactor appear to be uncontrollable throughout the experiment, while biomass growth in CPS fed reactors has constrained and influenced a bit by CPS doses. The addition of CPS in feed has slightly lowered SVI which ranged 104-114 mL/g for CPS fed reactor biomasses, while reactor R_{cps0} biomass has shown SVI of 136 mL/g. ISV has been observed in the range of 2.42-2.52 m/h during stressed phase operation for CPS fed reactor biomasses, while reactor R_{cps0} biomass has shown ISV of 2.13 m/h. The organic degradation capability has been represented by ASBA which has been 6.57, 4.30, and 2.04 mg COD removed/mg VSS d for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses – on a lower side compared to 7.42 mg COD removed/mg VSS d for control reactor R_{cps0} biomass. Fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses have been indicated 98.2%, 48.3%, 33.7%, and 21.8% viable cells and 0.7%, 44.9%, 58.4, and 72.2% dead cells, respectively. Inactive portion of aerobic biomass has increased with increased CPS concentration towards the end stressed phase operation. PS, PN, and EPS contents in reactor biomasses fed with CPS has varied slightly. The adverse effect of CPS on metabolic activity of aerobic sludge biomass has been confirmed by enzyme activity. $E_{Dehydrogenase}$ and E_{Urease} have been significantly affected due to CPS stress. As per ARDRA, CPS has stressed microorganisms, and 50 mg/L of CPS has altered the microbe population structure of aerobic sludge biomass. ARDRA has established the relation between control and CPS fed reactor biomasses and confirmed the microbial community changes (31-48%) during stressed phase operation. However, there is a need to study almost no impact on filamentous biomass under CPS stress.

During recovery phase operation, all reactors have shown significant COD removal (more than 88% during steady-state condition). The pH has been observed to be neutral, and the alkalinity has been observed to be nearly the same for all the reactors. The reactor fed with CPS during stressed phase operation has maintained biomass growth and has shown almost similar levels as the control reactor during recovery phase operation. SVI has increased for all reactors due to filamentous biomass, that also is a reason for decreased ISV. Reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses have shown SVI of 185, 152, 145, and 135 mL/g, respectively. ISV for all reactor biomasses have shown minor variation. The ISV for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has been 2.06, 2.08, and 2.28 m/h – on lower side as compared to control reactor biomass (1.48 m/h). The discontinuation of CPS allowed the

aerobic sludge biomass to regain its organic degradation capability and ASBA has improved from 7.42, 6.57, 4.30, and 2.04 mg COD removed/mg VSS d obtained during stressed phase operation to 8.13, 7.45, 7.13, and 7.10 mg COD removed/mg VSS d during recovery phase operation for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. Extremely dense and thick flocs with filaments have been observed and FI value has reached to 5 for all reactor biomasses. Fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses have been shown 94.8%, 85.7%, 67.6%, and 51.2% viable cells and 3.2%, 9.6%, 27.2, and 48.2% dead cells, respectively, towards the end of the recovery phase operation. The enzyme activity values for all the reactor biomasses have also improved, with values close to those observed for the control reactor biomass. CPS discontinuation in the feed during recovery phase has also allowed the biomass to recover its microbial communities. ARDRA has revealed that the reactor R_{cps50} , R_{cps100} and R_{cps200} biomasses has regained its microbial community structure and has shown 78-93% similarity compared to reactor R_{cps0} biomass. Overall, it appears that complete recovery of aerobic sludge biomass is possible after discontinuation of CPS stress in the feed.

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Impacts of Piggery Wastewater Stress on Aerobic Sludge Biomass

In comparison to other livestock species, pig rearing has a higher potential to contribute to economic gain for small and marginal farmers. In India, pig farming sector is highly unorganized in rural areas. The proper cleaning of pig farms is required to protect animals from infectious diseases, which results in a considerable volume of wastewater. Unorganized piggery farms are also emerging-up in urban areas which discharge organic and inorganic rich wastewater into a nearby drain/sewer line — ending up to the sewage treatment plant. This chapter studies the effect of piggery wastewater on the un-acclimatized aerobic sludge biomass.

5.1 Background

Pig farming, on the whole, need a lot of water. Piggery wastewater (PWW) includes pig body waste (urine and feces), food waste and water from cleaning operation, living quarters — a typical livestock farm wastewater contains high concentrations of suspended solids, organic matter, nitrogen, phosphorus, growth promoters, and drugs, (Liu et al., 2017; Sombatsompop et al., 2011; Wang et al., 2019; Yan et al., 2013; Zhou et al., 2016) but having poor biodegradability of organics (Sheng et al., 2017). In addition, trace amounts of metals are required as growth promoters for pigs and pharmaceuticals like antibiotics and antimicrobials are required for prevention and treatment of diseases (Fridrich et al., 2014). A total of 17 antibiotics have been reported in PWW, ranging from 13.70×10^4 ng/L to 16.60×10^4 ng/L (Zhou et al., 2013). The occurrence of drugs/antibiotics in PWW depends upon type of antibiotics, its doses, the population of pigs and application frequency, which can vary from farm to farm. The high levels of Zn(II) and Cu(II), both of which are essential micro-nutrients for pigs, are added to the feed as growth promoters (Abe et al., 2012; Suzuki et al., 2010). Since pigs can absorb only 10-20% of Cu(II) and Zn(II), the rest is excreted (Suzuki et al., 2010). As a consequence, PWW contains Cu(II) and Zn(II) in high concentrations.

Metals more often encountered in PWW are cadmium (0.01-0.35 mg/L), cobalt (0.18-1.28 mg/L), chromium (0.16-3.88 mg/L), copper (1.25-148.02 mg/L), Fe (1.64-447.58 mg/L), Ni (0.69-3.31 mg/L), and Zn (2.82-606.17 mg/L) (Creamer et al., 2010; Moral et al., 2008; Tilche et al., 2001; Zhang et al., 2011, 2012; Zheng et al., 2018).

Animal husbandry and livestock rearing sectors are critical for rural livelihood. In India, 70% of the pig population is reared under traditional small-holding, low-input demand-driven production system, except for a limited number of semi-commercial pig farms in Kerala, Punjab, and Goa (Chauhan et al., 2016). In developing countries such as India, piggery farm owners do not invest in constructing a proper treatment facility for wastewater generated from the farms. The generated PWW is directly discharged in the nearby drain/sewer line, which finds its way to a sewage treatment plant (STP). Even, Central Pollution Control Board (CPCB) does not have effluent standards regarding the treatment/discharge of PWW. Hence, most of the pig farms operators clean their farms and discharge PWW willingly/illegally to the nearby drain/sewer line without any treatment (Meng et al., 2016; Wang et al., 2019). Discharge of PWW to sewers are likely to impart toxic shock to the biological system of sewage treatment process (Zhou et al., 2016). STP receiving the PWW (which contain heavy metals, antibiotics, and high organic content) may result in hindrance in operation and reduction in the plant efficiency as such wastewater are not considered while designing the STP. The higher concentrations of metals and antibiotics may cause inhibitory or toxic effects to biomass, affecting cell growth, loss of flocs structure, changes in community structure, and decrease in treatment efficiency (Hao et al., 2016; Ong et al., 2004).

In reported literature, efforts and focus have been directed to the PWW treatment by biological processes (Edgerton et al., 2000; Obaja et al., 2005; Suzuki et al., 2010; Su et al., 1997; Wang et al., 2019; Wun-jern, 1989; Yan et al., 2013), whereas the impact on the biomass has been missing. The interests have mostly centered around reactor performance, nitrogen, phosphorus, metals, and antibiotics removals, etc. (Cheng et al., 2018; Daumer et al., 2007; Li et al., 2010; Liu et al., 2017; Lotti et al., 2012; Xu et al., 2019; Zhang et al., 2011). Therefore, a careful appraisal of PWW should be made mainly because of their high content of organic constituents together with heavy metals and antibiotics. Specific emphasis should be placed on COD removal along with biomass activity, morphology, community structure, and enzyme activity during the course of treatment process. Since the biomass quality and characteristics are the backbone of the biological process of wastewater treatment, the aim of this study is to understand the behavior and characteristics of biomass exposed to PWW. In addition, this study also evaluates recovery potential of the biomass when PWW addition is stopped as part of the feed.

5.2 Materials and methods

5.2.1 Chemicals and reagents

All chemicals and reagents used in this study are analytical or HPLC grade, manufactured by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals is presented in *Appendix-I*

(Table A-I.1).

5.2.2 Aerobic sludge biomass

The aerobic sludge biomass, withdrawn from the mother reactor (MR), has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in detail in *Appendix-II*.

5.2.3 Source of piggery wastewater

The raw piggery wastewater has been collected from a local piggery farm located at Kalakuchi (26°15'50.9"N 91°39'37.7"E), Assam, India. Crushed maize, wheat bran, soybean, refined oil, and salt have been fed to 76 pigs (including piglets), along with growth promoters and supplements (vitamin B3, vitamin B6, vitamin B7, vitamin B9, vitamin B12, copper, cobalt, zinc, iron, and calcium). Water is used to clean the pigs space twice in a day, which generates ~800 L/d of wastewater. The wastewater contains unconsumed feed, feces (a smaller portion remain after cleaning), and urine. The piggery wastewater (~20 L) has been collected from the outlet of the piggery farm and its characteristics has been presented in Table 5.1. Upon arrival at the laboratory, collected PWW has been divided into two parts. One part has been screened through a metallic sieve of 0.25 mm mesh to remove larger suspended solids that may resist biological degradation — termed as screened PWW (s). The other part, not subjected to any kind of pre-treatment, has been termed as raw PWW (r). The screened and raw PWWs have been stored in a cold storage at –20°C. When required, PWW has been withdrawn from the cold storage, left to equilibrate to room temperature, and fed into the reactor. Feed has been prepared by diluting the screened and raw PWWs (~500-750 mL) with tap water until the desired range of organic strength (COD ~900 mg/L) has been obtained. Since the organic strength of PWW differed on each collection day, therefore, COD has been estimated on every collected samples, before preparation of the feed.

Table 5.1 Characteristics of raw piggery wastewater collected from pig farm.

Parameters*	Concentration
pH	6.53±0.46 (4)
sCOD (mg/L)	1041.48±53.35 (4)
Suspended solids (mg/L)	1257.50±42.14(4)
Volatile suspended solids (mg/L)	946.50±32.28 (4)
Alkalinity (mg/L as CaCO ₃)	989.23±15.23 (4)
Ammonia (mg/L)	38.62±3.44 (4)
Zn (mg/L)	1.42±0.82 (4)
Cu (mg/L)	0.37±0.07 (4)
Ni (mg/L)	5.40±0.84 (4)
Fe (mg/L)	7.12±1.24 (4)
Cd (mg/L)	2.82±0.82 (4)

*Estimated in this study

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

5.2.4 Reactor setup and operation

The studies have been carried out in three different phases. The reactor setup and its operation in each of the phases are mentioned below.

5.2.4.1 Stressed phase I operation with PWW

The experimental set-up consists of two sets of three identical reactors (height 14.5 cm × diameter 12.5 cm) with a working volume of 1 L (Fig. 5.1). Reactors have been designated as R_{c_1} and R_{c_2} (control reactors with no PWW in the feed), R_{s_1} and R_{s_2} (screened PWW in the feed), and R_{r_1} and R_{r_2} (raw PWW in the feed). The control reactor R_c has been fed with Lactogen 3 as a source of carbon and nutrients. The composition of Lactogen 3 powder is presented in Table A-II.2. The reactors have been operated at ambient conditions without temperature and pH control for two cycles per day with a fill time of 15 min, a react time of 680 min, a settling time of 10 min, and a decant time of 15 min (as shown in Fig. 5.2). The reactors have been initially filled with ~435 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~3000 mg/L] withdrawn from mother reactor (MR) (Appendix-II). It is difficult to obtain the similar soluble COD (sCOD) values in collected PWW samples from a piggery farm on each of the collection day. Therefore, soluble COD has been estimated for each collected PWW samples during the study. Around 900 mg/L of soluble COD has been fed to the reactors R_s and R_r by diluting screened and raw PWW, respectively, with the tap water. The control reactor R_c has been fed with 0.90 g of Lactogen 3 (dissolved in 100 mL of lukewarm water). After addition of Lactogen 3, screened and raw PWW to reactors R_c , R_s , and R_r , respectively; the final volume has been made to 1 L using tap water. Aeration has been imparted for 1-2 min for proper mixing of the feed in the reactor. After collection of samples, aeration has been stopped and biomass is allowed to settle, and samples have been collected for analysis of initial parameters. After collection of samples, aeration has been

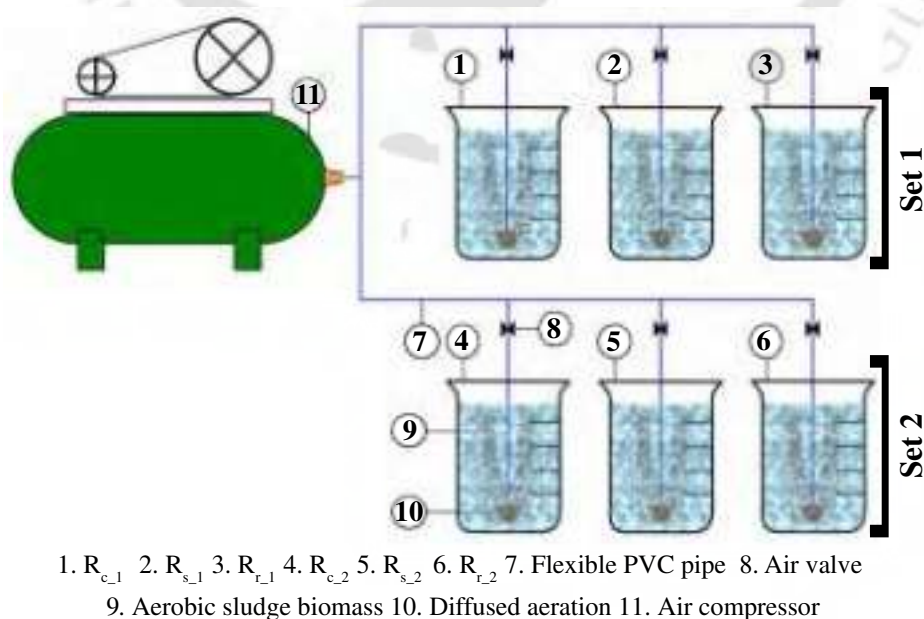


Fig. 5.1 Schematic diagram of reactor setup used for experiment during stressed phase I operation with PWW.

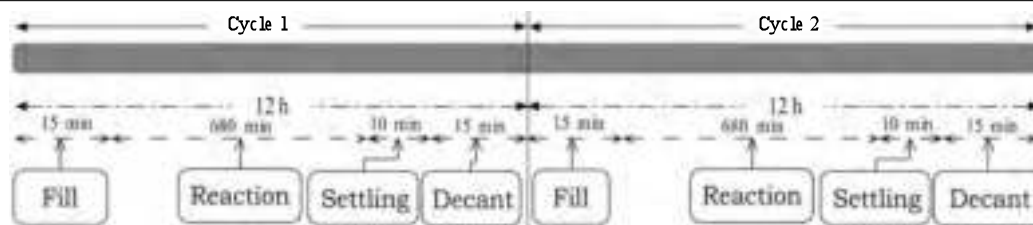


Fig. 5.2 Schematic representation of cycle time for reactors during stressed phase I operation with PWW.

restarted at a flow rate of 2.5 L/min through an air compressor. The 1st cycle has started around 9.30 am in the morning and initial room temperature has been recorded at that time, while room temperature during decanting has been recorded at 9.00 pm and then 2nd cycle started around 9.30 pm. This phase of the operation has been termed as stressed phase I operation.

5.2.4.2 Stressed phase II operation with PWW+Cu(II)

After stressed phase I operation with PWW feed in steady-state conditions, the mixed liquor concentration in reactors (R_c , R_s and R_r) have been observed in the range of 3500-4000 mg/L. The contents of each reactor R_{s_1} and R_{s_2} have now been evenly divided into two identical reactors containing mixed liquor of around 2000 mg/L. The reactors have been designated as R_{sCu40_1} , R_{sCu80_1} , R_{sCu40_2} and R_{sCu80_2} . Similarly, contents of each of the reactors R_{r_1} and R_{r_2} have also been evenly divided into two identical reactors containing mixed liquor of around 2000 mg/L designated as R_{rCu40_1} , R_{rCu80_1} , R_{rCu40_2} and R_{rCu80_2} as shown in Fig. 5.3. The reactors have been fed with Lactogen 3, screened, and raw PWWs as has been fed in stressed phase I operation (i.e. COD of ~900). Additionally, reactors R_{sCu40_1} , R_{sCu40_2} , R_{rCu40_1} and R_{rCu40_2} have to be fed with 40 mg/L of Cu(II), while reactors R_{sCu80_1} , R_{sCu80_2} , R_{rCu80_1} , and R_{rCu80_2} to be fed with 80 mg/L of Cu(II). The hydrated copper sulfate ($CuSO_4 \cdot 5H_2O$) has been used as the source of Cu(II) metal in feed. A stock solution of Cu(II) metal ion has been prepared by dissolving 3.92 g of hydrated copper sulfate in distilled water, and the final volume made to 100 mL. After feeding the reactors with Lactogen 3, screened, and raw PWW, Cu(II) metal stock solution of 0, 4, 4, 8, and 8 mL have been added, respectively, to reactors R_c , R_{sCu40} , R_{rCu40} , R_{sCu80} , and R_{rCu80} to yield the metal ion concentrations as 0, 40, 40, 80 and 80 mg/L, respectively. After addition of stock metal solution, the final volume of the reactor has been made to 1 L with tap water. The reactors have been operated at ambient conditions without pH and temperature control for two cycles per day with a fill time of 10 min, a react time of 665 min, a settling time of 10 min, a decant time of 15 min, and a wash time of 20 min (as shown in Fig. 5.4). The aeration has been imparted for 1-2 min for proper mixing of the feed in the reactor. Thereafter, aeration has been stopped and biomass has been allowed to settle, and samples have been collected for analysis of initial parameters. The 1st cycle has been started around 9.30 am and initial room temperature has been recorded at that time, while room temperature during decanting has been recorded at 8.45 pm and then 2nd cycle started around 9.30 pm. This phase of the operation has been termed as *stressed phase II operation*. The effluent samples have been collected during the decantation step at the end of a cycle.

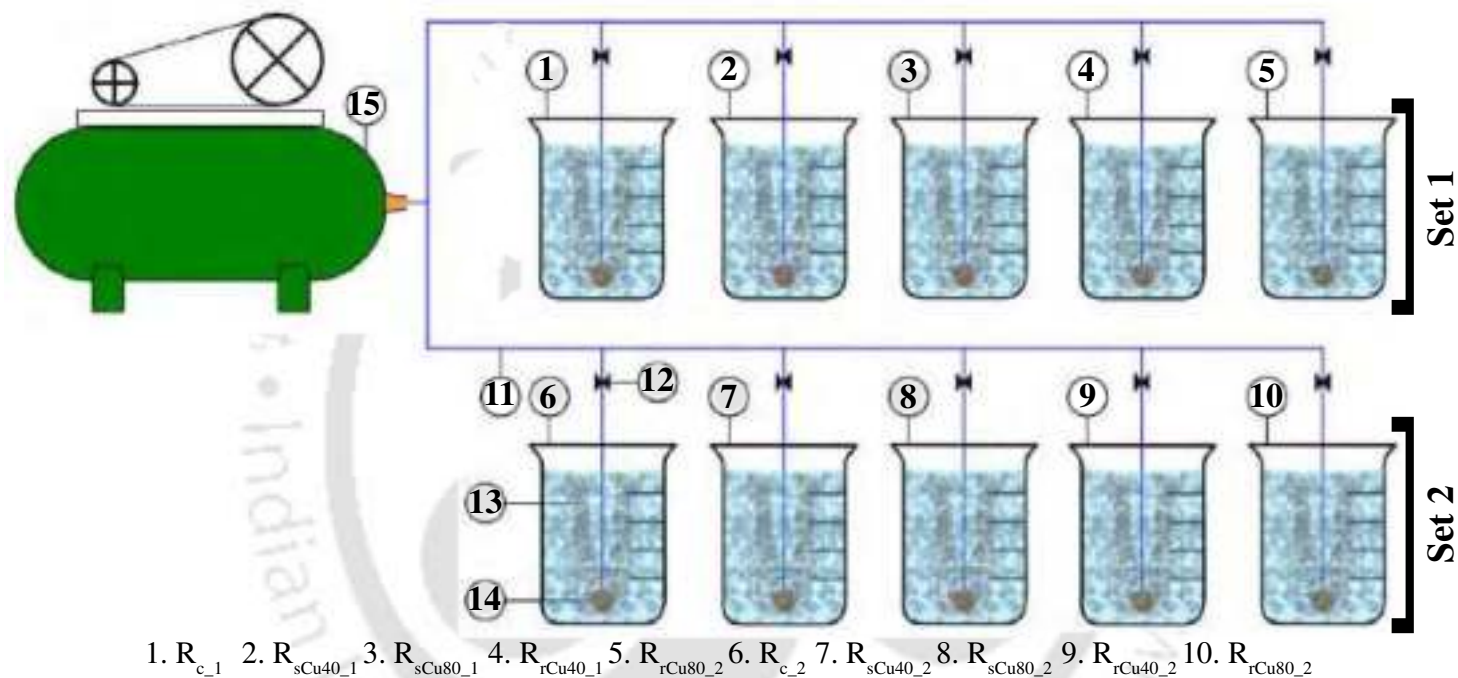


Fig. 5.3 Schematic diagram of reactor setup used for experiment during stressed phase II operation with PWW+Cu(II).

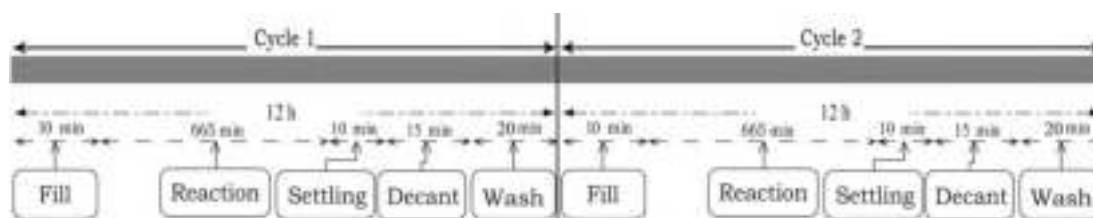


Fig. 5.4 Schematic representation of cycle time for reactors during stressed phase II operation with PWW+Cu(II).

Residuals from previous feedings and fresh addition in the subsequent feeding leads to progressive build-up of increasing Cu(II) concentration which exposes the reactor biomass to more than the planned Cu(II) doses, and hence the retained settled mixed liquor in the reactor after decantation is washed with tap water before starting the next feeding. The washing is carried out by refilling the decanted reactors with tap water up to 1 L volume and followed by gentle mixing of the content for 1-2 min. After that, the content is allowed to settle for 5 min before the supernatant is decanted. This process has been repeated two times. After washing, the reactor is again filled with tap water up to a 1 L volume. The clear liquid is sampled to estimate background (or residual) Cu(II) concentration left after the washing which is designated as 'BG_{Cu}'. During a settling time of 10 min, the settled mixed liquor (sludge biomass + liquid) has occupied volume up to 550 mL for poorly settling biomass and 180 mL for rapidly settling biomass. The volume exchange ratio for all the reactors have been in the range of 45-82% throughout the study. The aerobic sludge biomass has been washed thrice with tap water at the end of the stressed phase II operation but before starting the recovery phase operation.

5.2.4.3 Recovery phase operation

After steady-state stressed phase II operation with PWW+Cu(II) in the feed, the addition of PWW and Cu(II) both have been discontinued in the feed to reactors R_{sCu40}, R_{rCu40}, R_{sCu80}, and R_{rCu80}; and thereafter Lactogen 3 has been fed to serve as the sole carbon source to all the reactors. The reactors have been operated at ambient conditions without pH and temperature control for two cycles per day with a fill time of 15 min, a react time of 680 min, a settling time of 10 min, and a decant time of 15 min. The cycle time shown in Fig. 5.2 has been followed during recovery phase operation. The synthetic feed has been prepared using Lactogen 3 (0.90 g for each reactor) and lukewarm water (~100 mL) followed by proper mixing using magnetic stirrer. After addition of Lactogen 3 to the reactors, final volume has been made up to 1 L using tap water. For keeping the biomass in suspension and imparting mixing, the air has been supplied using an air compressor at a flow rate of 2.5 L/min through an air diffuser. The aeration has been initially imparted for 1-2 min for proper mixing of the feed in the reactor. Thereafter, aeration has been stopped and biomass has been allowed to settle, and samples have been collected for analysis of initial parameters. The 1st cycle has started around 9.30 am and initial room temperature has been recorded at that time, while room temperature during decanting has been recorded at 9.00 pm and then 2nd cycle started around 9.30 pm. This phase of the operation has been termed as recovery phase operation.

5.2.5 Analytical methods

The frequencies of initial and effluent sample analysis are presented in Table 5.2. The analytical parameters have been grouped into four different categories, as explained earlier. The analytical techniques for estimation of temperature, pH, alkalinity, COD, MLSS, MLVSS, Cu(II) concentration, interface settling velocity (ISV), sludge volume index (SVI), photographic observation, microscopic analysis, aerobic sludge biomass activity (ASBA) test, enzymes activity, and ARDRA, used in this study, have already been explained in Chapter 2. The cell viability test, estimation of polysaccharides (PS), proteins (PN), and extracellular polymeric substance (EPS) contents have been explained in Chapter 3.

Table 5.2 Analysis frequency of initial and effluent sample parameters.

Category	Parameters	Frequency
Environmental related parameters	Room temperature	Daily
	Initial pH	
	Initial Cu(II) concentration	
Reactor related performance parameters	Effluent pH	
	Effluent Cu(II) concentration	
	Chemical oxygen demand (COD)	
	Alkalinity	
Biomass related general parameters	Mixed liquor suspended solids (MLSS)	Every 7 th day
	Mixed liquor volatile suspended solids (MLVSS)	
	Sludge volume index (SVI)	
	Interface settling velocity (ISV)	
	Aerobic sludge biomass activity (ASBA)	Towards end of the each phases
Biomass related microbiological parameters	Microscopic observation after Gram's staining	Every 7 th day
	Photographic observation	
	Filament index (FI)	
	Cell viability test	
	Polysaccharides (PS), proteins (PN) and extracellular-polymeric substances (EPS)	Towards end of the each phases
	Catalase activity	
	Dehydrogenase activity	
	Protease activity	
	Urease activity	
	Amplified ribosomal DNA restriction analysis (ARDRA)	

5.3 Results and Discussion

The reactors have been operated during 0-41 d with PWW in the feed — termed as *stressed phase I operation* and afterward for 42-72 d with PWW+Cu(II) in the feed — termed as *stressed phase II operation*. In addition, reactors have been further operated for 73-120 d without PWW and Cu(II) in the feed — termed as *recovery phase operation*. The results obtained are discussed in subsequent subsections. The average values of duplicate reactors (average of R_{c_1} and R_{c_2} have been represented as R_c , R_{s_1} and R_{s_2} as R_s , R_{r_1} and R_{r_2} as R_r , R_{sCu40_1} and R_{sCu40_2} as R_{sCu40} , R_{sCu80_1} and R_{sCu80_2} as R_{sCu80} , R_{rCu40_1} and R_{rCu40_2} as R_{rCu40} , R_{rCu80_1} and R_{rCu80_2} as R_{rCu80}) along with the standard deviation have been used in the results.

5.3.1 Stressed phase I operation with PWW

5.3.1.1 Environmental and performance parameters

Variation in environmental and performance related parameters is shown in Fig. 5.5 to 5.7 and summary of data are presented in Table 5.3. The reactors have been operated under ambient conditions throughout the operation; hence, pH and reactor temperature have not been adjusted. The variation in room temperature has been mainly observed due to weather changes. The temperature variation has been shown in Fig. 5.5 and data has been summarized in Table 5.3. Since all the reactors have been operated at room temperature, variation in room temperature recorded at 9.30 am, and 9.00 pm have been presented in Fig. 5.5.

Variations in the initial and effluent pH for reactors have been shown in Fig. 5.5 to 5.7. The average initial and effluent pH for reactor R_c during 0-41 d of operation has been observed as 7.35 ± 0.22 and 7.36 ± 0.22 , respectively. However, pH for PWW fed reactors (R_s and R_r) have slightly decreased as compare to reactor R_c during stressed phase I operation due to addition of PWW as feed. The average initial pH for reactors R_s and R_r have been observed as 6.47 ± 0.17 and 6.32 ± 0.28 , respectively, while the average effluent pH of the reactors have been obtained as 6.95 ± 0.16 and 7.18 ± 0.25 , respectively.

The Cu(II) concentration in initial and effluent samples have been shown in Fig. 5.5-5.7 and data has been summarized in Table 5.3. The average initial Cu(II) concentration have been observed in the range of 0.30-0.80 mg/L, while average effluent Cu(II) concentration have ranged 0.01-0.05 mg/L for reactors R_c , R_s , and R_r as presented in Table 5.3.

The variations in COD removal during the stressed phase I operation for all reactors have been shown in Fig. 5.5-5.7 and data has been summarized in Table 5.3. On 1st d of operation, COD removal for reactors R_c , R_s , and R_r have been observed as 90.4%, 21.7%, and 15.7%, respectively, while, it has increased to 90.4%, 68.9%, and 48.9% of COD removal, respectively, on 14th d of operation. Similarly, towards the end of stressed phase I operation (on 41st d), COD removal for reactors R_c , R_s , and R_r have been observed as 91.1%, 76.0%, and 62.5%, respectively. It can be observed that reactor R_c showed uniform COD removal from 1 to 41 d of operation (Fig. 5.5), whereas reactors fed with PWW (screened and raw) have shown variation in COD removals (Fig. 5.6 and 5.7).

The COD removal for reactors R_s , and R_r have been poor during 1-15 d of operation, which can be attributed to complex and refractory nature of organics present in PWW (Wang et al., 2019). The PWW contains complex protein and fibrous carbohydrates (cellulose and lignin) (Yan et al., 2013), which is not easily degradable by aerobic sludge biomass. The sudden addition of PWW to reactors has also changed the pH of reactors. The aerobic sludge biomasses get adapted to the changed feed with time and start degrading COD (provided by PWW) after 20 d of operation. Towards the end of the stressed phase I operation, reactor R_s has shown improved COD removal than reactor R_r . The reactor R_s has been fed with screened PWW while reactor R_r has been fed with raw PWW, which may probably be composed of solids and slowly biodegradable material such as cellulose and fiber in PWW. The PWW also

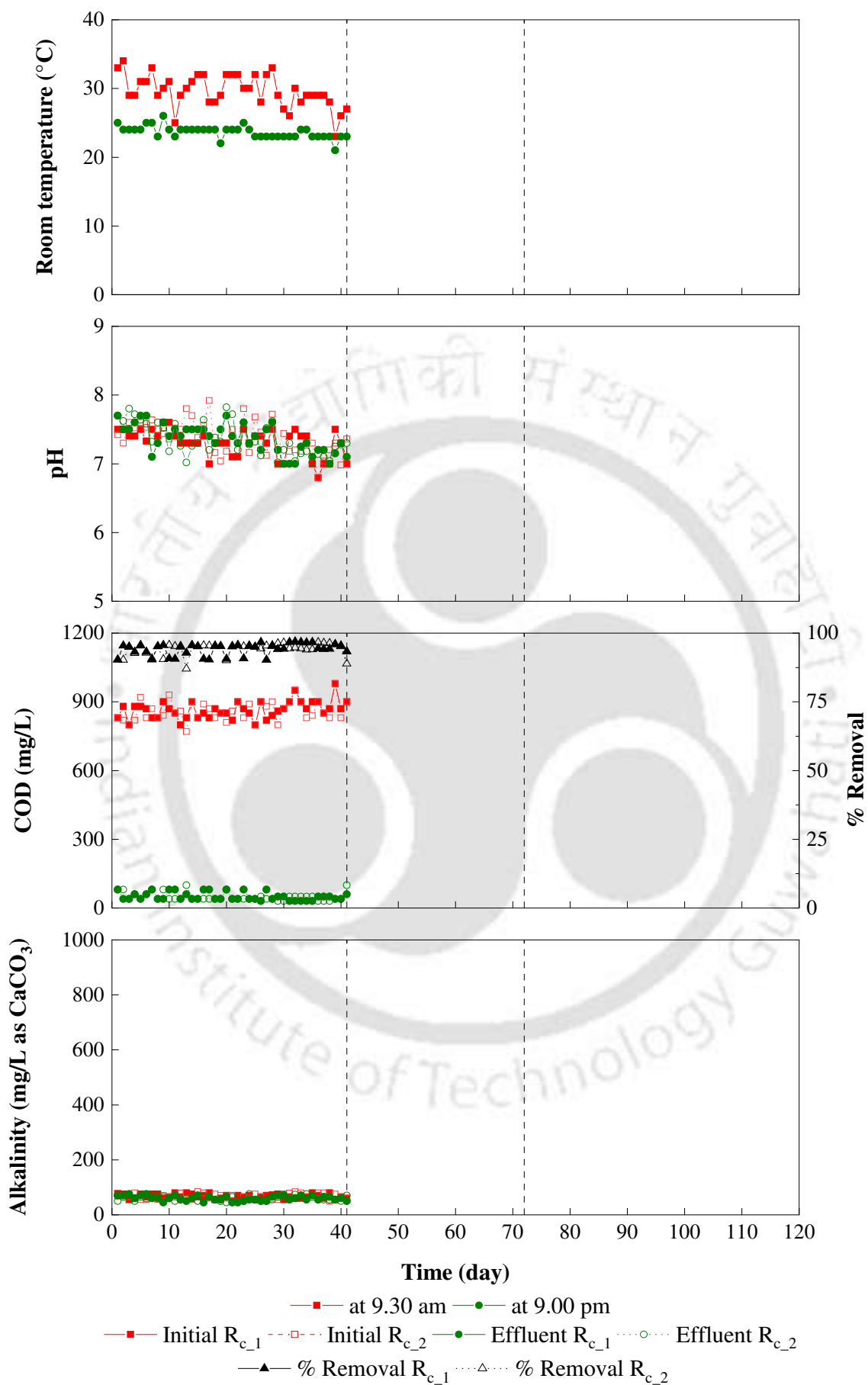


Fig. 5.5 Variation in environmental and performance related parameters for reactor R_c during 0-41 d of stressed phase I operation.

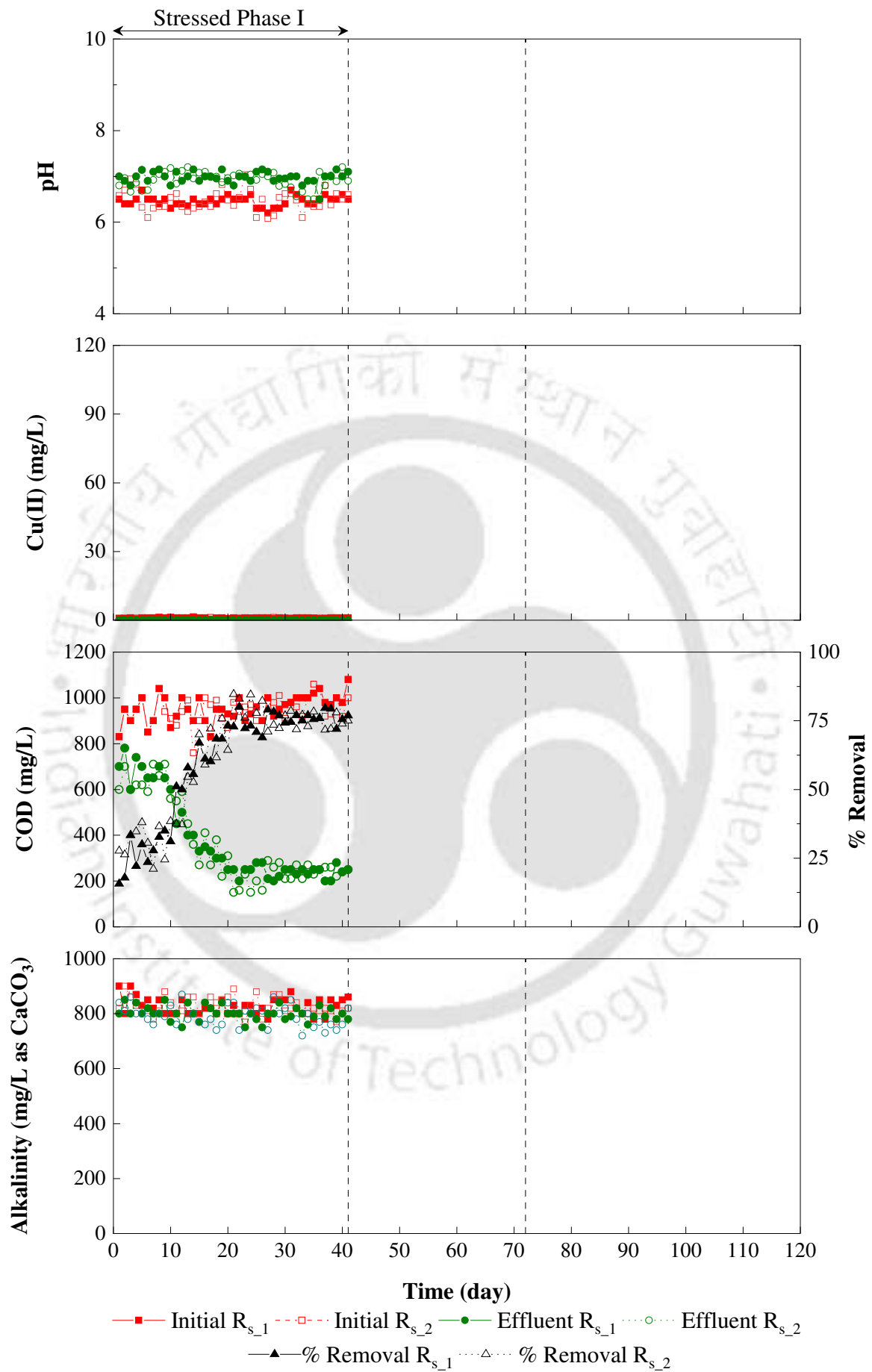


Fig. 5.6 Variation in environmental and performance related parameters for reactor R_s during 0-41 d of stressed phase I operation with PWW feed.

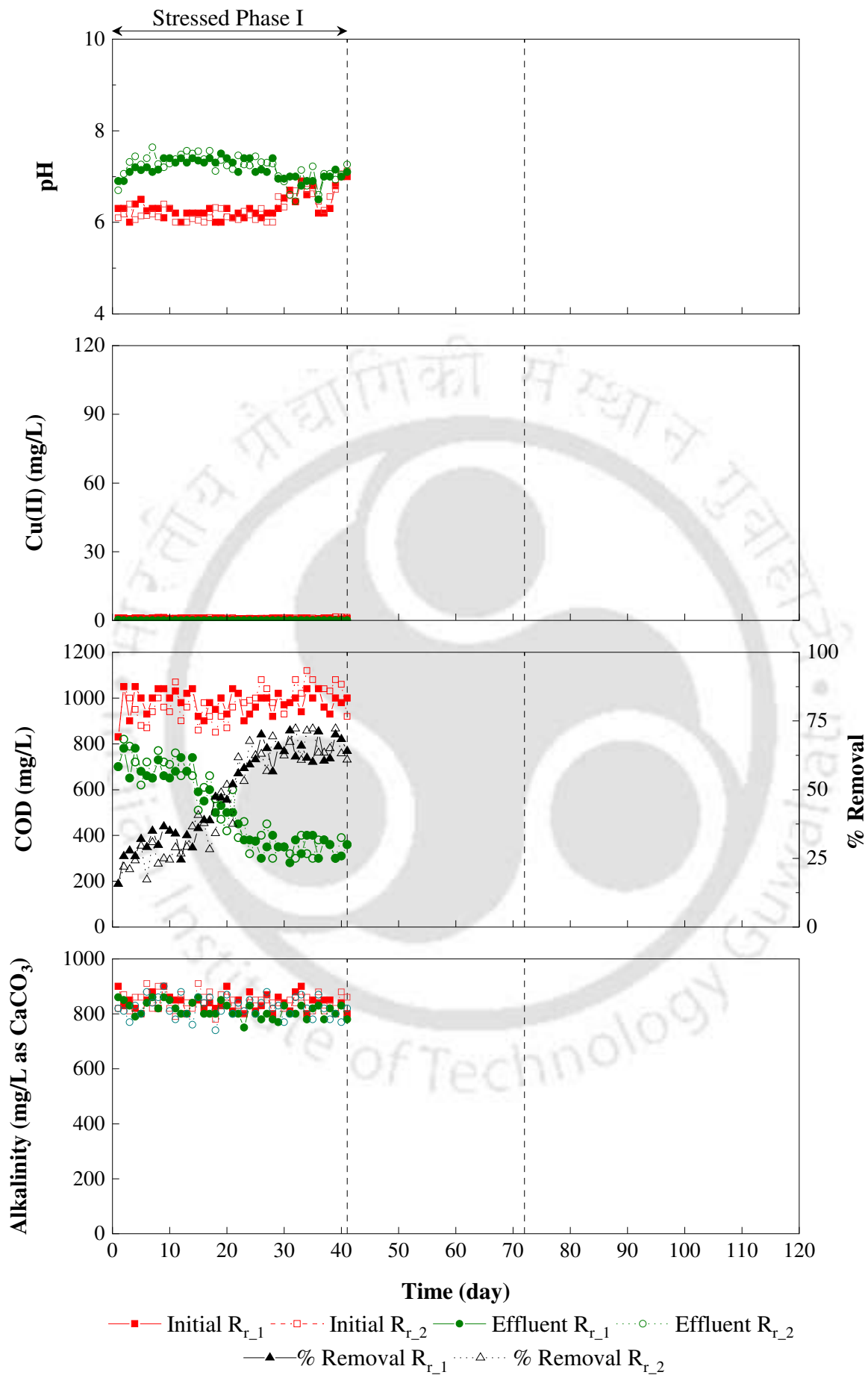


Fig. 5.7 Variation in environmental and performance related parameters for reactor R_r during 0-41 d of stressed phase I operation with PWW feed.

Table 5.3 Summary of environmental and performance related parameters for reactors operated during 0-41 d of stressed phase I operation with PWW feed.

Parameters	Day-wise	0-41		
	Reactor	R _c	R _s	R _r
	Lactogen 3 (g/L)	0.90	-	-
Stressed phase I	PWW (mL)	0	~500-750	~500-750
	Room temperature (°C) at 9.30 am	29.6±2.4 (41)	29.6±2.4 (41)	29.6±2.4 (41)
	Room temperature (°C) at 9.00 pm	23.6±0.9 (41)	23.6±0.9 (41)	23.6±0.9 (41)
	Initial pH	7.35±0.22 (82)	6.47±0.17 (82)	6.32±0.28 (82)
	Initial Cu(II) (mg/L)	0.03±0.01 (82)	0.80±0.20 (82)	0.79±0.25 (82)
	Effluent pH	7.36±0.22 (82)	6.95±0.16 (82)	7.18±0.25 (82)
	Effluent Cu(II) (mg/L)	0.01±0.01 (82)	0.03±0.01 (82)	0.05±0.05 (82)
	Initial soluble COD (mg/L)	862.68±41.48 (82)	949.76±58.61 (82)	981.22±62.26 (82)
	Effluent soluble COD (mg/L)	49.76±18.14 (82)	370.00±185.07 (82)	508.41±166.31 (82)
	COD removal (%)	94.19±2.23 (82)	60.61±20.46 (82)	47.78±17.75 (82)
	Initial alkalinity (mg CaCO ₃ /L)	67.09±9.06 (82)	828.29±31.52 (82)	843.90±30.91 (82)
	Effluent alkalinity (mg CaCO ₃ /L)	59.66±8.33 (82)	796.34±32.67 (82)	818.54±32.94 (82)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

contains a large fraction of lipids, the hydrolysis, as well as the metabolism of lipids, can be a limiting step in the removal of organic matter (Yan et al., 2013). The poor COD removal for reactor R_r has also been caused by solids in the feed, which has resulted in replacement of aerobic sludge biomass with feed solids during sludge wastage. The COD removal efficiency has gradually increased over the time for PWW fed reactors. The average COD removal has been observed as 94.2%, 60.6%, and 47.8% for reactors R_c, R_s, and R_r, respectively, as presented in Table 5.3.

The variations in alkalinity during the stressed phase I operation for all the reactors have been shown in Fig. 5.5-5.7 and data has been summarized in Table 5.3. The average initial alkalinity has been observed as 67.09±9.06, 828.29±31.52, and 843.90±30.91 mg/L as CaCO₃; while average effluent alkalinity has been observed as 59.66±8.33, 796.34±32.67, and 818.54±32.94 mg/L as CaCO₃ for reactors R_c, R_s, and R_r, respectively. The alkalinity for PWW fed reactors (R_s and R_r) have been observed much higher than reactor R_c due to addition of PWW which contains a portion of feces and urine.

The COD removal has appeared to be stabilized after 32-35 d of operation during stressed phase I operation and thereafter has started yielding steady performance. Summary of environmental and performance related parameters for reactors operated during steady-state (35-41 d) of stressed phase I operation are presented in Table 5.4. The data suggests minimum variation in reactors performance. The initial and effluent pH for reactors have been observed in the range of 6.51–7.16. During the steady-state condition, the average COD removal for reactors R_c, R_s, and R_r have been observed as 94.8%, 75.7%, and 65.4%, respectively. The variation in initial and effluent alkalinity for reactor R_c has been observed as 67.86±10.51 and 58.86±6.95 mg/L as CaCO₃, respectively, while for reactors R_s, and R_r have been observed in the range of 780.00–837.14 mg/L as CaCO₃ (Table 5.4), which are on higher side.

Table 5.4 Summary of environmental and performance related parameters for reactors operated during 35-41 d of steady-state condition of stressed phase I operation with PWW feed.

Parameters	Day-wise	35-41		
	Reactor	R _c	R _s	R _r
	Lactogen 3 (g/L)	0.90	-	-
	PWW (mL)	0	~500-750	~500-750
Stressed phase I	Environmental			
	Room temperature (°C) at 9.30 am	27.3±2.2 (7)	27.3±2.2 (7)	27.3±2.2 (7)
	Room temperature (°C) at 9.00 pm	22.7±0.8 (7)	22.7±0.8 (7)	22.7±0.8 (7)
	Initial pH	7.11±0.22 (14)	6.51±0.12 (14)	6.65±0.34 (14)
	Initial Cu(II) (mg/L)	0.03±0.01 (14)	0.81±0.11 (14)	0.85±0.32 (14)
	Effluent pH	7.16±0.11 (14)	6.93±0.22 (14)	6.99±0.22 (14)
	Effluent Cu(II) (mg/L)	0.01±0.00 (14)	0.03±0.01 (14)	0.08±0.01 (14)
	Performance			
	Initial soluble COD (mg/L)	885.71±48.50 (14)	995.71±49.01 (14)	1011.43±44.86 (14)
	Effluent soluble COD (mg/L)	45.71±17.42 (14)	241.43±22.12 (14)	348.57±39.25 (14)
	COD removal (%)	94.84±1.96 (14)	75.70±2.61 (14)	65.41±4.65 (14)
	Initial alkalinity (mg CaCO ₃ /L)	67.86±10.51 (14)	818.57±26.05 (14)	837.14±27.10 (14)
	Effluent alkalinity (mg CaCO ₃ /L)	58.86±6.95 (14)	780.00±24.32 (14)	807.14±28.25 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

5.3.1.2 Biomass related general parameters

The stress imparted by PWW on aerobic sludge biomass characteristics have been assessed grossly through MLSS, MLVSS, ISV, SVI, and ASBA tests.

Aerobic sludge biomass characteristics

The solids characteristics have been shown in Fig. 5.8 and data is summarized in Table 5.5 during stressed phase I operations (from 0 to 41 d). The zig-zag pattern has been observed due to wastage of excess sludge every week to maintained ~3000 mg/L of mixed liquor concentration in each of the reactors. During stressed phase I operation, reactor R_c biomass has shown continuous growth [Fig. 5.8(a)] with a gradual increase in SVI due to abundance of filamentous biomass in the reactor. While PWW fed reactor R_s has shown slow growth during stressed phase I operation, as shown in Fig. 5.8(b). The addition of PWW has slowed down biomass growth in reactor R_s biomass as shown in Fig. 5.8(b) compared to reactor R_c biomass [Fig. 5.8(a)]. The MLSS concentration in reactor R_c has increased from 3162 mg/L (on 1st d of operation) to 3653 mg/L (on 8th d) in a week and has yielded almost same growth throughout the operation. Similarly, reactor R_s biomass has shown slow increase in biomass growth in a week (from 3053 mg/L to 3323 mg/L) [Fig. 5.8(b)]. On the other hand, the reactor fed with raw PWW (R_r) have shown increase in MLSS concentration (from 3082 to 5689 mg/L in a week) as compared to reactor R_c biomass [Fig. 5.8(c)]. The raw PWW has contained high concentration of solids (comprised of unconsumed food and feces), which might be an attributing factor in increased MLSS concentration for reactor R_r biomass. Initially, a sudden change in feed composition has disturbed the ambient environment unfavorable for microbial growth resulting in slow growth, while towards the end of the stressed phase I operation, aerobic sludge biomass of reactor R_s has adapted on the PWW and has started growing. In the case of reactor R_r biomass, it has been fed with raw PWW, which consisted of suspended

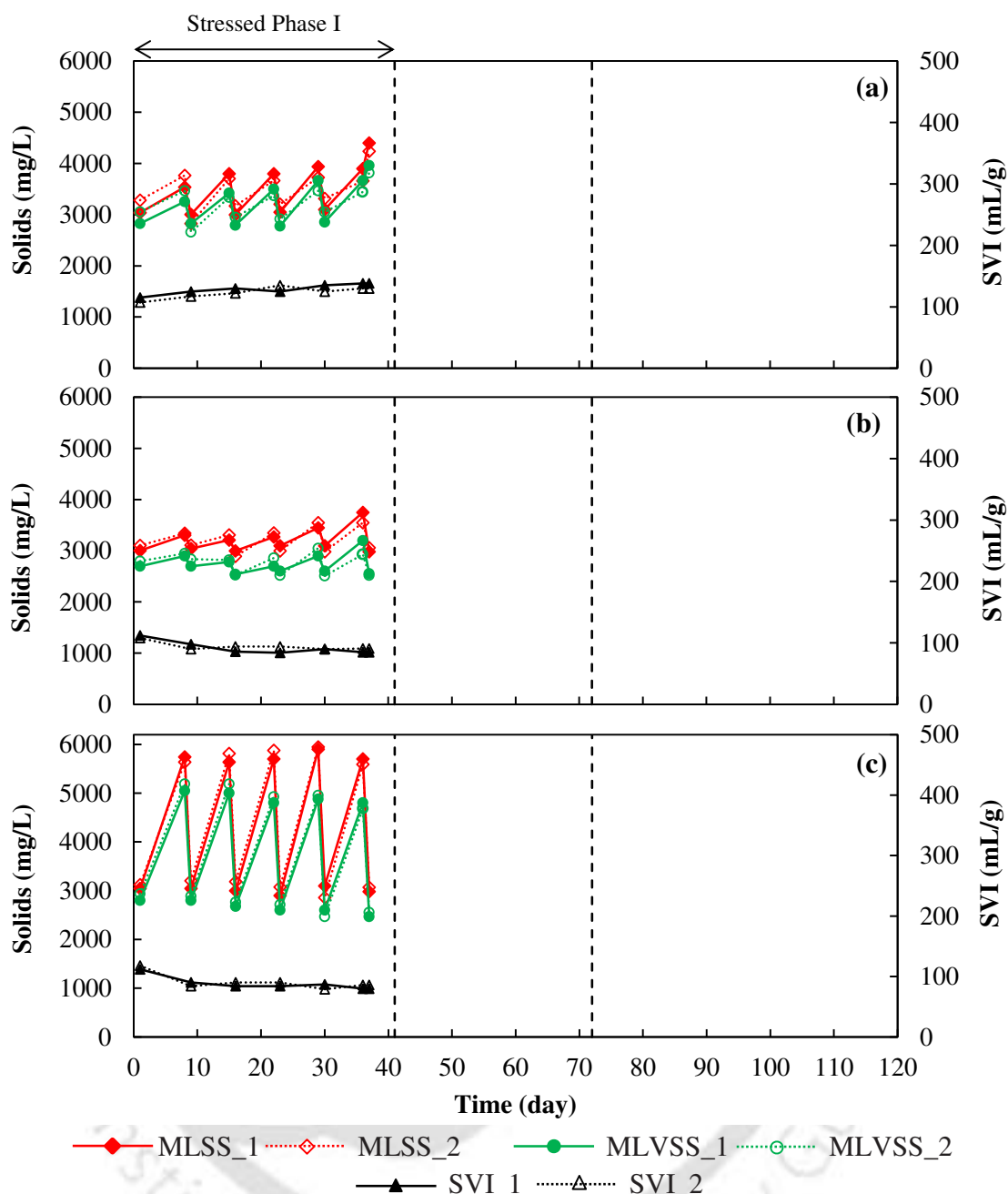


Fig. 5.8 Variation in MLSS, MLVSS, and SVI values for reactors (a) R_c , (b) R_s , and (c) R_r during stressed phase I operation with PWW feed.

solids resulting in increased solids concentration into the reactor. The MLVSS/MLSS ratio has ranged between 0.86-0.92 for the reactors during stressed phase I operation (Table 5.5). The SRT for reactors R_c , R_s , and R_r has been observed in the range of 7-35 d during stressed phase I operation.

The SVI of aerobic sludge biomass for all reactors have been shown in Fig. 5.8 and data has been summarized in Table 5.5. The SVI on 1st, 16th, and 30th d of operation for reactor R_c biomass has been observed as 111, 126, and 130 mL/g, respectively (Fig. 5.8), while the same has been obtained as 110, 90, and 90 mL/g for reactor R_s biomass; 115, 87, and 83 mL/g for reactor R_r biomass, respectively. The addition of PWW to the reactors have disintegrated the flocs; hence the SVI for reactors have significantly decreased in the initial

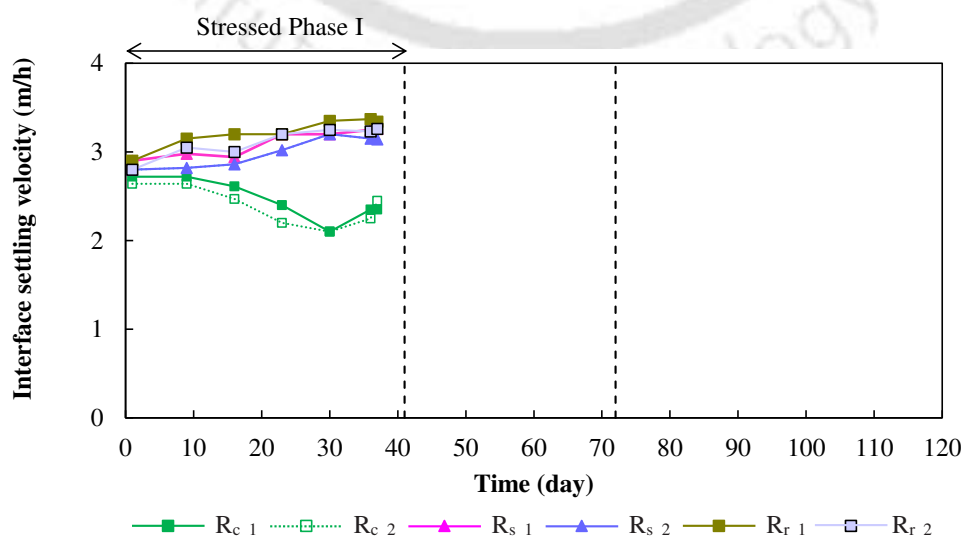
Table 5.5 Summary of solids characteristics estimated during stressed phase I operation with PWW feed.

Gross parameters	Stressed phase I operation			
	Day	0-41		
	Reactor	R _c	R _s	R _r
MLSS (mg/L)		3370.86±505.80 (14)	3119.00±242.46 (14)	3419.14±985.42 (14)
MLVSS (mg/L)		3112.43±439.47 (14)	2680.91±206.53 (14)	2981.89±789.23 (14)
MLVSS/MLSS ratio		0.92±0.02 (14)	0.86±0.03 (14)	0.88±0.04 (14)
SVI (mL/g)		126.57±8.93 (14)	92.71±8.35 (14)	89.29±11.92 (14)
ISV (m/h)		2.43±0.22 (14)	3.05±0.16 (14)	3.16±0.17 (14)

[The data is presented in $xx \pm yy$ (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

day of stressed phase I operation. The average SVI for reactor R_c, R_s, and R_r biomasses have been observed as 126.57 ± 8.93 , 92.71 ± 8.35 , and 89.29 ± 11.92 mL/g, respectively, during stressed phase I operation (Table 5.5).

The interface settling velocity (ISV) of reactors operated during stressed phase I operation has been shown in Fig. 5.9 and data has been presented in Table 5.5. On the 1st, 23rd, and 37th d of stressed phase I operation, the ISV for reactor R_c biomass has been observed as 2.84, 2.30, and 2.40 m/h, respectively (Fig. 5.9). The reactor R_c biomass has shown a decrease in ISV due to uncontrolled growth of filamentous microorganisms. However, ISV has slightly increased for reactors biomasses fed with PWW (R_s and R_r). On the 1st, 23rd, and 37th d of stressed phase I operation, the ISV for reactor R_s biomass has been observed as 2.85, 3.11, and 3.20 m/h, respectively, while 2.85, 3.20, 3.30 m/h of ISV has been observed for reactor R_r biomass. The loose flocs have been converted to small pin-point flocs resulting in slight improvement in ISV. Suspended solids present in raw PWW have also contributed in improved settling velocity. The average ISV for reactor R_c, R_s, and R_r biomasses have been observed as 2.43 ± 0.22 , 3.05 ± 0.16 , and 3.16 ± 0.17 m/h, respectively, during stressed phase I operation (Table 5.5).

**Fig. 5.9** Variation in interface settling velocities for reactor biomasses during stressed phase I operation with PWW feed.

Aerobic sludge biomass activity test

Aerobic sludge biomass activity (ASBA) test has been carried out towards the end of the stressed phase I operation (on 41st d), when reactors have achieved the steady-state condition. The results have been shown in Fig. 5.10 and data has been represented in Table 5.6. ASBA test is a key test that evaluates microbe metabolic ability. ASBA for reactor R_c, R_s and R_r biomasses have been obtained as 7.03, 5.87, and 5.05 mg COD removed/mg VSS d, respectively, towards the end of stressed phase I operation (Fig. 5.10 and Table 5.6). The addition of PWW to the reactors have caused a change in the influent composition and pH of the reactor. The change in feed composition created an unfavorable condition for microbial growth and activity. It is also reported that change in pH can also affect microbial activity (Yan et al., 2013). Each of the microorganisms has an optimum range of pH in which both

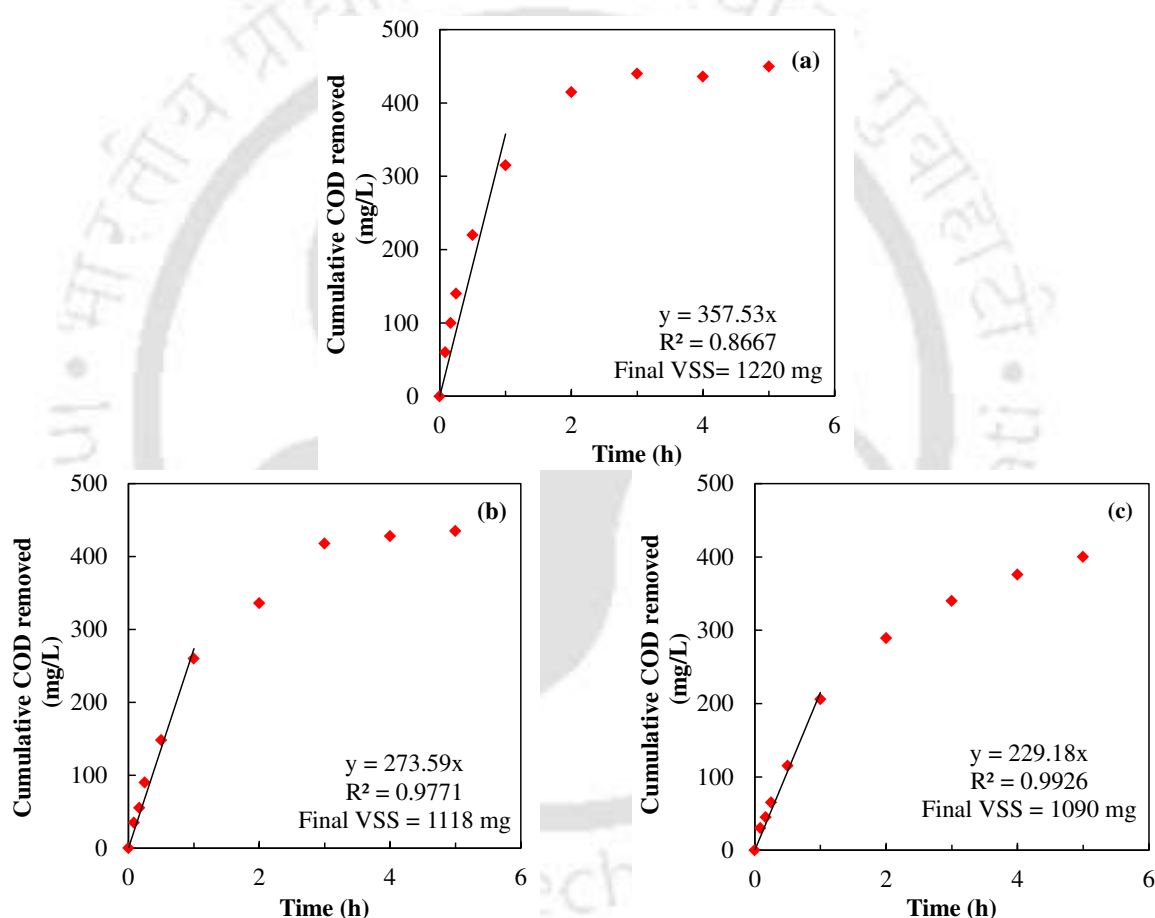


Fig. 5.10 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactors (a) R_c, (b) R_s, and (c) R_r on 41st d of stressed phase I operation with PWW feed.

Table 5.6 Aerobic sludge biomass activity test results carried out towards the end of stressed phase I operation with PWW feed.

Stressed phase I operation			
Biomass withdrawn on	41 st d		
Reactor	R _c	R _s	R _r
ASBA (mg COD removed/mg VSS d)	7.03	5.87	5.05

the specific growth rate and substrate utilization rate reach a maximum. This could be the possible reason for decreased ASBA under stressed phase I operation.

Re-looking at biomass related general parameters viz. MLSS, MLVSS, ISV, SVI and ASBA obtained during stressed phase I operation; it appears that the average values of MLSS and MLVSS do not reflect the impact of imposed stresses as the ratio of MLVSS/MLSS varies in a narrow range of 0.86 to 0.92. However, the average values of SVI have decreased while average values of ISV have increased for reactor R_s and R_r biomasses in comparison to control reactor R_c biomass. Apparently, decreased SVI and increased ISV indicate improved settling properties of sludge biomasses from reactors R_s and R_r . But the estimated ASBA values for reactor R_s and R_r biomasses are 5.87 and 5.05 mg COD removed/mg VSS d, respectively, which is on a lower side compared to 7.03 mg COD removed/mg VSS d for control reactor R_c biomass. It has indicated deterioration in bio-degradation capability of organics by reactor R_s and R_r biomasses under stressed phase I operation. The reduced COD removals of ~76% and 65% by reactor R_s and R_r biomasses, respectively, in comparison to around 95% by control reactor R_c biomass have clearly indicated deterioration in bio-degradation capability of reactor biomasses during steady state condition under stressed phase I operation, which has to be visualized as an undesirable situation for efficient organics degradation by the reactor biomasses — considered the primary objective of biomasses. Therefore, taking into consideration SVI, ISV and ASBA parameters together, and rather than attributing the decreased SVI and increased ISV of biomasses to improvement in settling properties, it may indirectly be taken as an indication for deterioration in bio-degradation capability of biomasses as observed with decreased values of ASBA under stressed phase I operation.

5.3.1.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

The flocs structure and morphology of the sludge have been examined through microscopic observation. The images of aerobic sludge biomass samples have been taken at 5× and 100× magnifications after being stained (Gram's staining), and the result has been presented in [Table 5.7](#). The changes in biomass morphology during stressed phase I operation have also been observed through the naked eye (photographic observation) as shown in [Table 5.8](#). The aerobic sludge biomass has been stained and observed under a fluorescence microscope for cell viability, and results have been presented in [Table 5.9](#). The filament index (FI) has also been estimated during stressed phase I operation, and data has been summarized in [Table 5.10](#).

The microscopic observation during stressed phase I operation have been shown in [Table 5.7](#). The reactor R_c biomass has exhibited continuous increase in size and filament abundance has been observed (from 1st to 36th d of operation), whereas disintegrated flocs structure has been observed for PWW fed reactors, during stressed phase I operation. The microscopic images taken at 100× magnification suggest that filaments have always

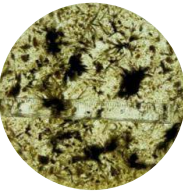
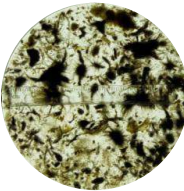
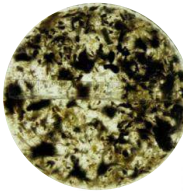
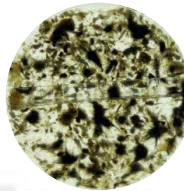
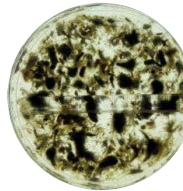
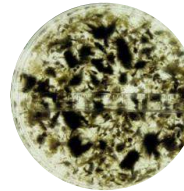
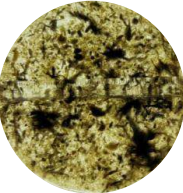
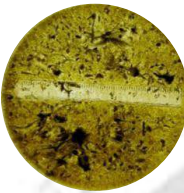
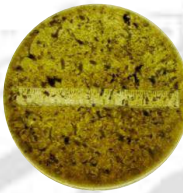
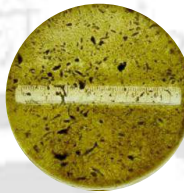
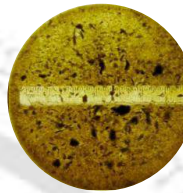
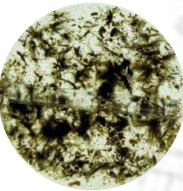
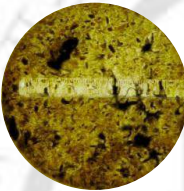
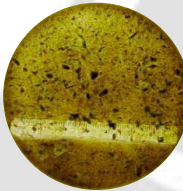
Table 5.7 Microscopic examination of Gram’s stained aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.

Reactor	Mag.	Aerobic sludge biomass samples withdrawn on					
		Stressed phase I operation					
		1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d
R_c	5×						
	100×						
R_s	5×						
	100×						
R_r	5×						
	100×						

been present in reactor R_c biomass (from 1st to 36th d of operation). Images taken at 5× magnification for reactor R_s biomass suggest that flocs disintegration has started after 8th d of operation and completely disintegrated towards the end of the stressed phase I operation (on 36th d of operation). While, images at 100× magnification of reactor R_s biomass suggest that density of filaments have reduced after 8th d. Similar observations have also been observed for reactor R_r biomass (Table 5.7). Addition of PWW to reactors R_s and R_r have damaged filamentous biomass and formed a net-like structure. It can be said that the addition of PWW has changed the flocs morphology of aerobic sludge biomass resulting in disintegrated and pinpoint flocs.

The results of photographic observations during stressed phase I operation has been presented in Table 5.8. The floc size has been measured using a transparent plastic scale with minimum division of 1.0 mm placed in the Petri dish. On the 1st d of operation, the flocs size of reactor R_c , R_s , and R_r biomasses have ranged between 1.0-12.0 mm (Table 5.8). The flocs size remained approximately same for reactor R_c biomass, while a decrease in flocs size have been observed due to addition of PWW to reactor R_s and R_r biomasses. The aerobic sludge biomass in reactors R_s and R_r have disintegrated and converted into smaller flocs on 8th d as compared to 1st d of operation. The disintegration of flocs has resulted in damaged filaments with grains like structure (node connected to filaments). The possible

Table 5.8 Photographic images of aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.

Reactors	Aerobic sludge biomass samples withdrawn on					
	Stressed phase I operation					
	1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d
R_c						
R_s						
R_r						

reason for disintegration could be unfavorable condition of microorganisms provided by PWW composition and pH. The flocs size for reactor R_s and R_r biomasses have further decreased from 8th d and remained the same till the end of the stressed phase I operation (till 41th d) as shown in Table 5.8.

Fluorescence microscopic images distinguish the live and dead portion of aerobic sludge biomass as presented in Table 5.9. The analysis has been carried out on the 1st d and 41st d of operation. The bright-field images show the overall flocs structure, green color represents the active cell and red color shows the inactive cells of aerobic sludge biomass. On the 1st d of operation, all reactor biomasses have shown more active portion of aerobic sludge biomass (Table 5.9). The % live and % dead cells have been estimated using ImageJ software. On the 1st d of operation, live cells for reactor R_c , R_s , and R_r biomasses have been estimated as 94.1%, 92.5%, and 96.2%, respectively. On the other hand, dead cells for reactor R_c , R_s , and R_r biomasses have been estimated as 0.2%, 1.2%, and 1.1%, respectively. However, towards the end of the stressed phase I operation (41st d), reactor R_s biomass has shown slight decrease in active portion and corresponding increase in inactive portion of aerobic sludge biomass. Similarly, reactor R_r biomass has exhibited significant red color portion as compared to reactor R_c and R_s biomasses. On the 41st d of operation, live cells for reactor R_c , R_s , and R_r biomasses have been estimated as 94.1%, 74.3%, and 67.2%, respectively. On the other hand, dead cells for reactor R_c , R_s , and R_r biomasses have been estimated as 1.7%, 21.0%, and 26.4%, respectively. This result suggests that continued addition of PWW till 41st d has adversely affected the microbial cells (due to composition of PWW or pH); hence, the

Table 5.9 Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactor R_c , R_s , and R_r during stressed phase I operation with PWW feed.

Reactors	Aerobic sludge biomass samples withdrawn on					
	Stressed phase I operation					
	1 st d			41 st d		
	Bright field (20×)	Live cells (20×)	Dead cells (20×)	Bright field (20×)	Live cells (20×)	Dead cells (20×)
R_c						
R_s						
R_r						

active portion has decreased as compared to the 1st d of operation.

FI during stressed phase I operation has been presented in Table 5.10, which exhibit the abundance of filaments in reactor biomasses. Reactor R_c , R_s , and R_r biomasses have shown abundance of filamentous microorganisms, hence the FI for reactor biomasses have been observed as 5 on 1st d of the operation. The reactor R_c biomass has shown FI of 5 for 0-41 d of operation. In addition, on 8th d, FI for reactor R_s and R_r biomasses have been observed as 5 and 4, respectively. FI for reactor R_s and R_r biomasses have decreased over the time during stressed phase I operation and has reached to 3 and 2, respectively, on 36th d of operation. The FI and microscopic observations suggest that addition of PWW has adverse impact on filamentous biomass.

Table 5.10 Filament index of aerobic sludge biomass estimated during stressed phase I operation with PWW feed.

Biomass withdrawn on	Stressed phase I operation			
Day	R _c	R _s	R _r	
1 st d	5	5	5	
8 th d	5	5	4	
15 th d	5	4	4	
22 nd d	5	4	3	
29 th d	5	4	3	
36 th d	5	3	2	

Polysaccharide, protein and extracellular polymeric substance

Polysaccharide (PS), protein (PN) and extracellular polymeric substance (EPS) are secreted by microbial cells (Zhou et al., 2016), which is involved in agglomeration of cells (flocs formation) and also protect microbial cells from toxic effects of pollutants (Sheng et al., 2013). PS, PN, and EPS contents in aerobic sludge biomass have been estimated on 1st and 41st d of stressed phase I operation. The results have been shown in Fig. 5.11 and data

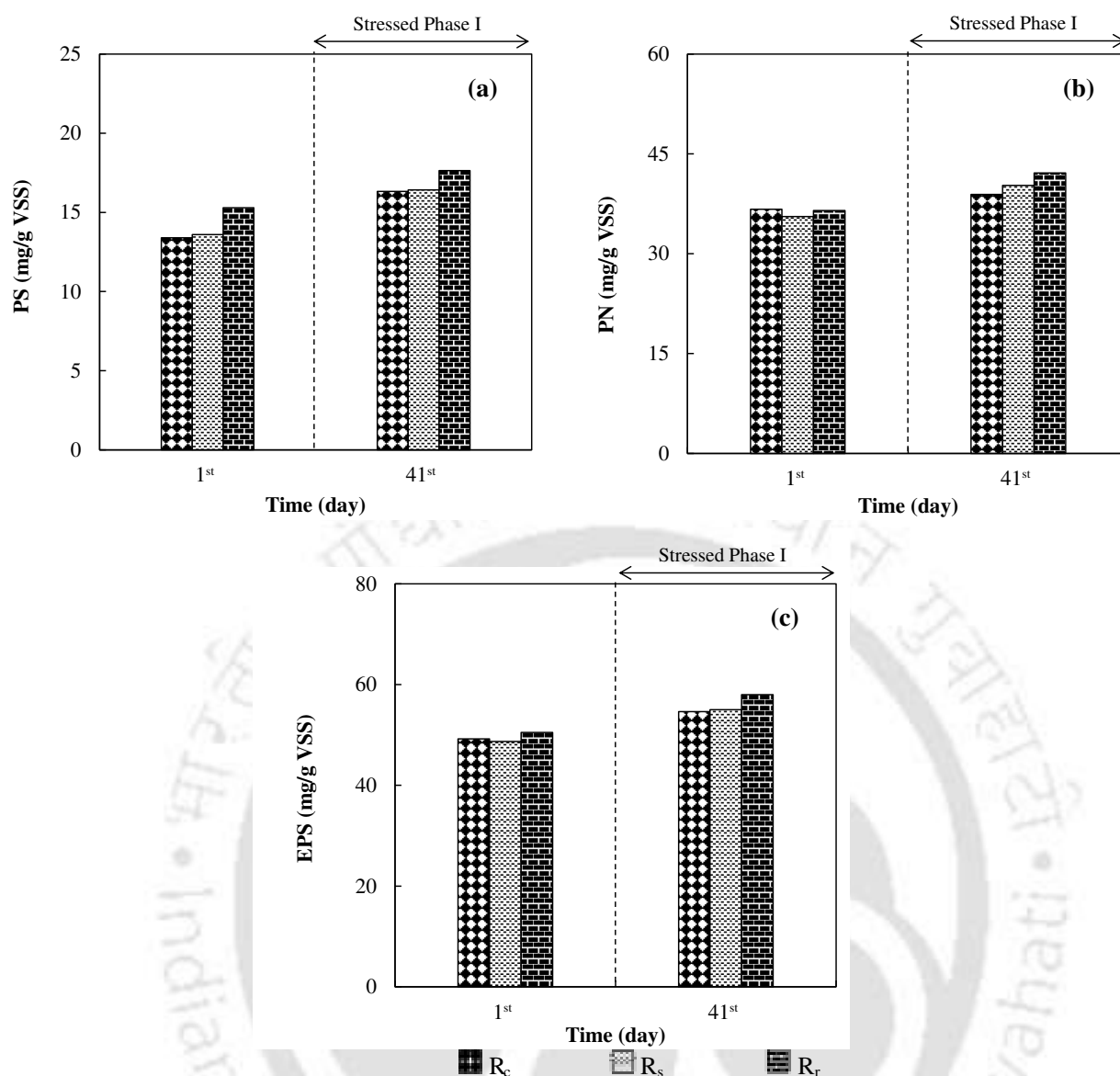


Fig. 5.11 Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase I operation with PWW feed.

Table 5.11 PS, PN and EPS contents in aerobic sludge biomass operated under stressed phase I operation with PWW feed.

Biomass withdrawn on	Reactor biomass	PS	PN (mg/g VSS)	EPS
Stressed phase I operation				
1 st d	R_c	13.42±1.51	36.71±3.04	49.22±4.18
	R_s	13.60±1.22	35.62±2.31	48.71±3.27
	R_r	15.31±1.10	36.50±3.21	50.51±2.88
41 st d	R_c	16.32±1.42	38.92±2.40	54.62±4.64
	R_s	16.42±1.62	40.26±1.42	55.02±3.42
	R_r	17.64±1.32	42.14±2.32	58.02±3.20

[The data is presented in $xx \pm yy$ format where xx is the average value, yy is the standard deviation.]

has been presented in Table 5.11. On the 1st d of operation, PS content in reactor R_c, R_s, and R_r biomasses have been observed as 13.42±1.51, 13.60±1.22, and 15.31±1.10 mg/g VSS, respectively [Fig. 5.11(a)], which has slightly increased to 16.32±1.42, 16.42±1.62, and 17.64±1.32 mg/g VSS, respectively, on 41st d of operation. The suspended solids present in raw PWW might have contributed slightly higher PS content in reactor R_r biomass. Similarly, PN content in reactor R_c, R_s, and R_r biomasses have been observed as 36.71±3.04, 35.62±2.31, and 36.50±3.21 mg/g VSS, respectively [Fig. 5.11(b)], on the 1st d of operation, which has increased to 38.92±2.40, 40.26±1.42, and 42.14±2.32 mg/g VSS, respectively, on 41st d of operation. The EPS content in reactor R_c, R_s, and R_r biomasses have also increased from 49.22±4.18, 48.71±3.27, and 50.51±2.88 mg/g VSS to 54.62±4.64, 55.02±3.42, and 58.02±3.20 mg/g VSS, respectively [Fig. 5.11(c)], from 1st d to 41st d of stressed phase I operation (Table 5.11).

Enzyme activity

The bio-degradation of organic matter is associated with many biochemical reactions, which are catalyzed by key enzymes. In this study, four key enzyme activities have been estimated during the stressed phase I operation. The result has been shown in Fig. 5.12 and data has been presented in Table 5.12. On the 1st d of operation, catalase activity (E_{Catalase}) for reactor R_c, R_s, and R_r biomasses have been observed as 0.35, 0.36, and 0.36 mg/g MLSS h, respectively [Fig. 5.12(a)], while on 41st d, 0.41, 0.27, and 0.21 mg/g MLSS h of E_{Catalase} has been observed. A slight decrease in E_{Catalase} has been observed due to addition of PWW as feed. The addition of PWW has shown % inhibition of 34.6% and 49.1% for reactor R_s and R_r biomasses, respectively, as compared to reactor R_c biomass on 41st d of operation.

The dehydrogenase activity ($E_{\text{Dehydrogenase}}$) has been shown in Fig. 5.12(b) and data is presented in Table 5.12. On the 1st d of operation, $E_{\text{Dehydrogenase}}$ for reactor R_c, R_s, and R_r biomasses have been observed as 25.67, 25.87, and 25.11 µg/g MLSS h, respectively, and it has further decreased to 27.87, 21.87, and 20.76 µg/g MLSS h on 41st d due to continuous addition of PWW to reactors excluding reactor R_c. The % inhibition has been estimated as 21.5%, and 25.5% for reactor R_s and R_r biomasses, respectively.

The protease activity (E_{Protease}) has been shown in Fig. 5.12(c) and data is presented in Table 5.12. E_{Protease} for reactor R_c, R_s, and R_r biomasses have been observed as 1.87, 1.78, and 1.87 mg/g MLSS h on the 1st d of operation; while 2.21, 1.53, and 1.21 mg/g MLSS h, respectively, on 41st d of operation. The % inhibition has been obtained as 30.8% and 45.2% for PWW fed reactor R_s and R_r biomasses, respectively. The results suggest that long term addition of PWW (0-41 d) could inhibit E_{Protease} .

The urease activity (E_{Urease}) has been shown in Fig. 5.12(d) and data is presented in Table 5.12. On the 1st d of operation, E_{Urease} for reactor R_c, R_s, and R_r biomasses have been observed as 14.23, 15.12, and 14.46 g/g MLSS h, while 16.23, 6.51, and 4.34 g/g MLSS h of E_{Urease} has been observed on 41st d of operation, respectively. E_{Urease} for reactor R_s biomass has less inhibited (10.9%) as compared to reactor R_r biomass (73.3%).

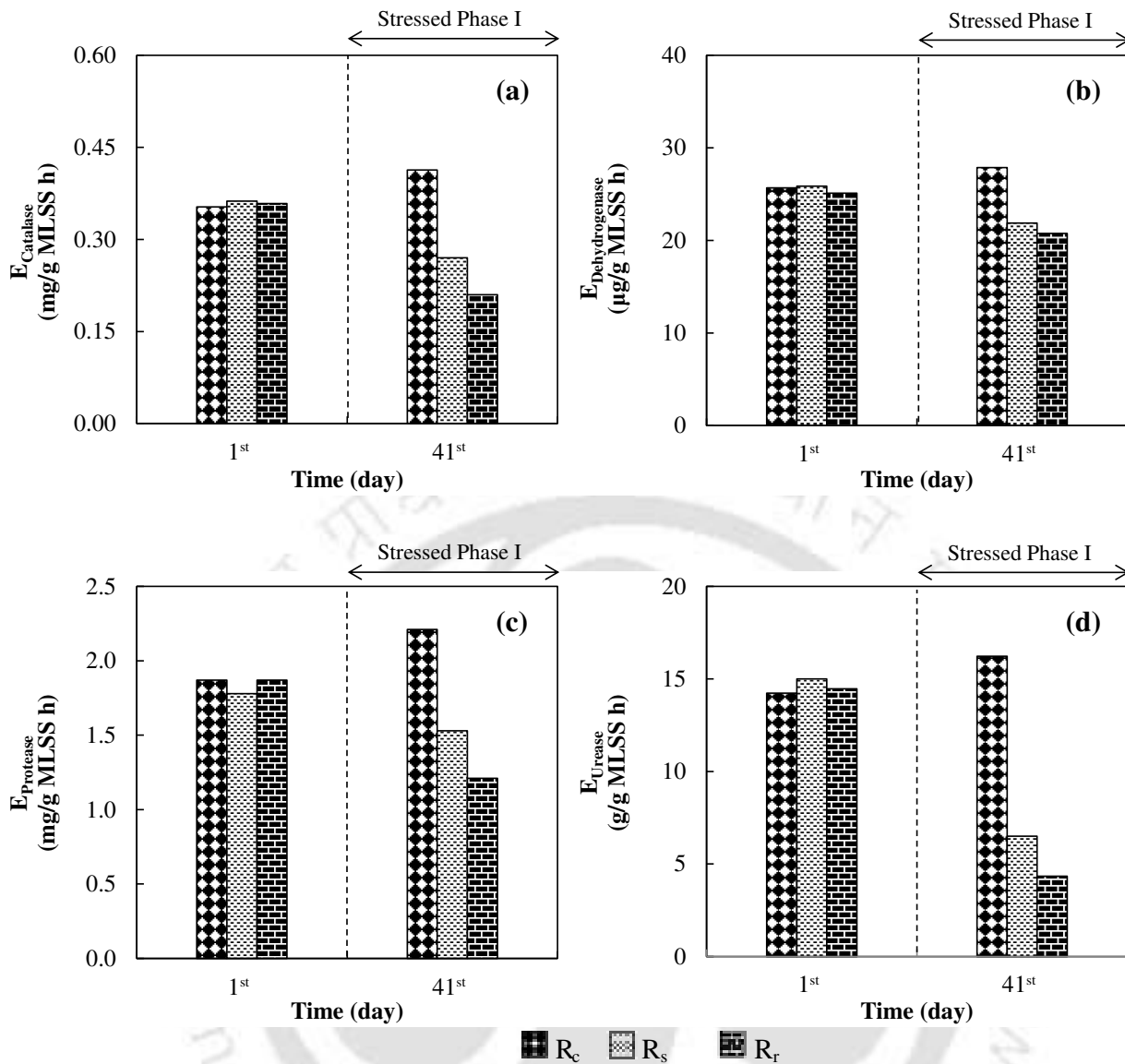


Fig. 5.12 Enzyme activities of aerobic sludge biomass (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} assessed during stressed phase I operation with PWW feed.

Table 5.12 Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase I operation with PWW feed.

Biomass withdrawn on	Reactor biomass	E_{Catalase} (mg/g MLSS h)	$E_{\text{Dehydrogenase}}$ (μg/g MLSS h)	E_{Protease} (mg/g MLSS h)	E_{Urease} (g/g MLSS h)
Stressed phase I operation					
1st d	R_c	0.35±0.03	25.67±1.88	1.87±0.17	14.23±1.10
	R_s	0.36±0.03	25.87±1.57	1.78±0.19	15.04±1.28
	R_r	0.36±0.02	25.11±2.14	1.87±0.12	14.46±1.17
41st d	R_c	0.41±0.02	27.87±2.23	2.21±0.12	16.23±0.92
	R_s	0.27±0.03	21.87±1.81	1.53±0.10	6.51±0.92
	R_r	0.21±0.03	20.76±1.72	1.21±0.12	4.34±1.11

[The data is presented in $xx \pm yy$ format where xx is the average value, yy is the standard deviation.]

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA has been performed with biomass samples withdrawn towards the end of the stressed phase I (on 41st d) operation to assess the possible changes in microbial community structure of aerobic sludge biomass due to PWW exposure. Fig. 5.13 shows the digestion pattern and dendrogram of aerobic biomass samples digested with *AluI*, *HhaI*, and *MspI* during stressed phase I operation. The dendrogram has been prepared using GelJ software, and a similarity table has been generated based on the gel image is presented in Table 5.13.

The results obtained after gel run (Fig. 5.13) implies that the reactor biomasses show different banding patterns for each restriction enzyme. The dendrogram has demonstrated the relationship among all the reactor biomasses. For reactor R_c , R_s , and R_r biomasses, the *AluI* restriction enzyme has exhibited 11, 9, and 9 bands with small differences in their banding positions, respectively. Similarly, *HhaI* has exhibited 10, 7, and 10 bands, while *MspI* has shown 9, 7, and 9 bands at different positions for reactor R_c , R_s , and R_r biomasses, respectively. Fig. 5.13(a) exhibits that the biomass from reactor R_s and R_r has very less difference in microbial compositions and are closest to reactor R_c biomass. Similarly, *HhaI* [Fig. 5.13(b)] and *MspI* [Fig. 5.13(c)] have also shown very less difference in microbial composition between two reactor R_s and R_r biomasses; and also has closest to reactor R_c biomass. A slight shift in the microbial community has been observed due to the long-term (0-41 d) addition of PWW to the reactors as compared to reactor R_c biomass. The reactor R_s

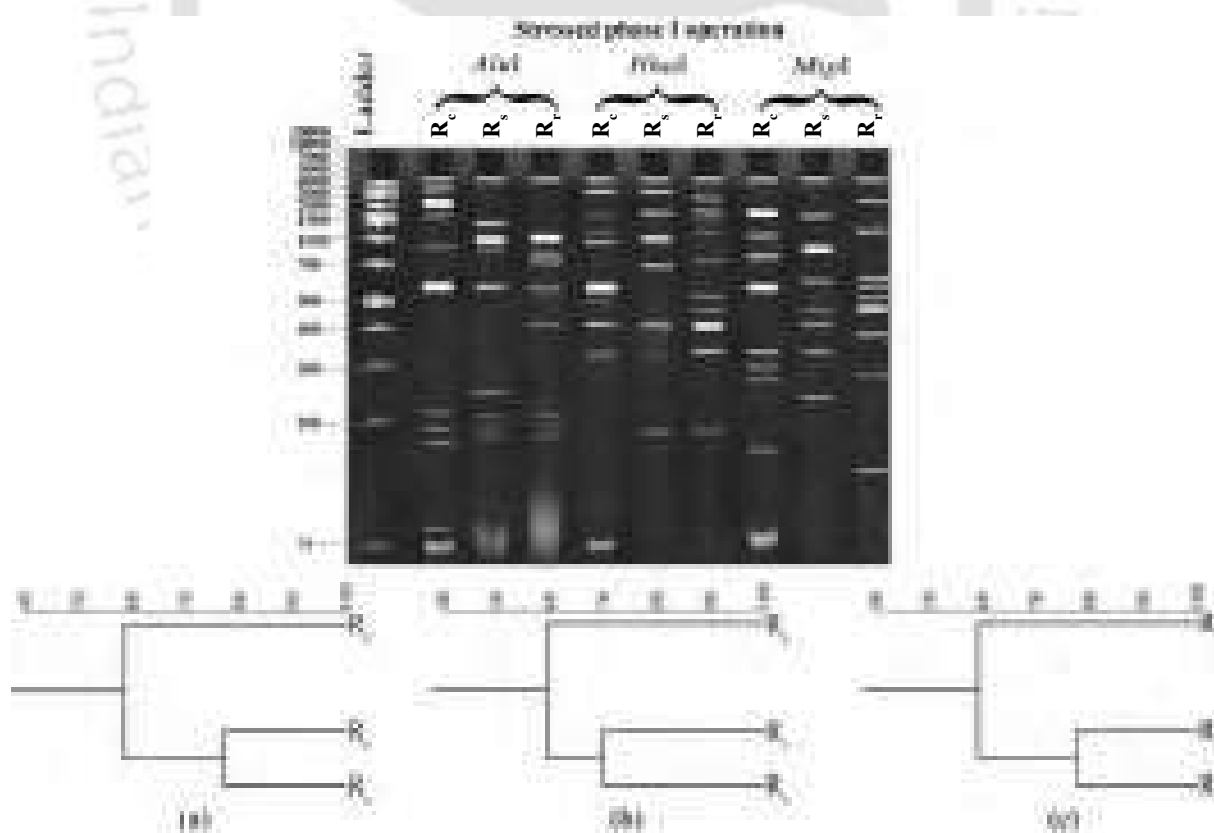


Fig. 5.13 Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase I operation with PWW feed treated with (a) *AluI*, (b) *HhaI*, and (c) *MspI* restriction enzymes.

Table 5.13 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase I operation with PWW feed.

Stressed phase I operation									
Reactor	<i>AluI</i>			<i>HhaI</i>			<i>MspI</i>		
	R _c	R _s	R _r	R _c	R _s	R _r	R _c	R _s	R _r
R _c	1	0.9223	0.8845	1	0.9003	0.8410	1	0.8912	0.8712
R _s	0.9223	1	0.9461	0.9003	1	0.9543	0.8912	1	0.9105
R _r	0.8845	0.9461	1	0.8410	0.9543	1	0.8712	0.9105	1

and R_r biomasses have exhibited similarity of 92.2% and 88.5% with *AluI* digestion; 90.0% and 84.1% with *HhaI* digestion; 89.1% and 87.1% with *MspI* digestion, as compared to reactor R_c biomass. The PWW is a complex substrate (containing lignin, cellulose, and fibers) that is not easily biodegradable by microbes. The long-term addition of PWW has depleted the microbial community that become unable to tolerate the PWW stress. It might be the probable reason for a slight change in microbial community structure.

The impacts of stress on sludge biomass morphology and its viability have been assessed through visual and microscopic examinations. The microscopic observations for reactor R_s and R_r biomasses have indicated severe adverse impact of imposed stresses leading to complete disintegration of flocs with heavy reduction in filament density after 8th d of stressed phase I operation, whereas presence of filaments with abundance throughout has been observed for the control reactor R_c biomass. The photographic observations have also indicated severe negative impact with disintegration of filamentous flocs into grains like structure for reactor R_s and R_r biomasses after 8th d of stressed phase I operation, while the floc sizes for the control reactor R_c biomass has ranged between 1.0-12.0 mm throughout. The FI has shown consistently decreasing values due to adverse impact of the imposed stresses for reactor R_s and R_r biomasses from an initial FI value of 5 to 3 and 2 towards the end of stressed phase I operation. The fluorescence microscopic observations carried out towards the end of stressed phase I operation for reactor R_c, R_s and R_r biomasses have indicated lower proportions of red color and higher proportion of green color (indicating presence of more active cells) for reactor R_c and R_s biomasses, while the reactor R_r biomass has shown a higher proportion of red color indicating presence of more inactive cells due to imposed stresses. The impact of stresses on sludge biomass has also been investigated using biochemical techniques. The assessed contents of PS, PN, and EPS contents in the biomass of all the three reactors, viz. control reactor R_c and stressed reactors R_s and R_r in the beginning as well as towards the end of stressed phase I operation have not shown any drastic variations, and therefore these biochemical parameters do not reflect the changes in biomass characteristics due to imposed stresses. The enzyme activities viz. E_{Catalase}, E_{Dehydrogenase}, and E_{Protease} have shown very sublime variations for control and stressed reactor biomasses (E_{Catalase} of 0.2-0.3 mg/g MLSS h for reactor R_s and R_r biomasses compared to 0.4 mg/g MLSS h for reactor R_c biomass; E_{Dehydrogenase} of 21-22 µg/g MLSS h for reactor R_s and R_r biomasses compared to 28 µg/g MLSS h for reactor R_c biomass; E_{Protease} of 1.2-1.5 mg/g MLSS h for reactor R_s and R_r

biomasses compared to 2.2 mg/g MLSS h for reactor R_c biomass) in its contents except E_{Urease} content which has shown drastic changes (E_{Urease} of 4.3-6.5 g/g MLSS h for reactor R_s and R_r biomasses compared to 16.2 g/g MLSS h for reactor R_c biomass) estimated towards the end of stressed phase I operation. Hence, the enzyme activity, E_{Urease} reflects the changes in biomass characteristics due to imposed stresses. The gel run of the amplified ribosomal DNA restriction analysis (ARDRA) carried out with three different restriction enzymes towards the end of stressed phase I operation has shown different banding patterns for stressed reactor R_s and R_r biomasses compared with the control reactor R_c biomass indicating changes in microbial communities in reactor biomasses. The dendrogram of stressed reactor R_s and R_r biomasses have shown close similarity (in the range of 84% to 92%) with each other when digested with the three restriction enzymes, but these have been definitely different from the control reactor R_c biomass indicating slight changes in microbial community structures in stressed reactor biomasses.

5.3.2 Stressed phase II operation with PWW+Cu(II)

After completing stressed phase I operation, reactor R_s and R_r biomasses have been evenly divided into reactors named as $R_{s\text{Cu}40}$, and $R_{s\text{Cu}80}$; $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$, respectively to be fed with 40 and 80 mg/L of Cu(II), respectively, in addition to PWW. Since the collected PWW did not have Cu(II) in higher concentration, hence, it has been added externally for this phase of the study and further operated for 31 d — termed as stressed phase II operation.

5.3.2.1 Environmental and performance parameters

Variation in environmental and performance-related parameters is shown in Fig. 5.14 to 5.18 and summary of data are presented in Table 5.14. Similar to the stressed phase I operation, reactors have been operated under ambient conditions throughout the operation; hence pH and reactor temperature have not been adjusted. The temperature variation has been shown in Fig. 5.14 and data has been summarized in Table 5.14. Since all the reactors have been operated at room temperature, variation in room temperature recorded at 9.30 am and 8.45 pm have been presented in Fig. 5.14.

Variations in the initial and effluent pH for reactors have been shown in Fig. 5.14 to 5.18. The average initial and effluent pH for reactor R_c during 42-72 d of operation has been observed as 7.24 ± 0.20 and 7.23 ± 0.23 , respectively. Whereas, the average initial pH has been observed as 6.13 ± 0.26 , 5.83 ± 0.27 , 5.81 ± 0.19 , and 5.42 ± 0.20 for reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$, respectively, while average effluent pH has been observed as 6.21 ± 0.34 , 6.06 ± 0.23 , 6.11 ± 0.21 , and 5.74 ± 0.18 for reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$, respectively (Table 5.14). The addition of Cu(II) along with PWW has slightly increased the reactor pH.

The variation in effluent Cu(II) and its retention have been shown in Fig. 5.15 to 5.18 and data has been summarized in Table 5.14. On the 42nd d of operation from the start, Cu(II) retention for reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ have been observed as 67.7%, 58.4%, 67.7%, 58.4%, 79.2%, and 67.3%, respectively. While 14.2%, 10.3%, 54.5%, and

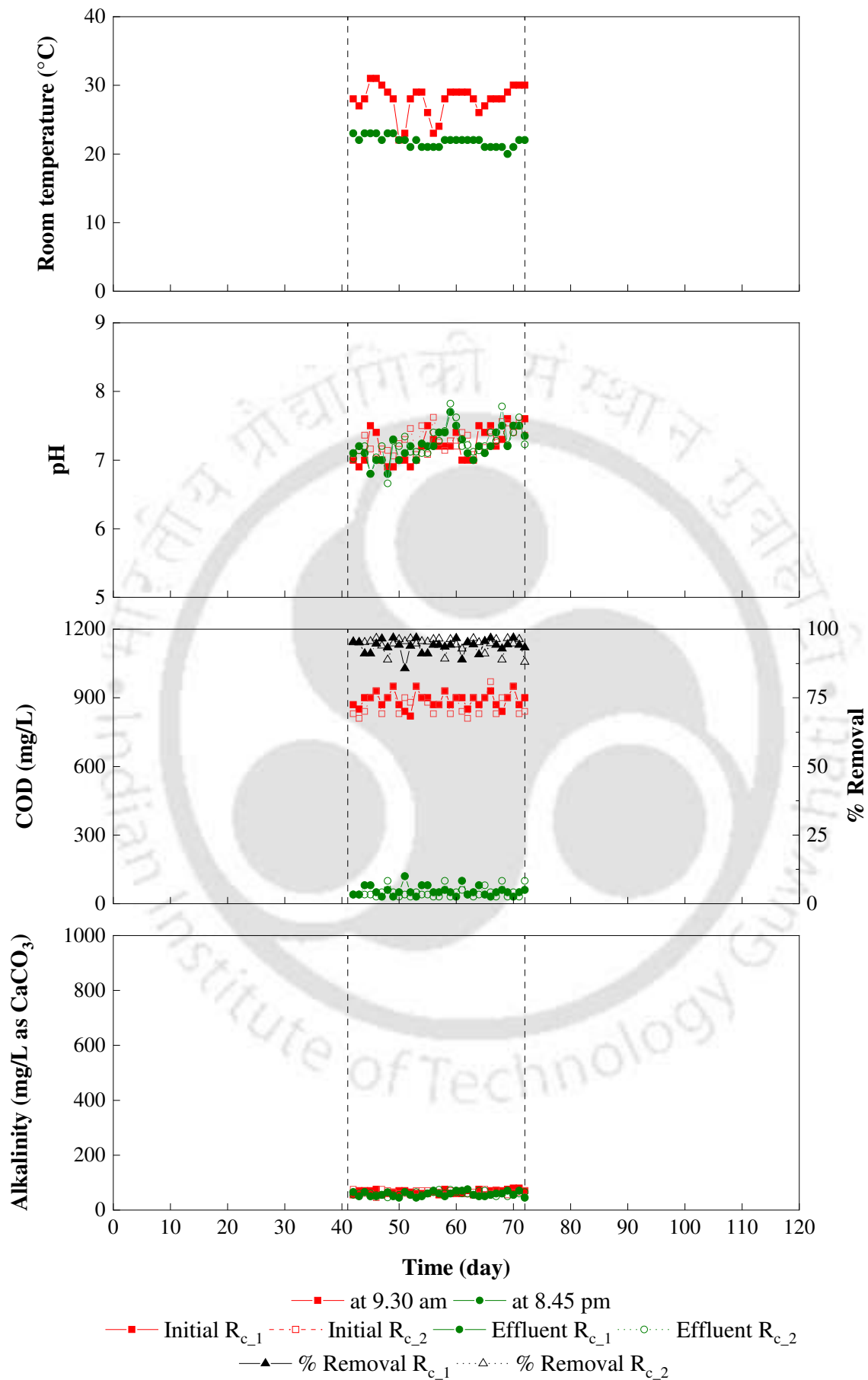


Fig. 5.14 Variation in environmental and performance related parameters for reactor R_c during 42-72 d of stressed phase II operation.

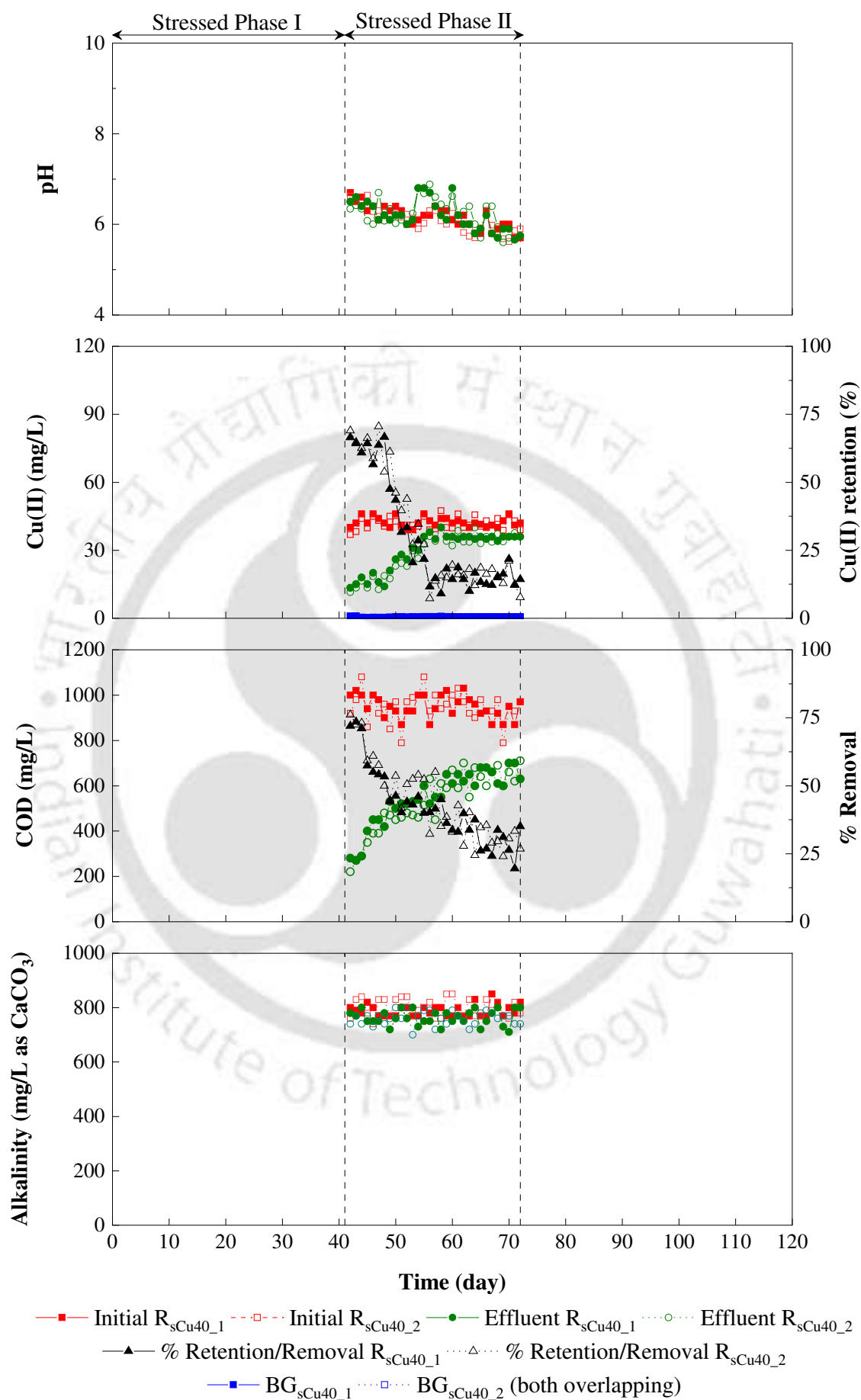


Fig. 5.15 Variation in environmental and performance related parameters for reactor R_{sCu40} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.

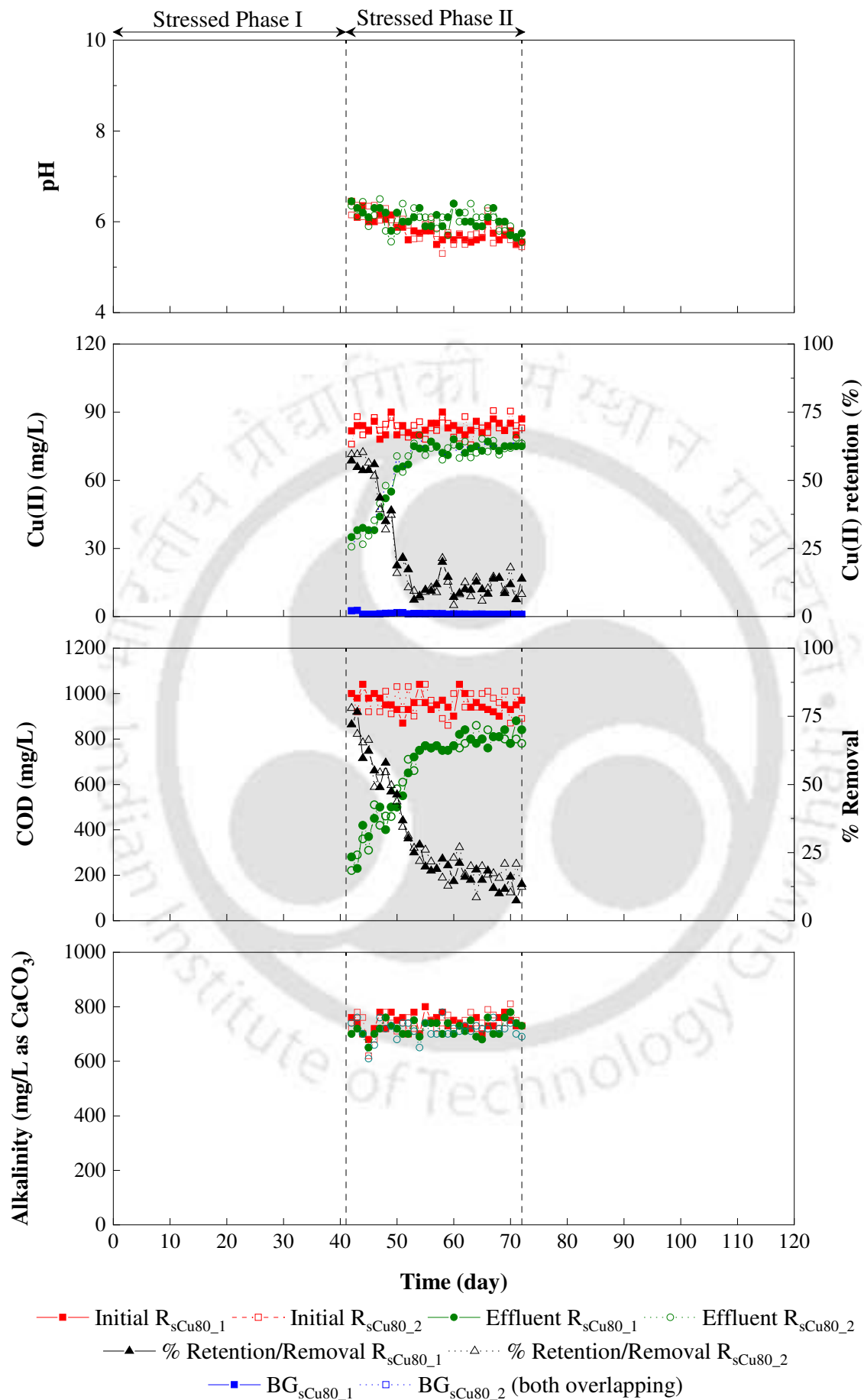


Fig. 5.16 Variation in environmental and performance related parameters for reactor R_{sCu80} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.

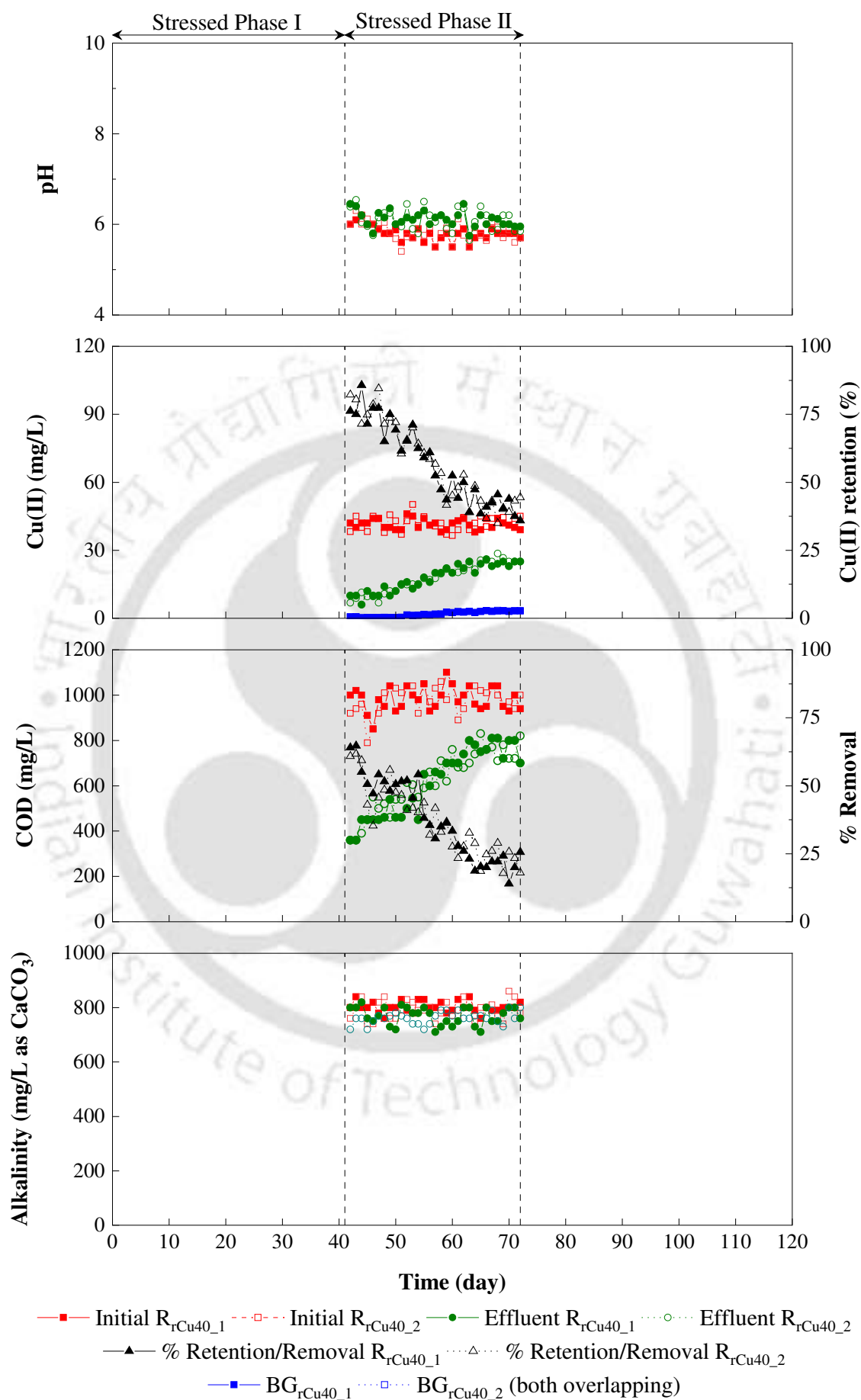


Fig. 5.17 Variation in environmental and performance related parameters for reactor R_{Cu40} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.

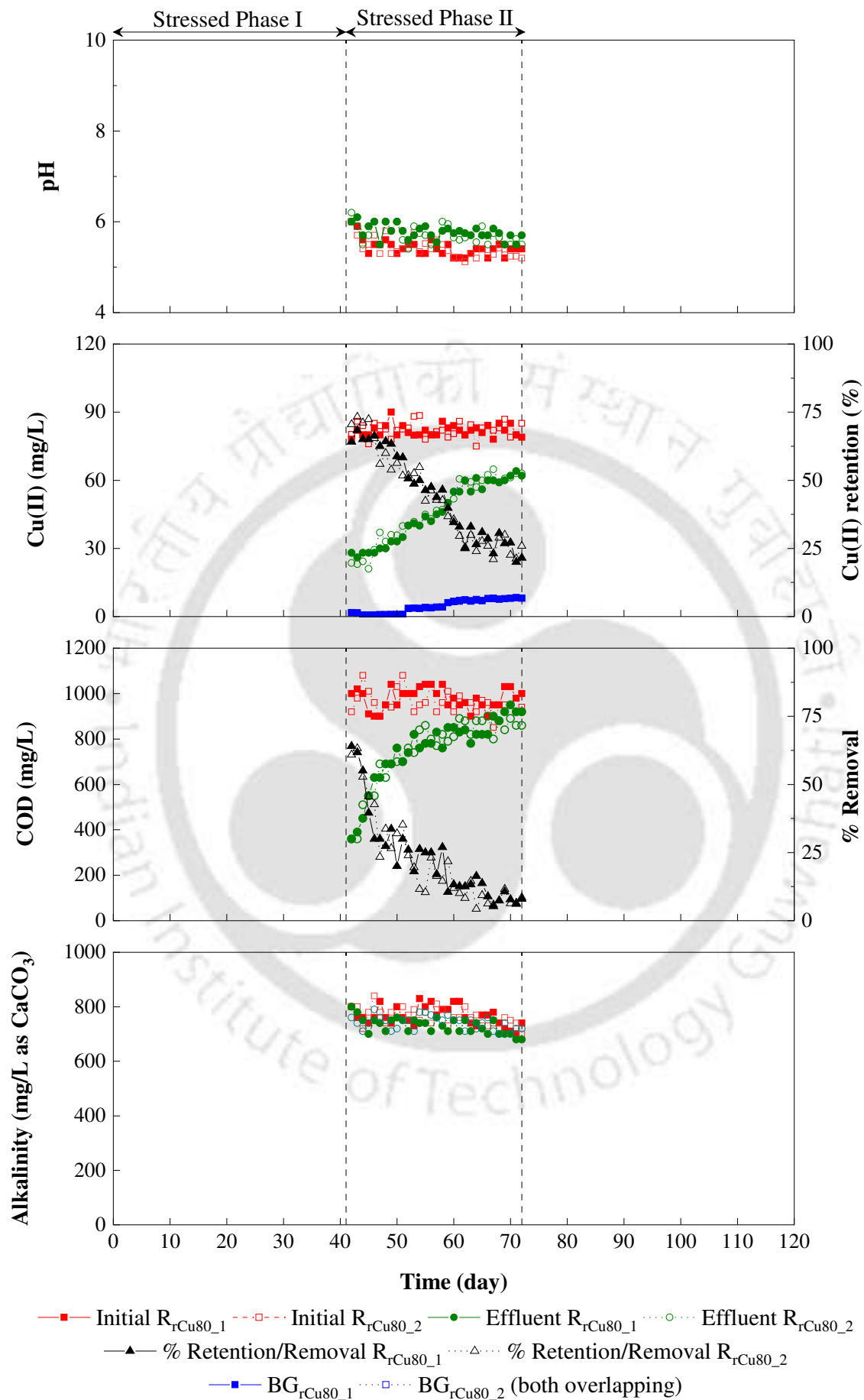


Fig. 5.18 Variation in environmental and performance related parameters for reactor R_{Cu80} during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.

Table 5.14 Summary of environmental and performance related parameters for reactors operated during 42-72 d of stressed phase II operation with PWW+Cu(II) feed.

Parameters	Day-wise	42-72				
	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
	Lactogen 3 (g/L)	0.90	-	-	-	-
	Cu(II) (mg/L)	0	40	80	40	80
	PWW (mL)	0	~500-750	~500-750	~500-750	~500-750
Stressed phase II	Environmental					
	Room temperature (°C) at 9.30 am	27.9±2.3 (31)	27.9±2.3 (31)	27.9±2.3 (31)	27.9±2.3 (31)	27.9±2.3 (31)
	Room temperature (°C) at 8.45 pm	21.8±0.8 (31)	21.8±0.8 (31)	21.8±0.8 (31)	21.8±0.8 (31)	21.8±0.8 (31)
	Initial pH	7.24±0.20 (62)	6.13±0.26 (62)	5.83±0.27 (62)	5.81±0.19 (62)	5.42±0.20 (62)
	Initial Cu(II) (mg/L)	0.03±0.01 (62)	42.04±2.40 (62)	83.18±3.48 (62)	41.71±2.70 (62)	82.05±2.94 (62)
	Effluent pH	7.23±0.23 (62)	6.21±0.34 (62)	6.06±0.23 (62)	6.11±0.21 (62)	5.74±0.18 (62)
	Effluent Cu(II) (mg/L)	0.01±0.01 (62)	29.16±8.87 (62)	65.42±14.83 (62)	17.87±6.23 (62)	46.04±13.48 (62)
	Cu(II) retention (%)	-	30.58±20.98 (62)	21.29±17.90 (62)	56.99±15.09 (62)	43.83±16.53 (62)
	Background Cu(II) (mg/L)	-	0.59±0.15 (62)	1.22±0.42 (62)	1.79±1.19 (62)	4.53±2.91 (62)
	Performance					
	Initial soluble COD (mg/L)	883.22±40.10 (62)	950.32±57.87 (62)	961.61±47.02 (62)	982.90±58.74 (62)	970.65±47.90 (62)
	Effluent soluble COD (mg/L)	51.61±22.43 (62)	537.16±127.30 (62)	664.16±185.91 (62)	618.65±140.94 (62)	753.71±149.98 (62)
	COD removal (%)	94.15±2.54 (62)	43.15±14.18 (62)	30.68±19.82 (62)	37.09±13.76 (62)	22.10±16.11 (62)
	Initial alkalinity (mg CaCO ₃ /L)	65.19±7.40 (62)	794.84±27.45 (62)	740.00±32.72 (62)	796.77±27.85 (62)	765.48±33.22 (62)
	Effluent alkalinity (mg CaCO ₃ /L)	58.74±7.77 (62)	761.61±26.49 (62)	718.06±31.50 (62)	765.48±27.41 (62)	735.48±26.98 (62)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

43.3% of Cu(II) retention has been observed on 57th d of operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. In addition, towards the end of stressed phase II operation (on 72nd d), 11.0%, 11.0%, 40.2%, and 23.7% of Cu(II) retention has been obtained for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. It can be observed that Cu(II) retention for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have gradually decreased and reactors fed with lower Cu(II) concentration (R_{sCu40} and R_{rCu40}) has shown higher Cu(II) retention than higher Cu(II) doses fed reactors (R_{sCu80} and R_{rCu80}). The reactors fed with raw PWW has shown enhanced Cu(II) retention due to additional suspended solid contents in PWW, which has possibly provided additional adsorption site for metal ion attachment. The average Cu(II) retention for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as $30.58 \pm 20.98\%$, $21.29 \pm 17.90\%$, $56.99 \pm 15.09\%$, and $43.83 \pm 16.53\%$, respectively. The average BG_{Cu} concentration for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as 0.59 ± 0.15 , 1.22 ± 0.42 , 1.79 ± 1.19 , and 4.53 ± 2.91 mg/L, respectively, during stressed phase II operation. Usually, Cu(II) and Zn(II) are added to pig's feed at high concentrations as supplement to improve feed efficiency and the health of pigs (Suzuki et al., 2010). Hence, Cu(II) has been added externally in this experiment and its concentration has been adopted based on experimental results presented in Chapter 2.

The variations in COD removal during the stressed phase II operation for all the reactors have been shown in Fig. 5.14 to 5.18 and data has been summarized in Table 5.14. The reactor R_c has shown uniform COD removal during 42-72 of operation, whereas PWW+Cu(II) fed reactors have shown variations in COD removal. On 42nd d of operation from the start, reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have shown 95.3%, 74.0%, 75.0%, 62.4%, and 62.4%, of COD removal, respectively, while on 57th d of operation, 95.4%, 48.2%, 18.9%, 36.1%, and 16.6% of COD removal has been obtained for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. Towards the end of the stressed phase II operation (on 72nd d), 90.7%, 30.9%, 12.9%, 21.8%, and 8.3% of COD removal has been observed for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. The results suggest that presence of Cu(II) has affected the COD removal and drastically inhibited the bio-degradation of PWW with increased Cu(II) concentration in the feed. The average COD removal for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as $94.15 \pm 2.54\%$, $43.15 \pm 14.18\%$, $30.68 \pm 19.82\%$, $37.09 \pm 13.76\%$, and $22.10 \pm 16.11\%$, respectively (Table 5.14).

The variations in alkalinity during the stressed phase II operation for all the reactors have been shown in Fig. 5.14 to 5.18 and data has been summarized in Table 5.14. During stressed phase II operation, the average initial alkalinity has been observed as 65.19 ± 7.40 , 794.84 ± 27.45 , 740.00 ± 32.72 , 796.77 ± 27.85 , and 765.65 ± 33.22 mg/L as $CaCO_3$ for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively, while average effluent alkalinity has been observed as 58.74 ± 7.77 , 761.61 ± 26.49 , 718.06 ± 31.50 , 765.48 ± 27.41 , and 735.48 ± 26.98 mg/L as $CaCO_3$, respectively, for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} . It has been observed that addition of Cu(II) to PWW fed reactors (R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80}) have reduced alkalinity a little bit and this little reduction varies with Cu(II) doses in the reactors.

The COD removal has appeared to be stabilized after 60-65 d of operation during stressed phase II operation and thereafter has yielded steady performance. Summary of environmental and performance related parameters for reactors operated during steady-state condition (66-72 d) of stressed phase II operation are presented in Table 5.15. The data suggests minimum variation in reactor performance parameters. The initial and effluent pH for reactors have been observed in the range of 5.31–7.45. During the steady-state condition, the average COD removal for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as 94.2%, 29.0%, 14.5%, 22.3%, and 7.6%, respectively, while Cu(II) retention has been observed as 14.9%, 11.3%, 40.6%, and 25.2% for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. The reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have yielded 0.60, 0.91, 3.21, and 7.94 mg/L of Cu(II) as BG_{Cu} . The variation in initial and effluent alkalinity for reactor R_c has been observed as 68.71 ± 5.25 and 58.86 ± 7.21 mg/L as $CaCO_3$, while for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed in the range of 711.43–800.00 mg/L as $CaCO_3$ (Table 5.15).

5.3.2.2 Biomass related general parameters

The stress imparted by PWW+Cu(II) on aerobic sludge biomass characteristics have been assessed grossly through MLSS, MLVSS, ISV, SVI, and ASBA tests.

Aerobic sludge biomass characteristics

The variation in solids characteristics have been shown in Fig. 5.19 and data is summarized in Table 5.16 for stressed phase II operation (from 42 to 72 d). Reactor R_c has shown continuous aerobic biomass growth [Fig. 5.19(a)] with a gradual increase in SVI due to filamentous growth in the reactor. While reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have shown decrease in biomass growth during stressed phase II operation, as shown in Fig. 5.19(b) and (c), while having almost similar SVI throughout the operation [Fig. 5.19(b) and (c)], whereas reactor R_{rCu40} and R_{rCu80} biomasses have shown increased solids concentration while having almost similar SVI [Fig. 5.19(d) and (e)]. The MLVSS concentration in reactor R_{rCu40} and R_{rCu80} biomasses have appeared to decrease due to addition of Cu(II). In addition, the MLVSS concentration in reactor R_{rCu80} biomass has been observed lower than reactor R_{rCu40} biomass due to exposure of higher doses of Cu(II) (80 mg/L), which has inhibited biomass growth. The increase in solid concentration has been due to addition of raw PWW to these reactors, which consisted of a higher fraction of suspended solids. The zigzag pattern in Fig. 5.19(d) and (e) is due to withdrawal of solids from reactors R_{rCu40} and R_{rCu80} to maintain the solids concentration closer to 2000 mg/L, as study of stressed phase II has been started with 2000 mg/L of solids in the reactors. The results suggest that the presence of Cu(II) in the feeds of reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have adversely impacted biomass growth during stressed phase II operation. The MLVSS/MLSS ratio has ranged between 0.65-0.80 during stressed phase II operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses (Table 5.16). The SRT for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} has been observed in the range of 4-27 d during stressed phase II operation.

Table 5.15 Summary of environmental and performance related parameters for reactors operated during 66-72 d of steady-state condition of stressed phase II operation with PWW+Cu(II) feed.

Parameters	Day-wise	66-72				
	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
	Lactogen 3 (g/L)	0.90	-	-	-	-
	Cu(II) (mg/L)	0	40	80	40	80
	PWW (mL)	0	~500-750	~500-750	~500-750	~500-750
Stressed phase II	Environmental					
	Room temperature (°C) at 9.30 am	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)
	Room temperature (°C) at 8.45 pm	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)
	Initial pH	7.45±0.13 (14)	5.89±0.22 (14)	5.71±0.23 (14)	5.79±0.12 (14)	5.31±0.09 (14)
	Initial Cu(II) (mg/L)	0.03±0.01 (14)	41.96±2.34 (14)	84.55±3.28 (14)	42.35±1.89 (14)	82.36±2.84 (14)
	Effluent pH	7.40±0.17 (14)	5.88±0.26 (14)	5.90±0.25 (14)	6.01±0.13 (14)	5.62±0.11 (14)
	Effluent Cu(II) (mg/L)	0.01±0.01 (14)	35.66±1.16 (14)	74.94±1.90 (14)	25.13±1.25 (14)	61.51±1.80 (14)
	Cu(II) retention (%)	-	14.87±3.86 (14)	11.27±3.64 (14)	40.60±3.48 (14)	25.23±3.75 (14)
	Background Cu(II) (mg/L)	-	0.60±0.01 (14)	0.91±0.02 (14)	3.21±0.13 (14)	7.94±0.21 (14)
	Performance					
	Initial soluble COD (mg/L)	891.43±47.68 (14)	918.57±52.51 (14)	948.57±41.00 (14)	982.86±41.43 (14)	954.29±42.78 (14)
	Effluent soluble COD (mg/L)	51.43±22.04 (14)	651.43±42.47 (14)	810.00±30.44 (14)	762.86±42.78 (14)	881.43±37.84 (14)
	COD removal (%)	94.20±2.61 (14)	28.96±4.67 (14)	14.49±3.81 (14)	22.34±3.97 (14)	7.61±1.89 (14)
	Initial alkalinity (mg CaCO ₃ /L)	68.71±5.25 (14)	798.57±28.92 (14)	751.43±26.38 (14)	800.00±25.62 (14)	735.71±24.86 (14)
	Effluent alkalinity (mg CaCO ₃ /L)	57.86±7.21 (14)	765.71±28.16 (14)	730.00±27.09 (14)	773.57±25.34 (14)	711.43±22.67 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

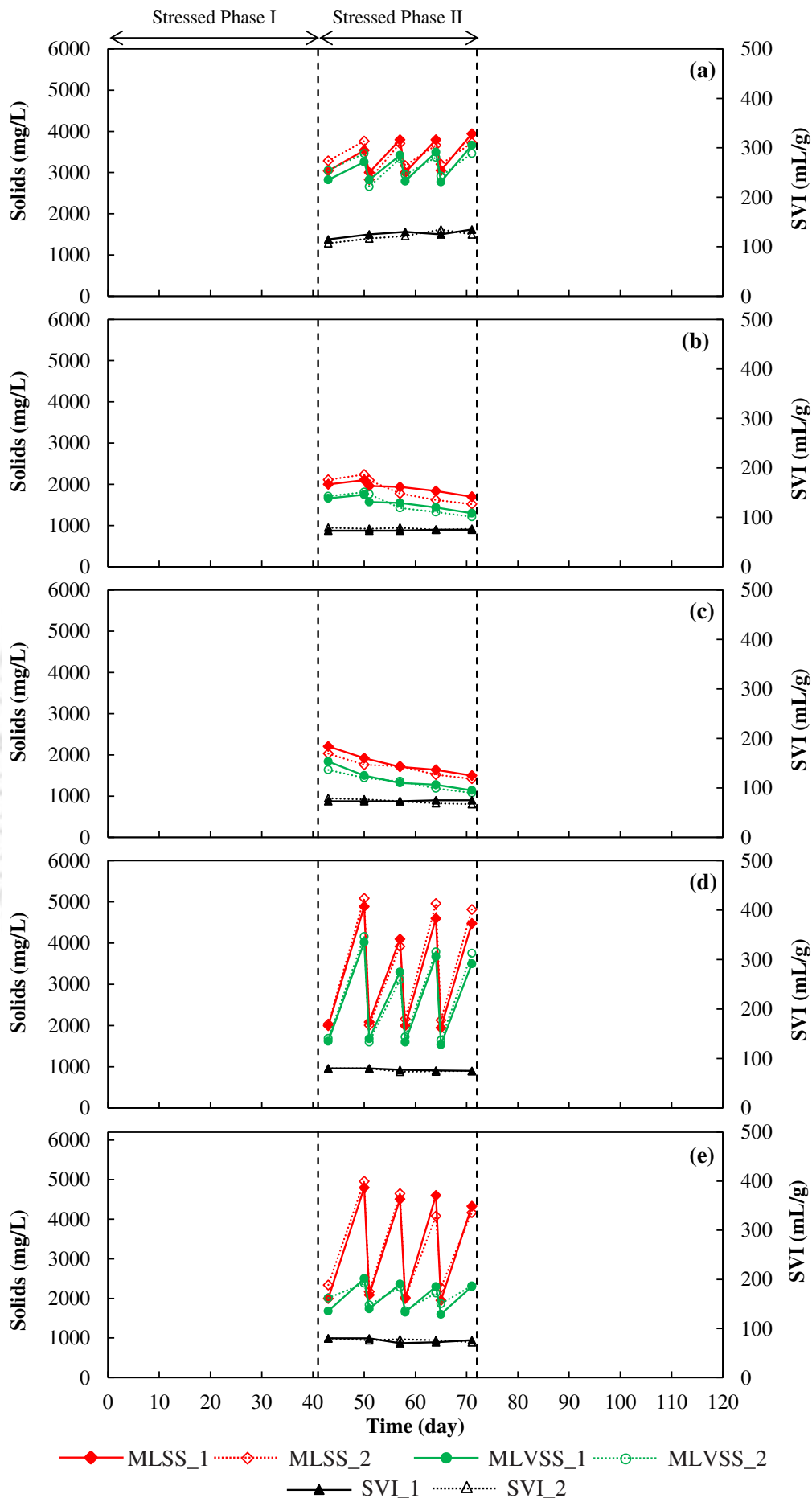


Fig. 5.19 Variation in MLSS, MLVSS, and SVI for reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.

Table 5.16 Summary of solids characteristics estimated during stressed phase II operation with PWW+Cu(II) feed.

Gross parameters	Stressed phase II operation					
	Day	42-72				
	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
MLSS (mg/L)		2337.40±556.49 (10)	1857.20±196.05 (10)	1744.00±256.59 (10)	3501.60±1374.54 (10)	3494.00±1240.03 (10)
MLVSS (mg/L)		2149.93±477.51 (10)	1495.66±188.92 (10)	1378.80±244.19 (10)	2771.63±1047.72 (10)	2096.63±267.05 (10)
MLVSS/MLSS ratio		0.92±0.02 (10)	0.80±0.02 (10)	0.79±0.03 (10)	0.80±0.02 (10)	0.65±0.18 (10)
SVI (mL/g)		111.60±8.46 (10)	75.60±1.38 (10)	73.40±3.10 (10)	77.03±2.79 (10)	76.00±3.76 (10)
ISV (m/h)		2.22±0.12 (10)	3.37±0.08 (10)	3.36±0.07 (10)	3.44±0.09 (10)	3.46±0.09 (10)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

The SVI of aerobic sludge biomass for all reactors have been shown in Fig. 5.19 and data has been summarized in Table 5.16. The average SVI for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 111.60 ± 8.46 , 75.60 ± 1.38 , 73.40 ± 3.10 , 77.03 ± 2.79 , and 76.00 ± 3.76 mL/g, respectively, under stressed phase II operation (Table 5.16). The SVI has decreased for PWW+Cu(II) fed reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses during stressed phase II operation as compared to stressed phase I operation. The possible reason might be addition of 40 and 80 mg/L of Cu(II) to the reactors, which has inhibited the filamentous biomass growth. The disintegration of aerobic sludge biomass flocs might also attribute to low SVI due to addition of PWW+Cu(II) feed in the reactors.

Minor variations have been observed in ISV during stressed phase II operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, which is reflected in Fig. 5.20. On the 43rd, 58th, and 71st d of stressed phase II operation, ISV for reactor R_c biomass has been observed as 2.34, 2.21, and 2.10 m/h, respectively, while 3.25, 3.42, and 3.40 m/h has been observed for reactor R_{sCu40} biomass; and 3.30, 3.40, and 3.40 m/h has been observed for reactor R_{sCu80} biomass, respectively. Similarly, ISV for reactor R_{rCu40} and R_{rCu80} biomasses

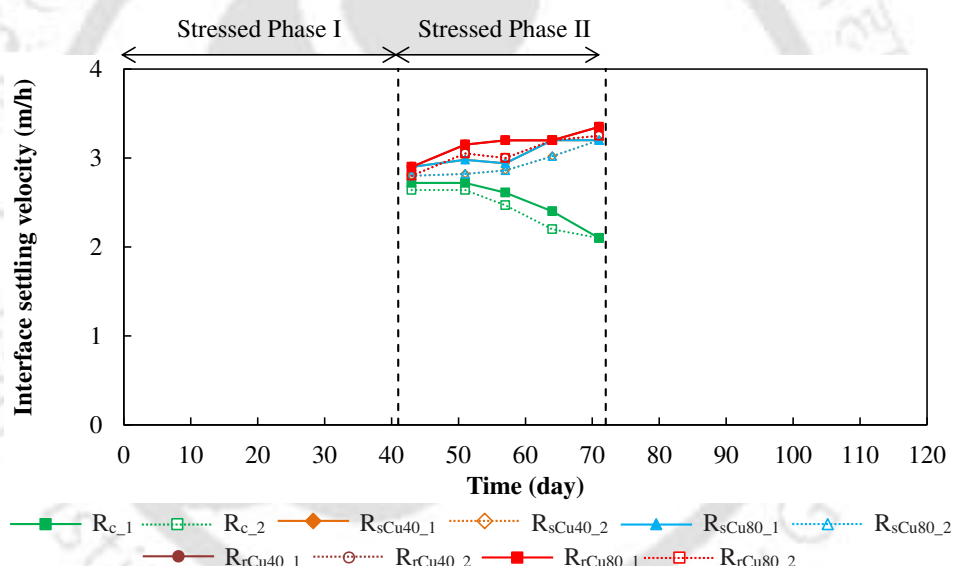


Fig. 5.20 Variation in interface settling velocities for reactor biomasses during stressed phase II operation with PWW+Cu(II) feed.

have been observed in the range of 3.30-3.51 m/h on the 43rd, 58th, and 71st d of stressed phase II operation, respectively. The average ISV for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses have been observed as 2.22 ± 0.12 , 3.37 ± 0.08 , 3.36 ± 0.07 , 3.44 ± 0.09 , and 3.46 ± 0.09 m/h, respectively (Table 5.16). The reactor R_c biomass has shown decreased settling velocity due to increase in filamentous microorganisms, whereas PWW+Cu(II) fed reactors have shown higher ISV with almost similar trends of settling.

Aerobic sludge biomass activity test

Aerobic sludge biomass activity (ASBA) test has been carried out towards the end of the stressed phase II operation (on 72nd d), when reactors have achieved steady-state condition. The results have been shown in Fig. 5.21 and data has been represented in Table 5.17. The

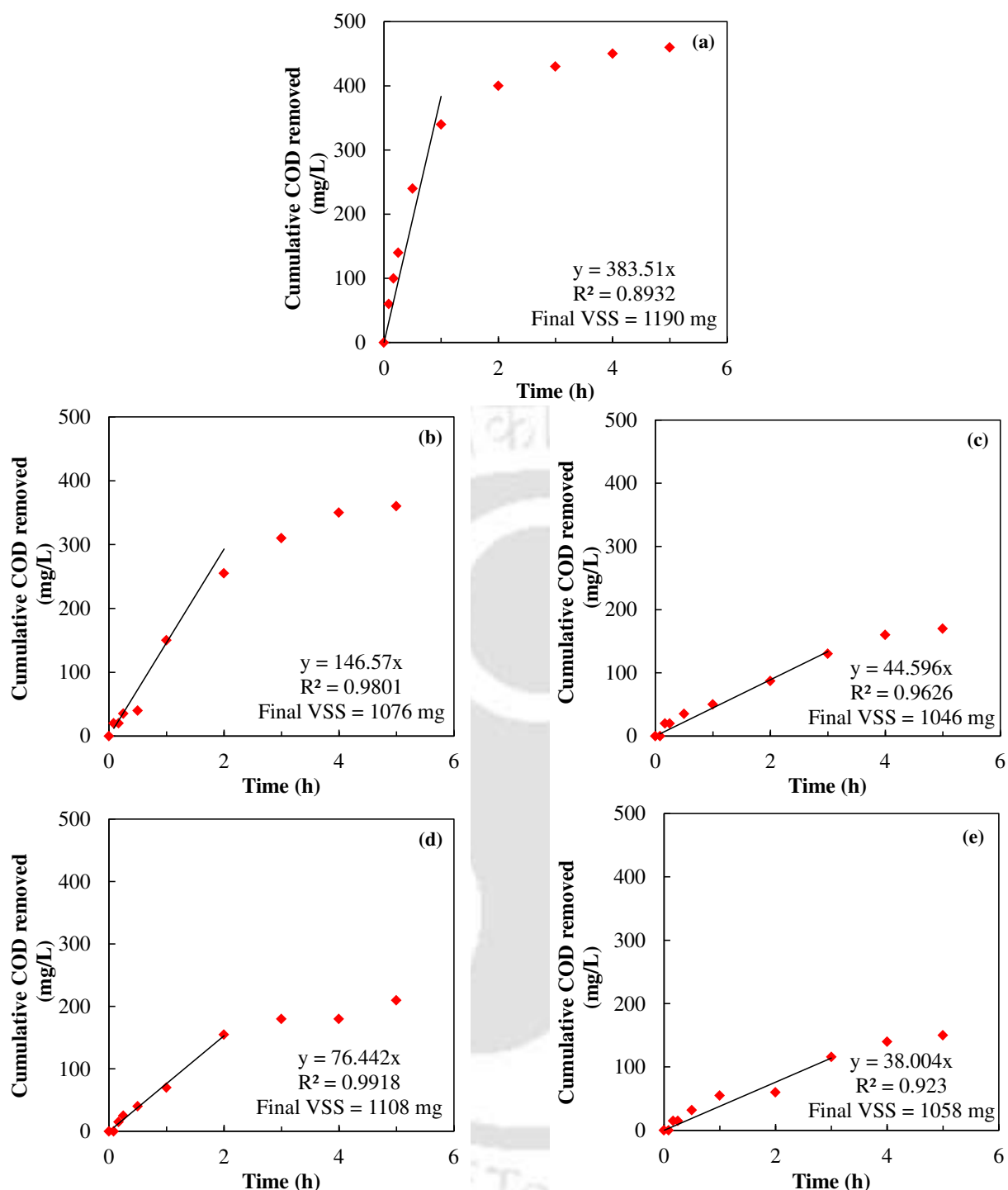


Fig. 5.21 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} on 72nd d of stressed phase II operation with PWW+Cu(II) feed.

Table 5.17 Aerobic sludge biomass activity test results carried out towards the end of stressed phase II operation with PWW+Cu(II) feed.

Biomass withdrawn on	72 nd d				
Reactor	R_c	R_{sCu40}	R_{sCu80}	R_{rCu40}	R_{rCu80}
ASBA (mg COD removed/mg VSS d)	7.75	2.89	1.02	1.65	0.86

ASBA for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has been estimated as 7.75, 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, respectively, towards the end of stressed phase II operation. The addition of Cu(II) along with PWW in feed has further decreased the ASBA due to Cu(II) stress. The presence of Cu(II) has also changed the reactor pH which might not be favorable for microbial activity. Adverse effect of Cu(II) on soluble oxygen uptake rate (sOUR) has also been reported in literature ([Madoni et al., 1999](#); [Oviedo et al., 2002](#)). Even 5 mg/L of Cu(II) has caused a drastic adverse effect on the bio-activity of aerobic sludge biomass ([Wang et al., 2010](#)). The information related to combined effect of PWW+Cu(II) in the feed to aerobic sludge biomass activity is unavailable till now and therefore these results could not be compared with.

Reconsidering biomass related general parameters estimated during stressed phase II operation; it appears that the average values of MLSS and MLVSS have decreased for reactor R_{sCu40} and R_{sCu80} biomasses, while the same appear to have increased for reactor R_{rCu40} , and R_{rCu80} biomasses (mainly due to presence of larger fraction of suspended solids in the feed) compared to control reactor R_c biomass. Also the ratio of MLVSS/MLSS appears to be constant in the range of 0.79-0.80 for reactor R_{sCu40} , R_{sCu80} , and R_{rCu40} biomasses, while the same is much lower — 0.65 for reactor R_{rCu80} biomass. However, MLSS and MLVSS values appear to have failed in reflecting the impacts of imposed stresses due to lack of a definite trend in its variations. Whereas the average values of SVI have decreased in the range of 73.4-77.0 mL/g for stressed reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses against 111.6 mL/g for control reactor R_c biomass; and correspondingly the average values of ISV have increased in the range of 3.36-3.46 m/h for stressed reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses against 2.22 m/h for control reactor R_c biomass. Apparently, decreased SVI and increased ISV indicate improved settling properties of sludge biomasses from stressed reactors. But ASBA estimated towards the end of stressed phase II operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses are 2.89, 1.02, 1.65 and 0.86 mg COD removed/mg VSS d, respectively, which is on a much lower side compared to 7.75 mg COD removed/mg VSS d for control reactor R_c biomass. It has indicated serious deterioration in bio-degradation capability of organics by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses under stressed phase II operation. The reduced COD removals of ~29%, 14%, 22% and 8% by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, in comparison to ~94% by control reactor R_c biomass have clearly indicated deterioration in bio-degradation capability of reactor biomasses during steady state condition under stressed phase II operation, which has to be visualized as a highly undesirable situation for organics degradation by the reactor biomasses — considered the primary objective of biomasses. Therefore, taking into consideration SVI, ISV and ASBA parameters together, the decreased SVI and increased ISV of biomasses may be taken as an indication for deterioration in bio-degradation capability of biomasses as observed with decreased values of ASBA under stressed phase II operation.

5.3.2.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

The microscopic observations during stressed phase II operation have been shown in Table 5.18. The addition of Cu(II) along with PWW in the feed has also significantly affected the flocs morphology, as shown in Table 5.18 (43-71 d). Whereas, continuous growth of filaments with increased sizes in aerobic sludge biomass have been recorded for reactor R_c biomass. However, addition of Cu(II) has adversely affected the filamentous microorganism in other reactors. Completely disintegrated flocs have been observed for reactor R_{sCu40} , R_{rCu40} , R_{sCu80} , and R_{rCu80} biomasses on 43rd d, which continued till 71st d of operation. The reactors fed with 40 mg/L of Cu(II) (reactors R_{sCu40} and R_{rCu40}) has exhibited significant disappearance of filamentous microbes due to Cu(II) stress, while at 80 mg/L of Cu(II) in reactors R_{sCu80} and R_{rCu80} , filaments have completely disappeared.

The photographic observations during stressed phase II operation have been shown in Table 5.19. During stressed phase II operation, the flocs size of aerobic biomass has been observed in the range of 1.0-14.0 mm for reactor R_c biomass, while the floc size for reactor R_{sCu40} , R_{rCu40} , R_{sCu80} , and R_{rCu80} biomasses have ranged in 1.0-5.0 mm, almost similar as stressed phase I operation. The photographic observation has suggested that addition of Cu(II) has not changed the flocs morphology; however, the morphology has changed (disappeared filaments) as could be seen through microscopic observations (Table 5.18). The granular flocs have been observed in reactors fed with 40 mg/L of Cu(II) (reactors R_{sCu40} and R_{rCu40}). In the case of reactor R_{sCu80} biomass, the granular flocs have also disappeared due to the stress of Cu(II) (Table 5.19). The possible reason could be the direct interaction with metal and microbial cells in reactor R_{sCu80} , which has not been possible in the case of reactor R_{rCu80} due to the presence of suspended solids in raw PWW. The additional suspended solids in reactor R_{rCu80} could have interfered with the interaction between metal and biomass; and the suspended solids might have adsorbed Cu(II) more than aerobic sludge biomass in reactor R_{rCu80} .

In addition, the presence of Cu(II) with PWW in the feed has significantly affected the existence of active and inactive cells towards the end of the stressed phase II operation (on 72nd d). The reactor R_{sCu80} and R_{rCu80} biomasses fed with 80 mg/L of Cu(II) have shown a significant decrease in active cells portion as compared to the reactor R_{sCu40} and R_{rCu40} biomasses fed with 40 mg/L of Cu(II) (Table 5.20). The fluorescence microscopic images has clearly shown decrease in green color portion with increase in Cu(II) doses in reactors, and consequently red color has increased with increased Cu(II) doses. On the 72nd d of operation, live cells for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been estimated as 93.8%, 36.4%, 23.5%, 28.7%, and 16.8%, respectively. On the other hand, dead cells for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been estimated as 1.4%, 46.8%, 62.0%, 61.2%, and 86.4%, respectively. The addition of Cu(II) along with PWW in the feed to the reactors could have inhibited the metabolic activities of aerobic sludge biomass resulting

Table 5.18 Microscopic examination of Gram’s stained aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.

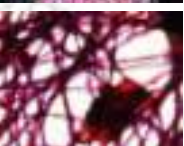
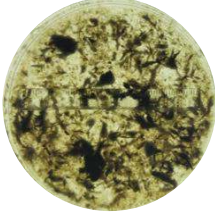
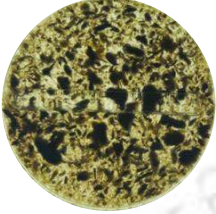
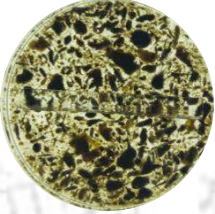
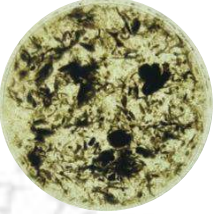
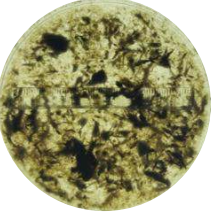
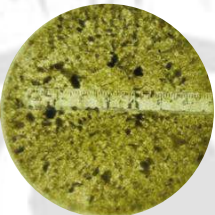
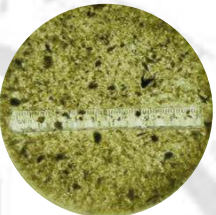
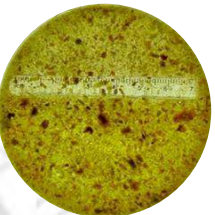
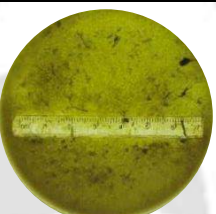
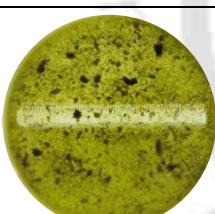
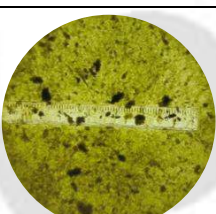
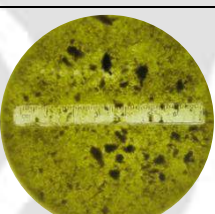
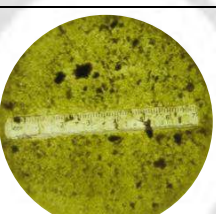
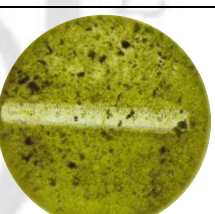
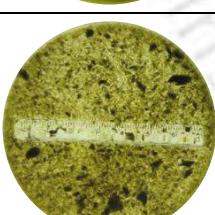
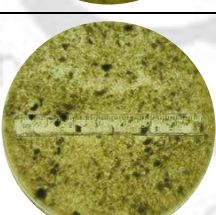
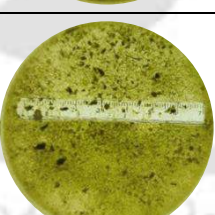
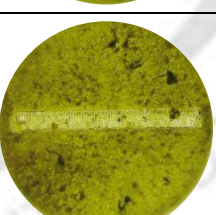
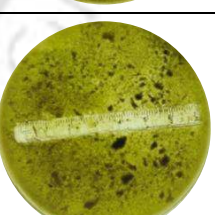
Reactors	Mag.	Aerobic sludge biomass samples withdrawn on				
		Stressed phase II operation				
		43 rd d	50 th d	57 th d	64 th d	71 st d
R_c	5×					
	100×					
R_{sCu40}	5×					
	100×					
R_{sCu80}	5×					
	100×					
R_{rCu40}	5×					
	100×					
R_{rCu80}	5×					
	100×					

Table 5.19 Photographic images of aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with $PWW+Cu(II)$ feed.

Reactors	Aerobic sludge biomass samples withdrawn on				
	Stressed phase II operation				
	43 rd d	50 th d	57 th d	64 th d	71 st d
R_c					
R_{sCu40}					
R_{sCu80}					
R_{rCu40}					
R_{rCu80}					

in decreased active cell population. The inactive portion of aerobic sludge biomass is much higher in reactor fed with a 80 mg/L dose of $Cu(II)$.

The FI for all the reactor biomasses have been presented in [Table 5.21](#). The reactor R_c biomass has shown FI of 5 for 42-72 d of operation. The addition of $Cu(II)$ to reactor R_{sCu40} , R_{rCu40} , R_{sCu80} , and R_{rCu80} have resulted in decrease in FI ([Table 5.21](#)). On 43rd d of operation, the FI for reactor R_{sCu40} , R_{rCu40} , R_{sCu80} , and R_{rCu80} biomasses have been estimated as 3, 3, 2, and 2, respectively, which has decreased further to 2, 1, 1, and 1, respectively, on 71st d of operation. The results have suggested that $Cu(II)$ along with PWW in the feed has been more toxic to filamentous biomass as compared to PWW alone in the feed.

Table 5.20 Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during stressed phase II operation with PWW+Cu(II) feed.

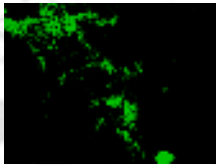
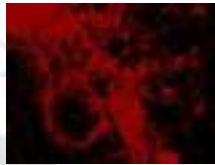
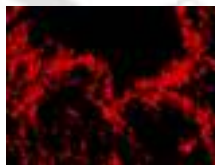
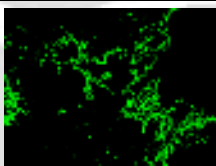
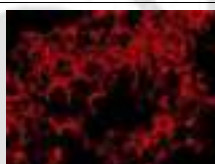
Reactors	Aerobic sludge biomass samples withdrawn on		
	Stressed phase II operation		
	72 nd d		
	Bright field (20×)	Live cells (20×)	Dead cells (20×)
R_c			
R_{sCu40}			
R_{sCu80}			
R_{rCu40}			
R_{rCu80}			

Table 5.21 Filament index of aerobic sludge biomass estimated during stressed phase II operation with PWW+Cu(II) feed.

Biomass withdrawn on	Stressed phase II operation				
Day	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
43 rd d	5	3	3	2	2
50 th d	5	3	2	2	2
57 th d	5	3	2	2	2
64 th d	5	3	2	1	1
71 st d	5	2	1	1	1

Polysaccharide, protein and extracellular polymeric substance

PS, PN, and EPS contents in aerobic biomass has also been estimated on 72nd d (towards the end of stressed phase II operation) and result has been shown in Fig. 5.22 and summarized data is presented in Table 5.22. The addition of Cu(II) to reactors has also enhanced PS, PN, and EPS secretion in aerobic sludge biomass. On 72nd d of operation, PS content in reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 16.54 ± 1.02 , 17.22 ± 0.80 ,

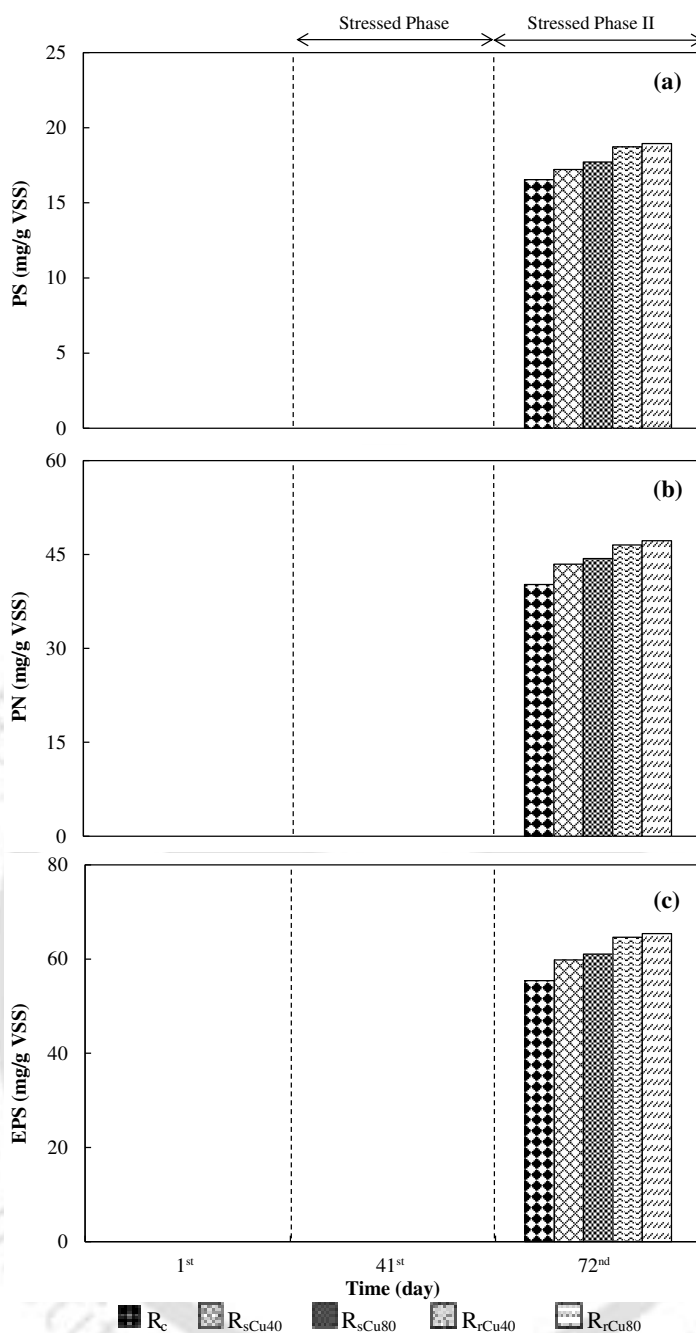


Fig. 5.22 Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during stressed phase II operation with PWW+Cu(II) feed.

Table 5.22 PS, PN and EPS contents in aerobic sludge biomass operated under stressed phase II operation with PWW+Cu(II) feed.

Biomass withdrawn on	Reactor biomass	PS	PN (mg/g VSS)	EPS
72 nd d	R_c	16.54±1.02	40.22±1.42	55.44±4.04
	R_{sCu40}	17.22±0.80	43.46±1.82	59.82±4.62
	R_{sCu80}	17.72±1.02	44.36±1.60	61.06±4.06
	R_{rCu40}	18.73±1.44	46.52±1.32	64.62±4.54
	R_{rCu80}	18.94±1.02	47.22±1.92	65.42±2.02

[The data is presented in $xx \pm yy$ format where xx is the average value, yy is the standard deviation.]

17.72±1.02, 18.73±1.44, and 18.94±1.02 mg/g VSS, respectively [Fig. 5.22(a)]. Similarly, PN content in reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 40.22±1.42, 43.46±1.82, 44.36±1.60, 46.52±1.32, and 47.22±1.92 mg/g VSS, respectively [Fig. 5.22(b)], on 72nd d of operation. The EPS content in reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have also been increased to 55.44±4.04, 59.82±4.62, 61.06±4.06, 64.62±4.54, and 65.42±2.02 mg/g VSS (Table 5.22), respectively, on 72nd d of operation.

EPS mainly consists of proteins, carbohydrates, and humic substances, etc. (Sheng et al., 2013), which are the major components of the activated sludge flocs in biological wastewater treatment systems. The increased PS, PN, and EPS content is a protective response of microorganisms that exist in aerobic sludge biomass (Chen et al., 2014; Wang et al., 2018). It is also reported that PN and PS contain some negatively charged functional groups, which could effectively bind heavy metal cation and act as a permeability barrier to hinder intracellular penetration of the metal (Wang et al., 2018). The increased PS, PN, and EPS concentrations could be caused by the enhanced microbial secretion in response to change in feed composition (PWW) and also due to the presence of Cu(II) in the feed (Xu et al., 2019).

Enzyme activity

The $E_{Catalase}$, $E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease} have also been assessed for all reactor biomasses towards the end of the stressed phase II operation (on 72nd d). The results have been shown in Fig. 5.23 and data has been summarized in Table 5.23. The $E_{Catalase}$ for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 0.42, 0.20, 0.10, 0.18, and 0.08 mg/g MLSS h, respectively [Fig. 5.23(a)]. The addition of PWW+Cu(II) in the feed to the reactors have significantly inhibited $E_{Catalase}$ and % inhibition has increased with increased dose of Cu(II). The % inhibition has been estimated as 52.7%, 76.7%, 57.8%, and 81.1% for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively. The reactors fed with high concentration of Cu(II) has shown increased inhibition in $E_{Catalase}$ than lower Cu(II) concentration because higher Cu(II) dose has increased stress to microbial cells and probability of enzymes denaturation has also increased.

Similarly, $E_{Dehydrogenase}$ for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 30.12, 19.23, 12.33, 16.53, and 8.03 µg/g MLSS h, respectively [Fig. 5.23(b)]. The % inhibition has been estimated as 36.2%, 59.1%, 45.1%, and 73.4% for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively. $E_{Dehydrogenase}$ has decreased with increased Cu(II) doses.

On the other hand, $E_{Protease}$ has been observed as 2.31, 0.72, 0.43, 0.52, and 0.21 mg/g MLSS h for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, on 72nd d of operation [Fig. 5.23(c)]. The % inhibition of $E_{Protease}$ has been obtained as 68.8%, 81.4%, 77.5%, and 90.9% for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively. The $E_{Protease}$ has significantly been decreased under stressed phase II operation. The long term addition of 40 mg/L of Cu(II) has less inhibition effect than 80 mg/L of Cu(II) on $E_{Protease}$.

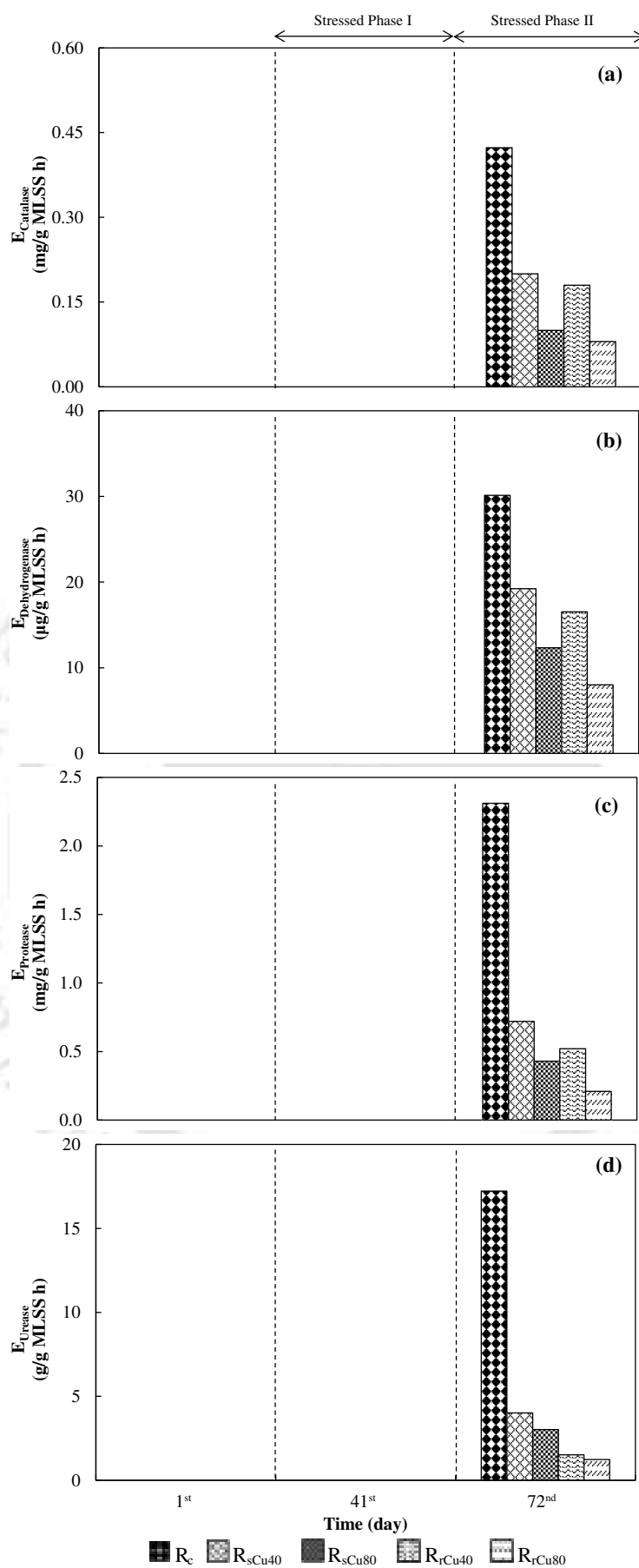


Fig. 5.23 Enzyme activities of aerobic sludge biomass (a) E_{Catalase} , (b) $E_{\text{Dehydrogenase}}$, (c) E_{Protease} , and (d) E_{Urease} assessed during stressed phase II operation with PWW+Cu(II) feed.

Table 5.23 Summary of enzyme activities of aerobic sludge biomass assessed during stressed phase II operation with PWW+Cu(II) feed.

Biomass withdrawn on	Reactor biomass	E _{Catalase} (mg/g MLSS h)	E _{Dehydrogenase} (μg/g MLSS h)	E _{Protease} (mg/g MLSS h)	E _{Urease} (g/g MLSS h)
72 nd d	R _c	0.42±0.03	30.12±1.81	2.31±0.11	17.23±0.80
	R _{sCu40}	0.20±0.01	19.23±1.73	0.72±0.07	4.02±0.81
	R _{sCu80}	0.10±0.01	12.33±2.12	0.43±0.10	3.02±1.04
	R _{rCu40}	0.18±0.01	16.53±2.05	0.52±0.08	1.52±0.64
	R _{rCu80}	0.08±0.03	8.01±2.04	0.21±0.05	1.24±0.51

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

Similarly, towards the end of stressed phase II operation (on 72nd d), E_{Urease} for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses have drastically been inhibited and observed as 17.23, 4.02, 3.02, 1.52, and 1.24 g/g MLSS h, respectively [Fig. 5.23(d)]. The E_{Urease} has been highly affected among all four enzymes due to PWW+Cu(II) stress. The % inhibition of E_{Urease} has been estimated as 76.7%, 82.5%, 91.2%, and 92.8% for reactor R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses, respectively.

From the results (Fig. 5.23 and Table 5.23), it can be observed that addition of Cu(II) along PWW in the feed has resulted in further inhibition in enzyme activities due to the toxic nature of Cu(II). The intensity of inhibition has increased with an increase in Cu(II) concentration. Cu(II) ions have been responsible for the denaturation of cellular proteins and enzymes, and the pH of the system has also significantly lowered due to Cu(II) doses. The enzyme activity might depend on several factors such as pH, temperature, substrate and metal concentration, etc., in addition to the presence of Cu(II) in feed, which might also be responsible for enzyme inhibition. Although Cu(II) in trace concentration is required as a co-factor for several enzymes but the higher concentration might damage the enzyme structure resulting in blockage or inhibition of activities.

Amplified ribosomal DNA restriction analysis (ARDRA)

Microbial growth depends upon the substrate concentration and operating factors, including the pH, dissolved oxygen, temperature and free ammonia concentration. In addition, Cu(II) has also been added along with PWW as feed, which might harm aerobic sludge quality and morphology. Under such circumstances, some microorganisms might adapt to PWW and high Cu(II) concentration and gradually became predominant bacteria. In contrast, others microorganisms lacking PWW- and Cu(II)-tolerance capacity might gradually deplete or weaken (Wang et al., 2015). Therefore, it is required to know the shift in microbial community structure of aerobic sludge biomasses exposed to PWW+Cu(II). The digestion pattern and dendrogram of aerobic biomass samples digested with restriction enzymes *AluI*, *HhaI*, and *MspI* during stressed phase II operations has been shown in Fig. 5.24. The dendrogram has been prepared using GelJ software, and a similarity table has been generated based on the gel image presented in Table 5.24. ARDRA has been performed with the samples withdrawn towards the end of the stressed phase II operation (on 72nd d) to assess the possible changes

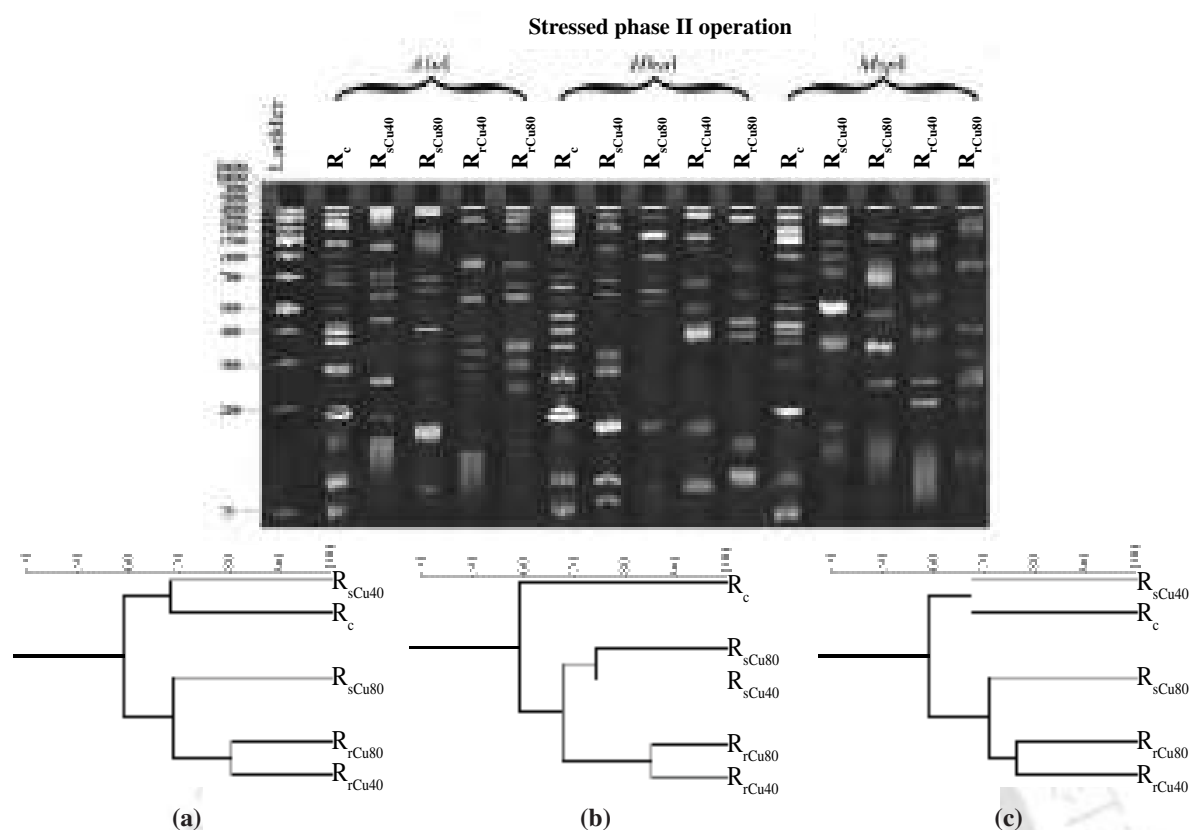


Fig. 5.24 Digestion pattern and dendrogram of aerobic sludge biomass under stressed phase II operation with PWW+Cu(II) feed treated with (a) *AluI*, (b) *HhaI*, and (c) *MspI* restriction enzymes.

in microbial community structure of aerobic sludge biomass due to PWW+Cu(II) stress. The gel image has shown different banding pattern when digested with *AluI*, *HhaI*, and *MspI* restriction enzymes. The restriction enzyme *AluI* has produced 19, 8, 10, 9, and 10 bands at different positions for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Table 5.24(a)) has shown that reactor R_{sCu40} and R_{sCu80} biomasses have been closer to the control reactor R_c biomass as compared to reactor R_{rCu40} , and R_{rCu80} biomasses. On the other hand, *HhaI* has produced 16, 10, 8, 8, and 7 bands at different positions for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Table 5.24(b)) has shown that reactor R_{sCu40} and R_{sCu80} biomasses have been closer to the control reactor R_c biomass as compared to reactor R_{rCu40} , and R_{rCu80} biomasses. Similarly, *MspI* restriction enzyme has produced 14, 8, 5, 6 and 7 bands at different positions for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Table 5.24(c)) has shown that reactor R_{sCu40} and R_{sCu80} biomasses have been closer to the control reactor R_c biomass as compared to reactor R_{rCu40} , and R_{rCu80} biomasses. The significant changes in microbial community structure has been observed towards the end of the stressed phase II operation (on 72nd d). The restriction enzyme *AluI* suggested 52.1%, 43.4%, 33.8%, and 29.4% of similarity, while *HhaI* exhibited 51.4%, 41.2%, 39.3%, and 31.0% of similarity for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, as compared to reactor R_c biomass. The samples digested with *MspI* has shown 62.5%, 58.5%, 54.8%, and 48.3% of similarity among PWW+Cu(II) fed reactors.

Table 5.24 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during stressed phase II operation with PWW+Cu(II) feed.

Stressed phase II operation															
<i>AluI</i>						<i>HhaI</i>					<i>MspI</i>				
Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
R _c	1	0.5213	0.4338	0.3384	0.2938	1	0.5143	0.4123	0.3927	0.3102	1	0.6249	0.5845	0.5476	0.4834
R _{sCu40}	0.5213	1	0.4831	0.4294	0.3217	0.5143	1	0.6215	0.4923	0.3793	0.6249	1	0.6471	0.4723	0.4367
R _{sCu80}	0.4338	0.4831	1	0.5653	0.4922	0.4123	0.6215	1	0.7343	0.5043	0.5845	0.6471	1	0.6875	0.6372
R _{rCu40}	0.3384	0.4294	0.5653	1	0.6967	0.3927	0.4923	0.7343	1	0.7756	0.5476	0.4723	0.6875	1	0.7217
R _{rCu80}	0.2938	0.3217	0.4922	0.6967	1	0.3102	0.3793	0.5043	0.7756	1	0.4834	0.4367	0.6372	0.7217	1

The results have suggested that PWW along with Cu(II) in the feed has an influence on the microbial community structure of aerobic sludge biomass. The addition of Cu(II) has drastically decreased similarity between the reactor biomasses. The % similarity has decreased with increase in Cu(II) concentration in the feed. In the case of reactors fed with raw PWW, disintegrated flocs have been washed-out or replaced with suspended solids during the decanting and washing step from the reactors and might have resulted in a decrease in % similarity. The possible reasons for microbial community change might be due to the composition of PWW, Cu(II) concentration and pH of reactors. It has also been reported that the organic loading rate might have influenced the changes in microbial community structures (Meng et al., 2016).

The impacts of stress observed through microscopic examination of biomasses have indicated complete disintegration of flocs with significant disappearance of filamentous growth at 40 mg/L of Cu(II) in feed for reactor R_{sCu40} and R_{rCu40} biomasses and complete disappearance of filaments at 80 mg/L of Cu(II) in feed for reactor R_{sCu80} and R_{rCu80} biomasses, but with continuous filamentous growth in control reactor R_c biomass during stressed phase II operation. The photographic examination has also shown serious impact with complete disappearance of even grains like structure of flocs from reactor R_{sCu80} biomass while fewer grains in the size range of 1.0-5.0 mm could be seen in reactor R_{sCu40} and R_{rCu40} biomasses, whereas reactor R_{rCu80} biomass has shown granular shaped particles which might be the retained suspended solids of the raw PWW feed. In contrast, the control reactor R_c biomass has shown flocs in the size range of 1.0-14.0 mm throughout the operation. The fluorescence microscopic examination conducted towards the end of stressed phase II operation for reactor biomasses has indicated much higher fraction of inactive cells in reactors fed with 80 mg/L dose of Cu(II), while the reactors fed with 40 mg/L of Cu(II) have shown relatively lower inactive cells. The FI has decreased to 2, 1, 1, and 1 for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, respectively, towards the end of stressed phase II operation from FI of 3, 3, 2 and 2 in the beginning of this phase for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, respectively. The biochemical analysis of biomasses of all the reactors (including the control reactor R_c) for polysaccharide (PS), protein (PN) and extracellular polymeric substance (EPS) contents have varied within the range of 16.54-18.94 mg/g VSS for PS, 40.22-47.22 mg/g VSS for PN and 55.44-65.42 mg/g VSS for EPS which fails to distinguish the impact of imposed stresses for reactor biomasses exposed to Cu(II) in the feed. The enzyme activities viz. $E_{Catalase}$, $E_{Dehydrogenase}$, and $E_{Protease}$ have shown variations in mg or μ g levels for control and stressed reactor biomasses ($E_{Catalase}$ of 0.2-0.1 mg/g MLSS h for stressed reactor biomasses compared to 0.4 mg/g MLSS h for reactor R_c biomass; $E_{Dehydrogenase}$ of 19-8 μ g/g MLSS h for stressed reactor biomasses compared to 30 μ g/g MLSS h for reactor R_c biomass; $E_{Protease}$ of 0.7-0.2 mg/g MLSS h for stressed reactor biomasses compared to 2.3 mg/g MLSS h for reactor R_c biomass) except E_{Urease} which has shown drastic changes (E_{Urease} of 4.0-1.2 g/g MLSS h for stressed reactor biomasses compared to 17.2 g/g MLSS h for reactor R_c biomass) estimated towards the end of stressed phase II operation. Hence, the enzyme activity, E_{Urease} reflects

the biochemical changes in biomass due to imposed stresses. The gel run of ARDRA carried out with three different restriction enzymes towards the end of stressed phase II operation has shown different banding patterns for the stressed reactor biomasses compared with the control reactor R_c biomass indicating changes in microbial communities in reactor biomasses. The dendrogram of stressed reactor biomasses have shown reduced similarity (in the range of 29% to 63%) with each other when digested with the three restriction enzymes, but these have been definitely different from the control reactor R_c biomass indicating significant changes in microbial community structures in stressed reactor biomasses.

5.3.3 Recovery phase operation

After completion of stressed phase II operation (on 72nd d), reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been operated for 73-120 d without PWW and Cu(II) in the feed, terming the operation as recovery phase operation. The reactors have been fed with Lactogen 3 as source of carbon for aerobic sludge biomass. To highlight the differences between observations and results of recovery phase operation with the stressed phase operations, most of the figures and tables have been reproduced with the recovery phase operation results along with stressed phase I and II operations.

5.3.3.1 Environmental and performance parameters

The reactors have been operated for 48 d (73-120 d) during recovery phase operation. The results obtained during recovery phase operation have been shown in Fig. 5.25-5.29 and summary of data has been presented in Table 5.25. The room temperature and pH have not been controlled during recovery phase operation. The variation in room temperature has been observed due to weather changes. The average variation in room temperature recorded at 9.30 am and 9.00 pm has been observed as 25.4 ± 2.5 and $17.7 \pm 2.7^\circ\text{C}$, respectively.

The average initial and effluent pH for reactor R_c have been obtained as 7.33 ± 0.20 and 7.32 ± 0.22 during recovery phase operation (Table 5.25). The reactor R_c has shown uniform pH throughout the operation (Table 5.25). However, pH for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have reached closer to neutral during recovery phase operation. The pH of reactors during recovery phase operation has increased from the stressed phase II operation. During steady-state condition of stressed phase II operation, average effluent pH for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as 5.88 ± 0.26 , 5.90 ± 0.25 , 6.01 ± 0.13 , and 5.62 ± 0.11 , respectively (Table 5.15). During recovery phase operation, the average initial pH for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been observed as 7.12 ± 0.20 , 7.06 ± 0.37 , 7.04 ± 0.19 , and 7.04 ± 0.44 , respectively, whereas effluent pH has been observed as 7.22 ± 0.20 , 7.08 ± 0.31 , 7.17 ± 0.19 , and 7.08 ± 0.50 , respectively (Table 5.25).

The residual Cu(II) in effluent has been detected during initial days of recovery phase operation (Fig. 5.26 to 5.29). On 73th d, the effluent Cu(II) concentration has been 3.24, 4.60, 6.54, and 12.34 mg/L for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively, whereas it has decreased with in six days (on 78th d) to less than 0.10 mg/L of Cu(II) for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , as shown in Fig. 5.26 to 5.29.

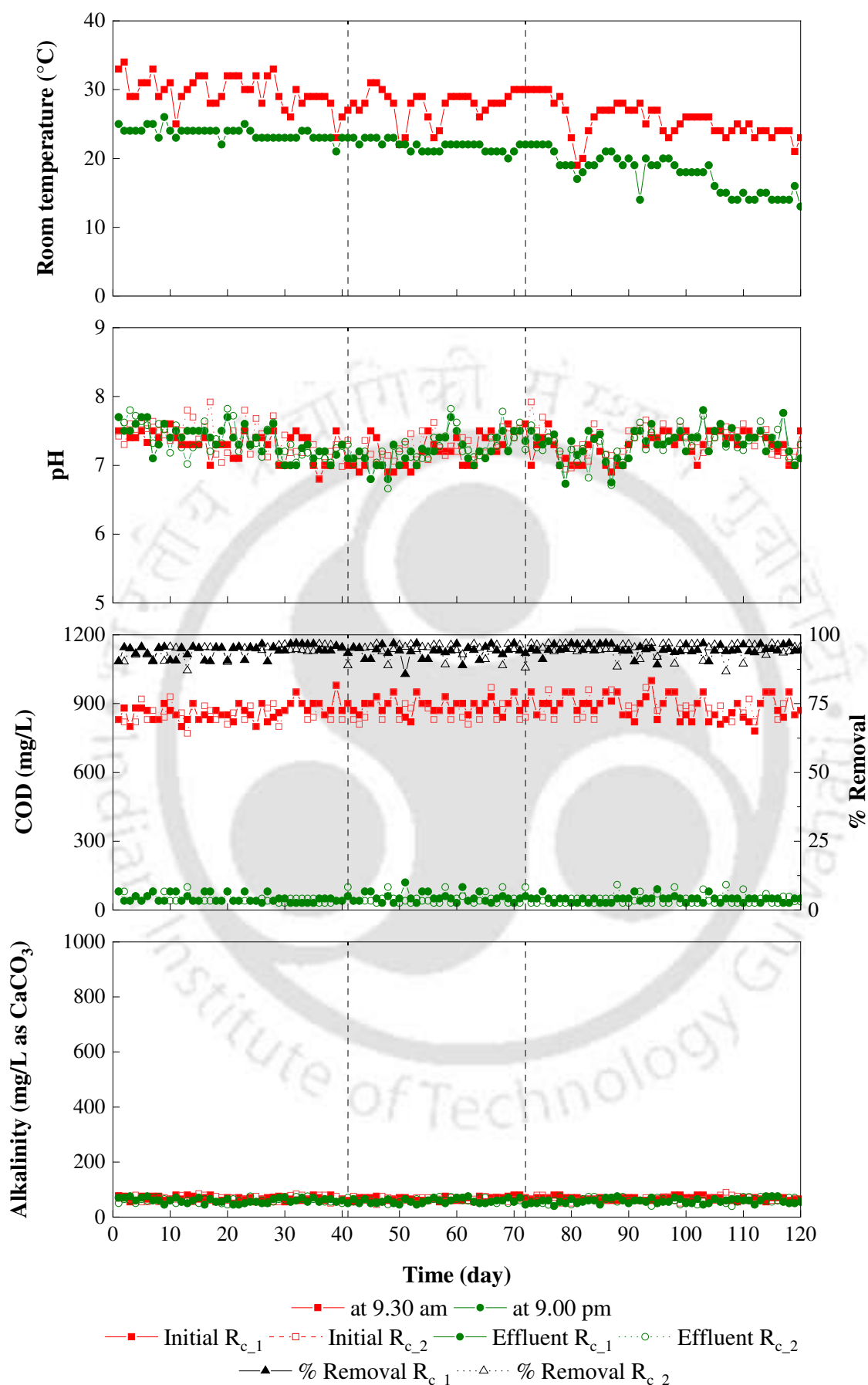


Fig. 5.25 Variation in environmental and performance related parameters for reactor R_c during 73-120 d of under recovery phase operation along with data for stressed phase I and II operations.

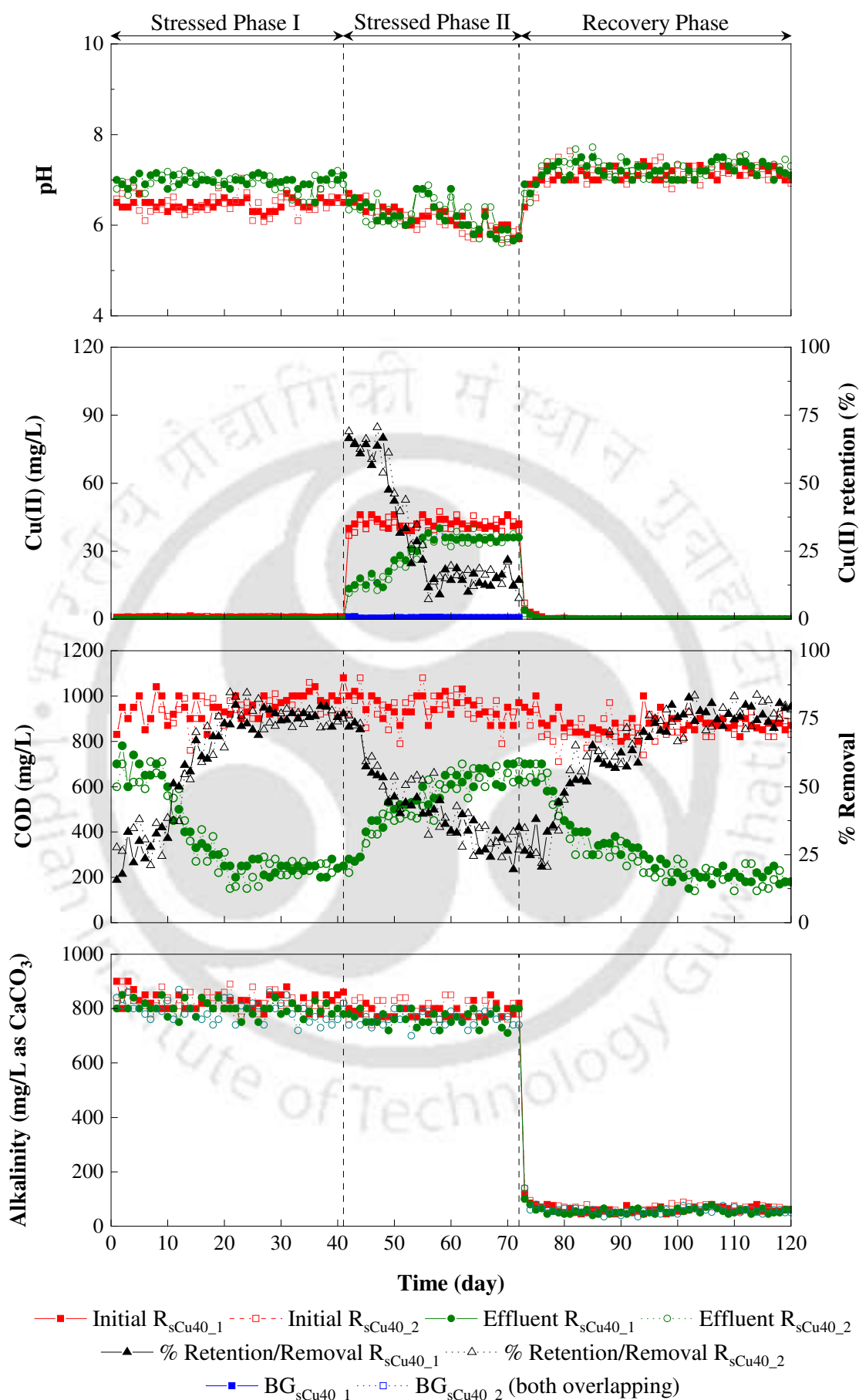


Fig. 5.26 Variation in environmental and performance related parameters for reactor R_{sCu40} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.

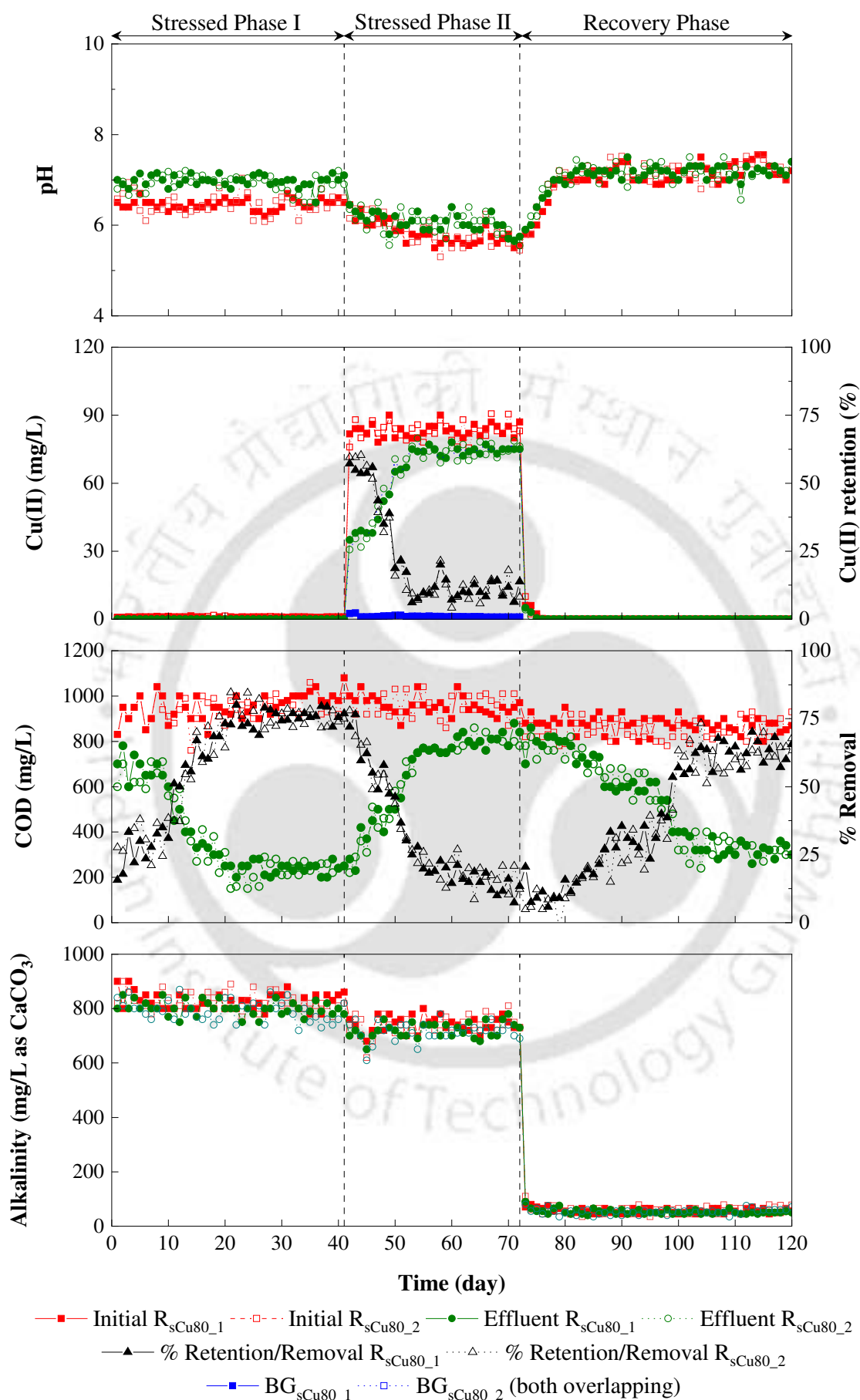


Fig. 5.27 Variation in environmental and performance related parameters for reactor R_{sCu80} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.

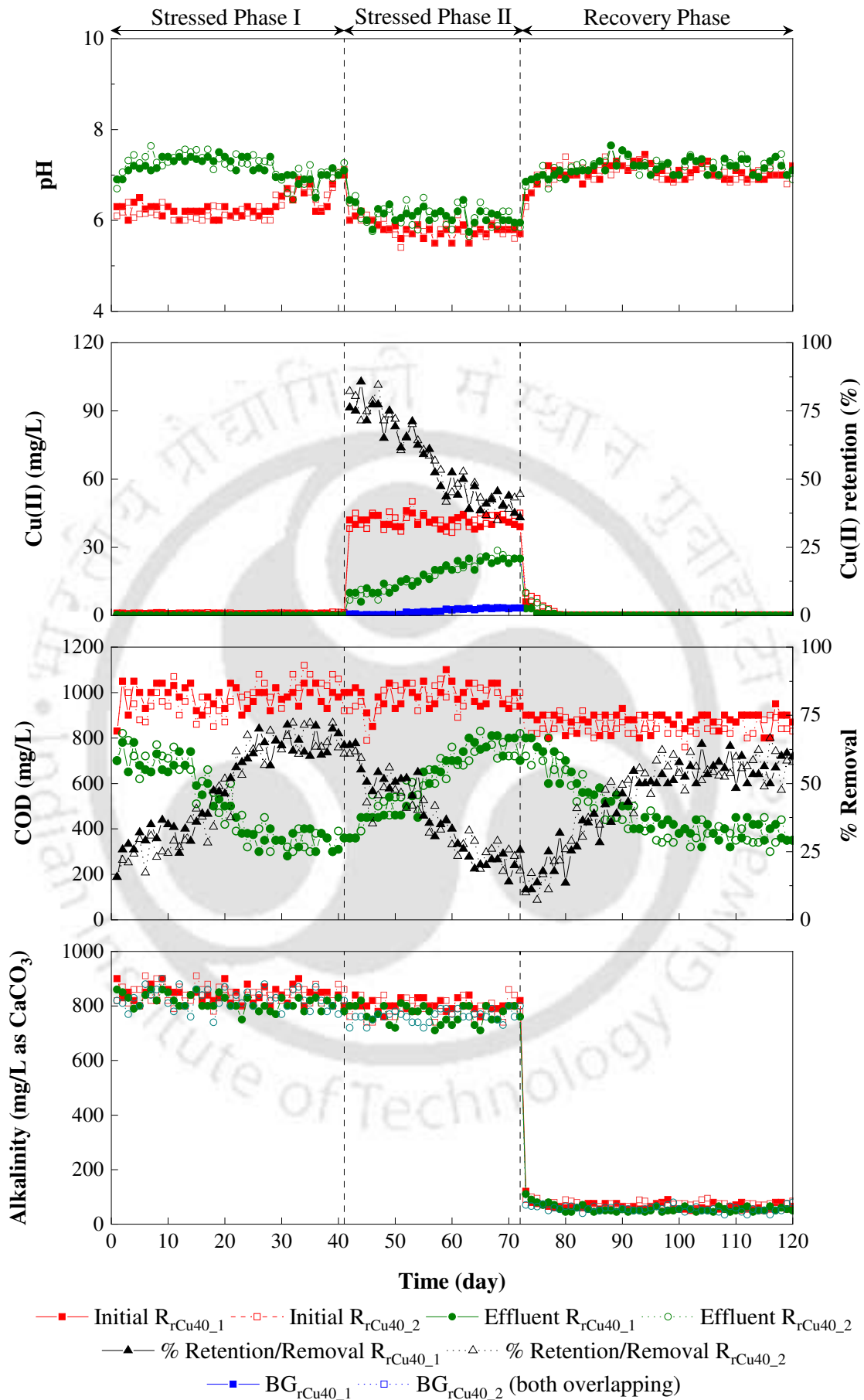


Fig. 5.28 Variation in environmental and performance related parameters for reactor R_{rCu40} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.

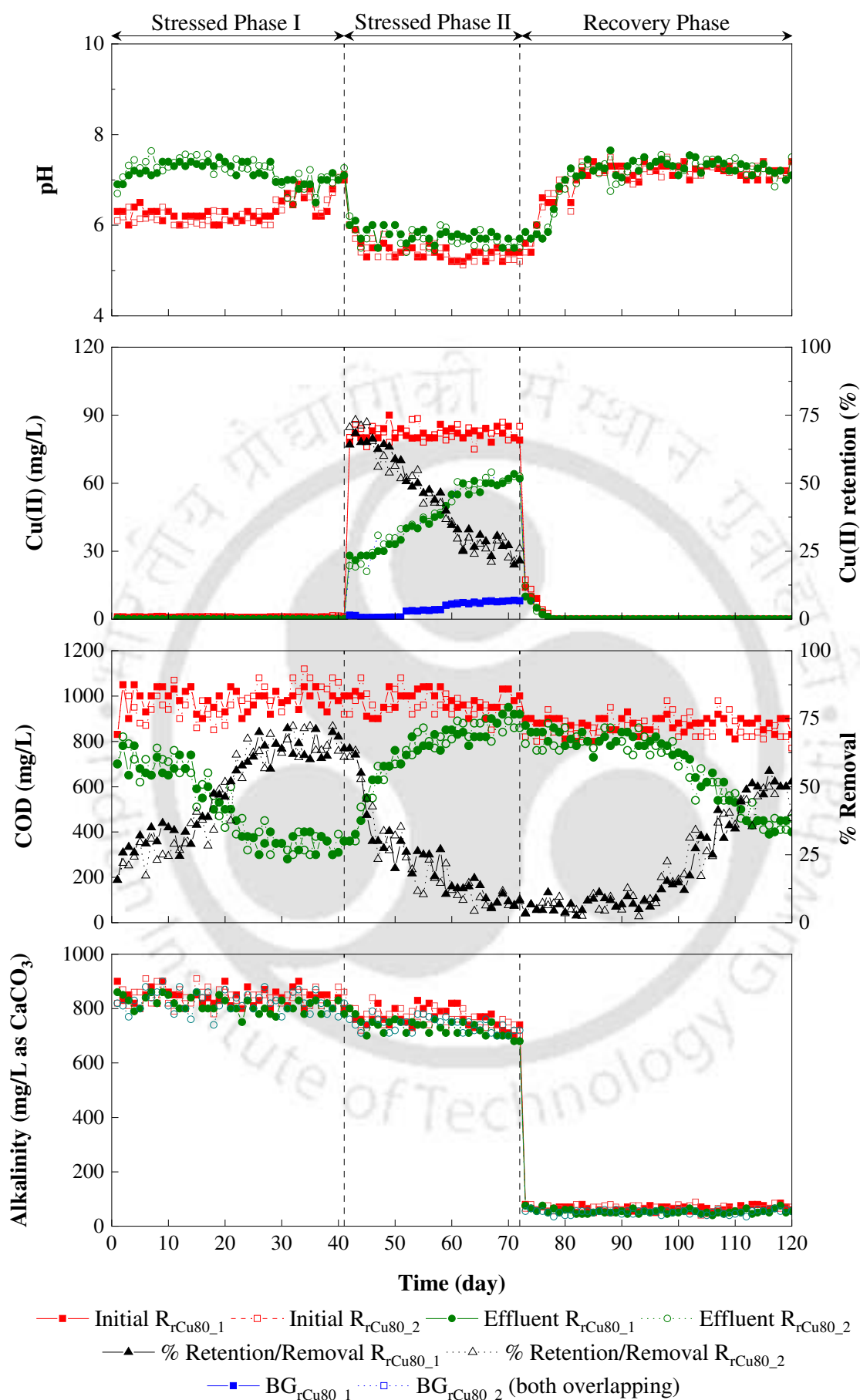


Fig. 5.29 Variation in environmental and performance related parameters for reactor R_{Cu80} operated during 73-120 d under recovery phase operation along with data for stressed phase I and II operations.

Table 5.25 Summary of environmental and performance related parameters for reactors operated during 73-120 d of recovery phase operation along with data for stressed phase I and II operations.

	Parameters	Day-wise	0-41		
		Reactor	R _c	R _s	R _r
Stressed phase I	Lactogen (g/L)	0.90	-	-	-
	PWW (mL)	0	~500-750	~500-750	~500-750
	Environmental	Room temperature (°C) at 9.30 am	29.6±2.4 (41)	29.6±2.4 (41)	29.6±2.4 (41)
		Room temperature (°C) at 9.00 pm	23.6±0.9 (41)	23.6±0.9 (41)	23.6±0.9 (41)
		Initial pH	7.35±0.22 (82)	6.47±0.17 (82)	6.32±0.28 (82)
		Initial Cu(II) (mg/L)	0.03±0.01 (82)	0.80±0.20 (82)	0.79±0.25 (82)
		Effluent pH	7.36±0.22 (82)	6.95±0.16 (82)	7.18±0.25 (82)
	Performance	Effluent Cu(II) (mg/L)	0.01±0.01 (82)	0.03±0.01 (82)	0.05±0.05 (82)
		Initial soluble COD (mg/L)	862.68±41.48 (82)	949.76±58.61 (82)	981.22±62.26 (82)
		Effluent soluble COD (mg/L)	49.76±18.14 (82)	370.00±185.07 (82)	508.41±166.31 (82)
		COD removal (%)	94.19±2.23 (82)	60.61±20.46 (82)	47.78±17.75 (82)
		Initial alkalinity (mg CaCO ₃ /L)	67.09±9.06 (82)	828.29±31.52 (82)	843.90±30.91 (82)
		Effluent alkalinity (mg CaCO ₃ /L)	59.66±8.33 (82)	796.34±32.67 (82)	818.54±32.94 (82)
Stressed phase II	Parameters	Day-wise	42-72		
		Reactor	R _c	R _{sCu40}	R _{rCu40}
	Lactogen (g/L)	0.90	-	-	-
	PWW (mL)	0	~500-750	~500-750	~500-750
	Cu(II) (mg/L)	0	40	80	40
	Environmental	Room temperature (°C) at 9.30 am	27.9±2.3 (31)	27.9±2.3 (31)	27.9±2.3 (31)
		Room temperature (°C) at 8.45 pm	21.8±0.8 (31)	21.8±0.8 (31)	21.8±0.8 (31)
		Initial pH	7.24±0.20 (62)	5.83±0.27 (62)	5.42±0.20 (62)
		Initial Cu(II) (mg/L)	0.03±0.01 (62)	83.18±3.48 (62)	82.05±2.94 (62)
		Effluent pH	7.23±0.23 (62)	6.06±0.23 (62)	6.11±0.21 (62)
	Performance	Effluent Cu(II) (mg/L)	0.01±0.01 (62)	65.42±14.83 (62)	46.04±13.48 (62)
		Cu(II) retention (%)	-	21.29±17.90 (62)	43.83±16.53 (62)
		Background Cu(II) (mg/L)	-	1.22±0.42 (62)	4.53±2.91 (62)
		Initial soluble COD (mg/L)	883.22±40.10 (62)	961.61±47.02 (62)	970.65±47.90 (62)
		Effluent soluble COD (mg/L)	51.61±22.43 (62)	664.16±185.91 (62)	753.71±149.98 (62)
		COD removal (%)	94.15±2.54 (62)	30.68±19.82 (62)	22.10±16.11 (62)
		Initial alkalinity (mg CaCO ₃ /L)	65.19±7.40 (62)	740.00±32.72 (62)	765.48±33.22 (62)
		Effluent alkalinity (mg CaCO ₃ /L)	58.74±7.77 (62)	718.06±31.50 (62)	735.48±26.98 (62)
Recovery phase	Parameters	Day-wise	73-120		
		Lactogen (g/L)	0.90	0.90	0.90
	PWW (mL)	0	0	0	0
	Cu(II) (mg/L)	0	0	0	0
	Environmental	Room temperature (°C) at 9.30 am	25.4±2.5 (48)	25.4±2.5 (48)	25.4±2.5 (48)
		Room temperature (°C) at 9.00 pm	17.7±2.7 (48)	17.7±2.7 (48)	17.7±2.7 (48)
		Initial pH	7.33±0.20 (96)	7.06±0.37 (96)	7.04±0.44 (96)
		Effluent pH	7.32±0.22 (96)	7.08±0.31 (96)	7.08±0.50 (96)
		Effluent Cu(II) (mg/L)	0.01±0.01 (96)	0.15±0.73 (96)	0.62±2.27 (96)
	Performance	Initial soluble COD (mg/L)	891.77±49.98 (96)	860.42±39.14 (96)	867.50±40.76 (96)
		Effluent soluble COD (mg/L)	46.58±18.44 (96)	523.13±193.19 (96)	687.29±153.25 (96)
		COD removal (%)	94.76±2.12 (96)	39.13±22.36 (96)	20.78±17.33 (96)
		Initial alkalinity (mg CaCO ₃ /L)	66.21±7.70 (96)	64.95±14.43 (96)	68.92±12.63 (96)
		Effluent alkalinity (mg CaCO ₃ /L)	58.79±9.16 (96)	52.96±9.70 (96)	52.81±7.91 (96)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

The variations in COD removal during the recovery phase operation have been shown in Fig. 5.25 to 5.29 and data has been summarized in Table 5.25. On 73rd d of operation (i.e., 1st d of recovery phase), the COD removal for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} has been obtained as 95.8%, 30.5%, 12.9%, 10.6%, and 3.5%, respectively, while on 96th d, 95.6%, 74.1%, 31.8%, 54.0%, and 8.0% of COD removal has been observed, respectively, for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} . Towards the end of operation (on 120th d), reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} have shown 95.0%, 78.8%, 66.7%, 58.8%, and 46.3% COD removal, respectively. The COD removal for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have improved during recovery phase operation. The average COD removals have improved from 29.0%, 14.5%, 22.3%, and 7.6% (COD removal for steady-state condition in stressed phase II operation, Table 5.15) to 64.4%, 39.1%, 44.9% and 20.8% (COD removal for recovery phase operation) for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} , respectively. The reactors R_{sCu40} and R_{rCu40} have indicated the recovery towards the end of recovery phase, whereas reactors R_{sCu80} and R_{rCu80} have been severely affected by presence of Cu(II) and has shown slow recovery.

The Cu(II) is toxic for aerobic sludge biomass and it has inhibited the activity of microorganisms in aerobic sludge biomasses. The Cu(II) has probably denatured the cellular proteins, which has resulted in decreased degradation of organic matter. When the stressed phase II operation ended, all reactors have been fed with Lactogen (as a sole carbon source) during recovery phase. The aerobic sludge biomasses have tried to repair its cellular machinery by synthesizing enzymes to start degradation of synthetic feed. Due to severe impact of Cu(II) on aerobic sludge biomasses, the COD removal process has been slowed down for reactors R_{sCu80} and R_{rCu80} during recovery phase operation. The pH has adverse influences on utilization rate and metabolic activity of microbial mass, which influences biomass yield and pollutant removal efficiency. Generally, the optimum pH for bacterial growth lies between 6.50 and 7.50. For carbonaceous substrate removal, the tolerable pH is in the range of 6.00-9.00, while the optimal performance occurs near a neutral pH (Deng et al., 2008).

The variations in alkalinity during the recovery phase operation have been shown in Fig. 5.25 to 5.29 and data has been summarized in Table 5.25. When PWW+Cu(II) stress have been stopped to the reactors, alkalinity has reached to the level of reactor R_c . The average initial alkalinity has been observed as 66.21 ± 7.70 , 64.95 ± 14.43 , 58.63 ± 11.42 , and 68.92 ± 12.63 mg/L as $CaCO_3$; whereas average effluent alkalinity has been observed as 58.79 ± 9.16 , 57.40 ± 13.31 , 52.96 ± 9.70 , and 56.48 ± 11.89 mg/L as $CaCO_3$ for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} , respectively, during recovery phase operation. The PWW has attributed to the higher alkalinity during stressed phase I and II operation. The discontinuation of PWW+Cu(II) in recovery phase operation has resulted in drastic decrease in alkalinity for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} .

The steady-state condition has been achieved after 105-110 d of recovery phase operation, and reactors have been operated in the steady-state condition during 114-120 d. The summary of steady-state performance data during recovery phase operation has

been presented in Table 5.26. The steady-state operation has shown stabilization in the performance-related parameters. Both initial and effluent pH for all reactors have been observed in the range of 7.04–7.31 for the recovery phase operation compared to the initial and effluent pH values have ranged between 5.31–7.45 for the stressed phase II operation (Table 5.26). The steady-state COD removal for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} have been obtained as 95.0%, 78.0%, 63.7%, 57.1%, and 49.2%, respectively, which has significantly improved from 94.2%, 29.0%, 14.5%, 22.3%, and 7.6%, respectively, of the stressed phase II steady-state COD removal (Table 5.26). The COD removal profile has suggested that reactors have recovered from PWW+Cu(II) impact and have shown significant organic removal. Minimum variation in alkalinity values have been obtained during the steady-state of the recovery phase operation, and it varied in the range of 55–70 mg/L as $CaCO_3$.

5.3.3.2 Biomass related general parameters

Aerobic sludge biomass characteristics

The variation in solids characteristics and SVI has been shown in Fig. 5.30 and data has been summarized in Table 5.27. The sudden decrease in solids concentration (just after 72nd d) in all reactors is due to biomass withdrawal for ASBA test during stressed phase II operation. The PWW+Cu(II) in feed has limited the aerobic sludge biomass growth during stressed phase II operation, whereas, termination of PWW+Cu(II) in the feed has allowed the reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses to recover in terms of their growth during recovery phase operation. The reactor R_c biomass has shown continuous growth during recovery phase operation. The MLSS concentration in reactor R_c has increased from 2569 mg/L to 2904 mg/L during 78th d to 85th d of operation [Fig. 5.30(a)]. Similarly, reactor R_{sCu40} has also shown increment in MLSS from 898 mg/L to 1230 mg/L during 78th d to 85th d of operation, which indicated that the reactor R_{sCu40} biomass has shown accelerated growth with an increased SVI (76 mL/g on 72nd d) [Fig. 5.30(b)]. The MLSS concentration in reactor R_{sCu80} has been observed as 670 mg/L on 78th d, which has slowly increased to 730 mg/L on 85th d operation [Fig. 5.30(c)]. On the other hand, reactor R_{rCu40} and R_{rCu80} biomasses have shown very slow growth trend and a minor increase in MLSS has been observed from 78th d to 85th d [Fig. 5.30(d)-(e)]. Also, with discontinuation of raw PWW in the feed, the zig-zag pattern in solids concentration has disappeared. Fig. 5.30 suggests that reactor R_{sCu40} biomass has shown significant biomass growth after 85th d, whereas, reactor R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses have shown slower biomass growth after 92nd d. The MLVSS/MLSS ratio has ranged between 0.52–0.92 for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses during recovery phase operation (Table 5.27). The SRT for reactors R_c has been observed around 26 d, while no sludge wastage has been carried out for reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} during the recovery phase operation.

During recovery phase operation, all reactor biomasses have exhibited a slight increase in SVI [Fig. 5.30, Table 5.27]. The SVI for reactor R_c biomass has been observed as 122, 135,

Table 5.26 Summary of environmental and performance related parameters for reactors operated during 114-120 d of steady-state condition of recovery phase operation along with data for stressed phase I and II operations.

	Parameters	Day-wise		35-41			
		Reactor	R _c	R _s	R _t		
		Lactogen 3 (g/L)	0.90	-	-		
		PWW (mL)	0	~500-750	~500-750		
Stressed phase I	Environmental	Room temperature (°C) at 9.30 am	27.3±2.2 (7)	27.3±2.2 (7)	27.3±2.2 (7)		
		Room temperature (°C) at 9.00 pm	22.7±0.8 (7)	22.7±0.8 (7)	22.7±0.8 (7)		
		Initial pH	7.11±0.22 (14)	6.51±0.12 (14)	6.65±0.34 (14)		
	Performance	Initial Cu(II) (mg/L)	0.03±0.01 (14)	0.81±0.11 (14)	0.85±0.32 (14)		
		Effluent pH	7.16±0.11 (14)	6.93±0.22 (14)	6.99±0.22 (14)		
		Effluent Cu(II) (mg/L)	0.01±0.00 (14)	0.03±0.01 (14)	0.08±0.01 (14)		
		Initial soluble COD (mg/L)	885.71±48.50 (14)	995.71±49.01 (14)	1011.43±44.86 (14)		
		Effluent soluble COD (mg/L)	45.71±17.42 (14)	241.43±22.12 (14)	348.57±39.25 (14)		
		COD removal (%)	94.84±1.96 (14)	75.70±2.61 (14)	65.41±4.65 (14)		
		Initial alkalinity (mg CaCO ₃ /L)	67.86±10.51 (14)	818.57±26.05 (14)	837.14±27.10 (14)		
Effluent alkalinity (mg CaCO ₃ /L)	58.86±6.95 (14)	780.00±24.32 (14)	807.14±28.25 (14)				
Stressed phase II	Parameters	Day-wise		66-72			
		Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{tCu40}	R _{tCu80}
		Lactogen (g/L)	0.90	-	-	-	-
		PWW (mL)	0	~500-750	~500-750	~500-750	~500-750
	Environmental	Cu(II) (mg/L)	0	40	80	40	80
		Room temperature (°C) at 9.30 am	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)	29.0±1.0 (7)
		Room temperature (°C) at 8.45 pm	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)	21.1±0.7 (7)
	Performance	Initial pH	7.45±0.13 (14)	5.89±0.22 (14)	5.71±0.23 (14)	5.79±0.12 (14)	5.31±0.09 (14)
		Initial Cu(II) (mg/L)	0.03±0.01 (14)	41.96±2.34 (14)	84.55±3.28 (14)	42.35±1.89 (14)	82.36±2.84 (14)
		Effluent pH	7.40±0.17 (14)	5.88±0.26 (14)	5.90±0.25 (14)	6.01±0.13 (14)	5.62±0.11 (14)
		Effluent Cu(II) (mg/L)	0.01±0.01 (14)	35.66±1.16 (14)	74.94±1.90 (14)	25.13±1.25 (14)	61.51±1.80 (14)
		Cu(II) retention (%)	-	14.87±3.86 (14)	11.27±3.64 (14)	40.60±3.48 (14)	25.23±3.75 (14)
		Background Cu(II) (mg/L)	-	0.60±0.01 (14)	0.91±0.02 (14)	3.21±0.13 (14)	7.94±0.21 (14)
		Initial soluble COD (mg/L)	891.43±47.68 (14)	918.57±52.51 (14)	948.57±41.00 (14)	982.86±41.43 (14)	954.29±42.78 (14)
		Effluent soluble COD (mg/L)	51.43±22.04 (14)	651.43±42.47 (14)	810.00±30.44 (14)	762.86±42.78 (14)	881.43±37.84 (14)
		COD removal (%)	94.20±2.61 (14)	28.96±4.67 (14)	14.49±3.81 (14)	22.34±3.97 (14)	7.61±1.89 (14)
		Initial alkalinity (mg CaCO ₃ /L)	68.71±5.25 (14)	798.57±28.92 (14)	751.43±26.38 (14)	800.00±25.62 (14)	735.71±24.86 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	57.86±7.21 (14)	765.71±28.16 (14)	730.00±27.09 (14)	773.57±25.34 (14)	711.43±22.67 (14)
Recovery phase	Parameters	Day-wise		114-120			
		Lactogen (g/L)	0.90	0.90	0.90	0.90	0.90
		PWW (mL)	0	0	0	0	0
		Cu(II) (mg/L)	0	0	0	0	0
	Environmental	Room temperature (°C) at 9.30 am	23.3±1.0 (7)	23.3±1.0 (7)	23.3±1.0 (7)	23.3±1.0 (7)	23.3±1.0 (7)
		Room temperature (°C) at 9.00 pm	14.3±1.0 (7)	14.3±1.0 (7)	14.3±1.0 (7)	14.3±1.0 (7)	14.3±1.0 (7)
		Initial pH	7.23±0.17 (14)	7.13±0.13 (14)	7.27±0.20 (14)	7.04±0.12 (14)	7.22±0.15 (14)
	Performance	Effluent pH	7.31±0.25 (14)	7.26±0.10 (14)	7.23±0.10 (14)	7.16±0.18 (14)	7.17±0.16 (14)
		Effluent Cu(II) (mg/L)	0.01±0.01 (14)	0.01±0.01 (14)	0.01±0.01 (14)	0.02±0.01 (14)	0.03±0.01 (14)
		Initial soluble COD (mg/L)	898.57±50.94 (14)	861.43±25.80 (14)	860.00±41.58 (14)	871.43±43.36 (14)	848.57±33.09 (14)
		Effluent soluble COD (mg/L)	45.13±12.95 (14)	190.00±29.92 (14)	311.43±22.73 (14)	372.86±44.64 (14)	430.00±26.15 (14)
		COD removal (%)	94.99±1.36 (14)	77.98±3.17 (14)	63.66±3.83 (14)	57.11±5.53 (14)	49.25±3.10 (14)
		Initial alkalinity (mg CaCO ₃ /L)	63.57±6.07 (14)	63.07±9.08 (14)	62.00±8.64 (14)	67.43±10.15 (14)	70.29±8.21 (14)
		Effluent alkalinity (mg CaCO ₃ /L)	61.29±9.49 (14)	55.00±7.07 (14)	55.29±3.55 (14)	55.00±11.00 (14)	57.86±6.65 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

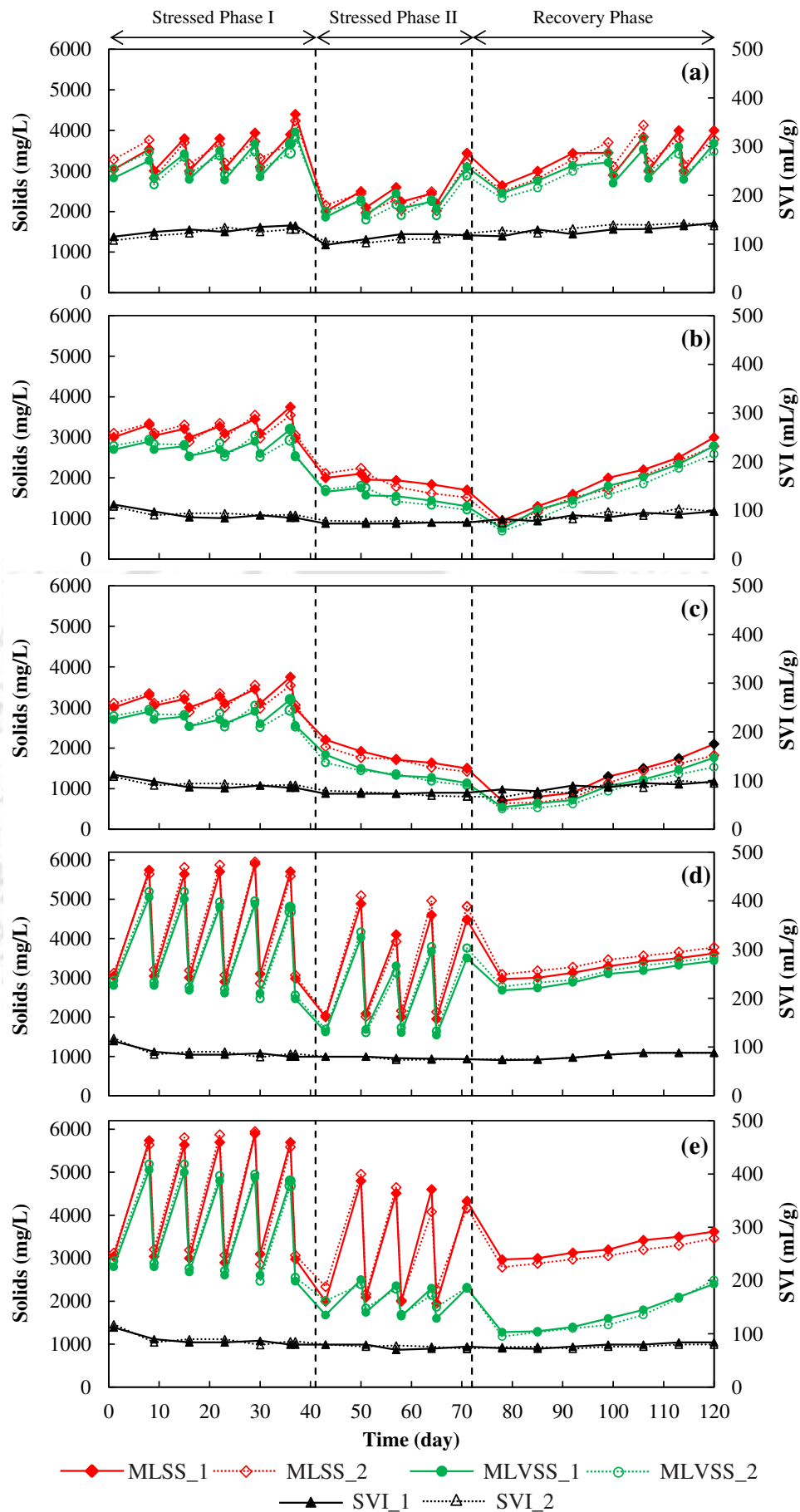


Fig. 5.30 Variation in MLSS, MLVSS, and SVI values for reactors (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , (e) R_{rCu80} during 73-120 d of recovery phase operation along with stressed phase I and II operations.

Table 5.27 Summary of solids characteristics estimated during recovery phase operation along with data for stressed phase I and II operations.

Gross parameters	Stressed phase I operation					
	Day	0-41				
	Reactor	R _c	R _s		R _r	
MLSS (mg/L)		3370.86±505.80 (14)	3119.00±242.46 (14)		3419.14±985.42 (14)	
MLVSS (mg/L)		3112.43±439.47 (14)	2680.91±206.53 (14)		2981.89±789.23 (14)	
MLVSS/MLSS ratio		0.92±0.02 (14)	0.86±0.03 (14)		0.88±0.04 (14)	
SVI (mL/g)		126.57±8.93 (14)	92.71±8.35 (14)		89.29±11.92 (14)	
ISV (m/h)		2.43±0.22 (14)	3.05±0.16 (14)		3.16±0.17 (14)	
Gross parameters	Stressed phase II operation					
	Day	42-72				
	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
MLSS (mg/L)		2337.40±556.49 (10)	1857.20±196.05 (10)	1744.00±256.59 (10)	3501.60±1374.54 (10)	3494.00±1240.03 (10)
MLVSS (mg/L)		2149.93±477.51 (10)	1495.66±188.92 (10)	1378.80±244.19 (10)	2771.63±1047.72 (10)	2096.63±267.05 (10)
MLVSS/MLSS ratio		0.92±0.02 (10)	0.80±0.02 (10)	0.79±0.03 (10)	0.80±0.02 (10)	0.65±0.18 (10)
SVI (mL/g)		111.60±8.46 (10)	75.60±1.38 (10)	73.40±3.10 (10)	77.03±2.79 (10)	76.00±3.76 (10)
ISV (m/h)		2.22±0.12 (10)	3.37±0.08 (10)	3.36±0.07 (10)	3.44±0.09 (10)	3.46±0.09 (10)
Gross parameters	Recovery phase operation					
	Day	73-120				
	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
MLSS (mg/L)		3130.57±422.41 (14)	1861.14±696.87 (14)	1224.29±502.33 (14)	3352.86±254.58 (14)	3178.29±246.11 (14)
MLVSS (mg/L)		2896.56±378.67 (14)	1690.83±681.42 (14)	1006.00±436.11 (14)	3097.60±287.20 (14)	1670.71±451.80 (14)
MLVSS/MLSS ratio		0.93±0.01 (14)	0.90±0.05 (14)	0.81±0.02 (14)	0.92±0.02 (14)	0.52±0.10 (14)
SVI (mL/g)		132.00±8.36 (14)	89.57±8.76 (14)	86.57±9.74 (14)	82.00±6.54 (14)	77.43±3.86 (14)
ISV (m/h)		1.86±0.13 (14)	3.10±0.16 (14)	3.17±0.12 (14)	3.31±0.14 (14)	3.36±0.10 (14)

[The data is presented in xx±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

and 140 mL/g on 78th, 99th, and 120th d, respectively. The SVI for reactor R_{sCu40} biomass has been observed as 78, 92, and 98 mL/g; and for reactor R_{sCu80} biomass has been observed as 82, 86, and 98 mL/g, respectively, on 78th, 99th, and 120th d, respectively. On the other hand, SVI for reactor R_{rCu40} biomass has been observed as 74, 84, and 88 mL/g, while SVI for reactor R_{rCu80} biomass has been observed as 74, 78, and 82 mL/g on 78th, 99th, and 120th d, respectively. The increase in SVI could be due to growth of filamentous microorganisms during recovery phase operation. The average SVI for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 132.00 ± 8.36 , 89.57 ± 8.76 , 86.57 ± 9.74 , 82.00 ± 6.54 , and 77.43 ± 3.86 mL/g, respectively, during recovery phase operation (Table 5.27).

The interface settling velocity (ISV) results have been shown in Fig. 5.31 and data has been summarized in Table 5.27. During recovery phase operation, ISV for reactor R_c biomass has been observed as 2.11, 1.90, and 1.81 m/h on 78th, 99th, and 120th d of operation, respectively. The ISV has decreased gradually for reactor R_c biomass till the end of the recovery phase operation as compared to stressed phase II operation. On 78th, 99th, and 120th d of operation, ISV for reactor R_{sCu40} biomass has been observed as 3.43, 3.00, and 3.07 m/h, respectively and for reactor R_{sCu80} biomass has been observed as 3.41, 3.12, and 3.10 m/h, respectively. In addition, ISV for reactor R_{rCu40} biomass has been observed as 3.51, 3.31, and 3.20 m/h, respectively and for reactor R_{rCu80} biomass has been observed as 3.51, 3.31, and 3.31 m/h, respectively, on 78th, 99th, and 120th d of operation. The reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have shown slight decrease in settling velocity. The tiny decrease in settling behavior is due to reappearance of filamentous microorganism in reactors. The average ISV has been observed as 1.86 ± 0.13 , 3.10 ± 0.16 , 3.17 ± 0.12 , 3.31 ± 0.14 , and 3.36 ± 0.10 m/h for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, during recovery phase operation.

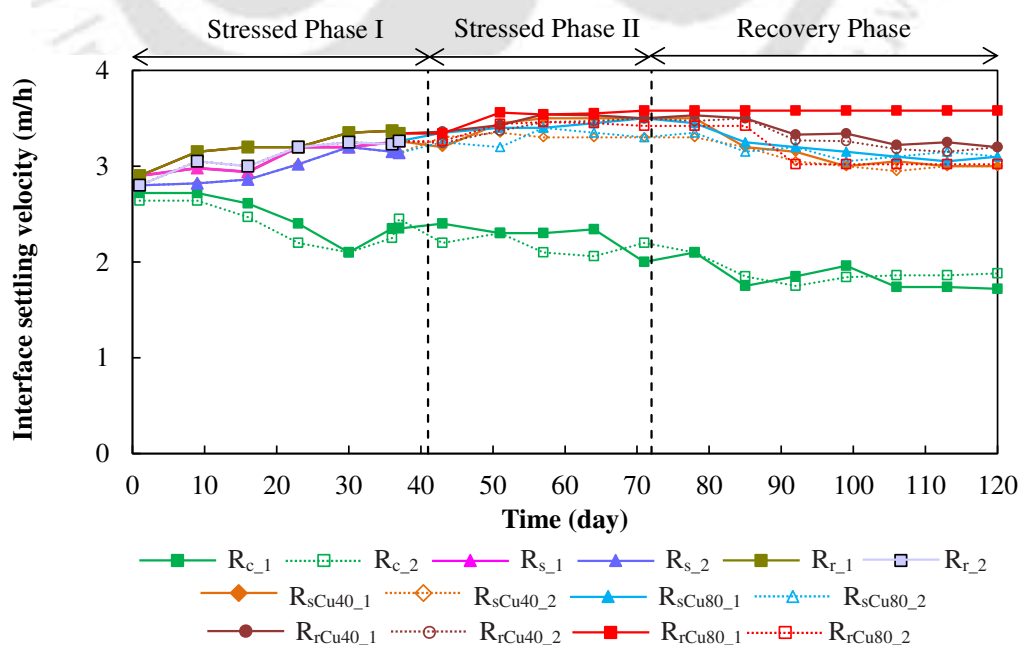


Fig. 5.31 Variation in interface settling velocities of reactor biomass during 73-120 d of recovery phase operation along with data for stressed phase I and II operations.

Aerobic sludge biomass activity test

When the reactors have achieved steady-state condition in the recovery phase operation, biomass has been withdrawn from all the reactors on 120th d and ASBA test has been performed. The test results have been shown in Fig. 5.32, while data has been presented in Table 5.28. The ASBA for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has been estimated as 7.80, 5.77, 2.82, 3.16, and 1.59 mg COD removed/mg VSS d, respectively, towards the end of recovery phase operation (Table 5.28), which has improved from 7.75, 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, respectively, obtained towards the

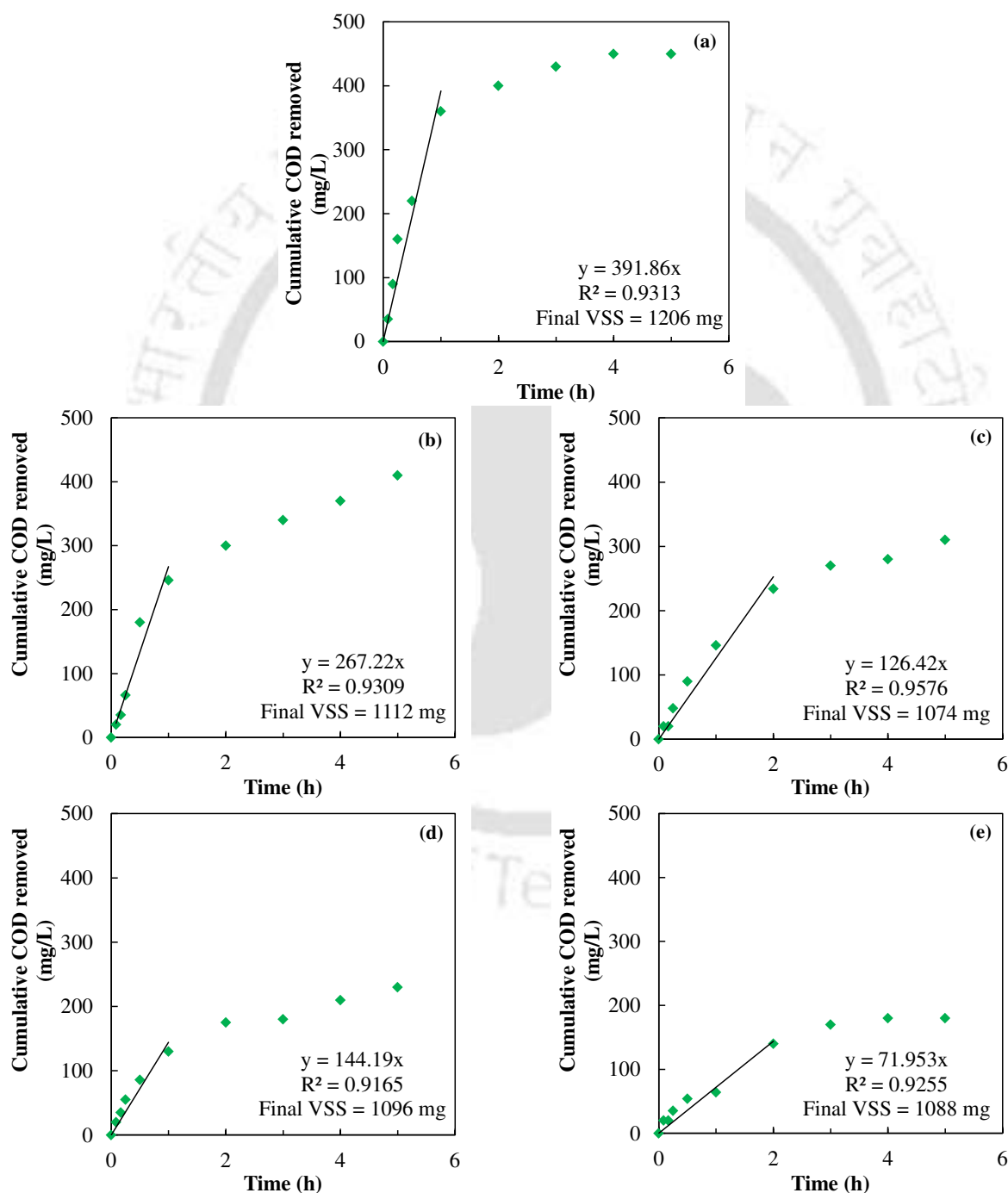


Fig. 5.32 Aerobic sludge biomass activity test results with sludge biomass withdrawn from reactor (a) R_c , (b) R_{sCu40} , (c) R_{sCu80} , (d) R_{rCu40} , and (e) R_{rCu80} on 120th d of recovery phase operation.

Table 5.28 Aerobic sludge biomass activity test results carried out towards the end of recovery phase operation along with data for stressed phase I and II operations.

Stressed phase I operation					
Biomass withdrawn on			41 st d		
Reactor	R _c		R _s	R _r	
ASBA (mg COD removed/mg VSS d)	7.03		5.87	5.05	
Stressed phase II operation					
Biomass withdrawn on			72 nd d		
Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
ASBA (mg COD removed/mg VSS d)	7.75	2.89	1.02	1.65	0.86
Recovery phase operation					
Biomass withdrawn on			120 th d		
Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
ASBA (mg COD removed/mg VSS d)	7.80	5.77	2.82	3.16	1.59

end of the stressed phase II operation. The discontinuation of PWW and Cu(II) in feed has shown recovery symptoms in aerobic biomass activity. The reactor R_{sCu40} and R_{rCu40} biomasses have tried to recover with improvement in ASBA. However, reactor R_{sCu80} and R_{rCu80} biomasses have been adversely affected by the PWW+Cu(II) in terms of the microbial activity and have resulted in slight improvement in ASBA during recovery phase operation. During this phase, aerobic sludge biomass has regained its metabolic capacity by repairing its enzymes and cellular machinery. It is also reported that presence of increasing concentrations and prolonged exposure to copper and zinc leads to a decreasing specific anammox activity (SAA) (Lotti et al., 2012).

Upon re-visiting biomass related general parameters estimated during recovery phase operation; it appears that the MLSS and MLVSS values have shown increasing trends for all previously stressed reactors. The reactor R_{sCu40} biomass has shown accelerated growth, while reactor R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have shown slower growth. Also, the ratio of MLVSS/MLSS appears to have increased for reactor R_{sCu40} , R_{sCu40} , and R_{rCu40} biomasses in the range of 0.81-0.92 from 0.79-0.80 of stressed phase II values. However, the MLVSS/MLSS ratio for reactor R_{rCu80} biomass appears to have decreased to 0.52 compared to 0.65 observed during stressed phase II operation, which might be attributed to absence of organic solids from raw PWW feed. The increasing trend in MLSS and MLVSS values for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses as well as increased MLVSS/MLSS ratio for reactor R_{sCu40} , R_{sCu80} , and R_{rCu40} biomasses have indicated regrowth of biomass in the absence of imposed stresses. SVI have also shown increasing trend for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses. The average SVI have increased in the range of 82.00-89.57 mL/g for reactor R_{sCu40} , R_{sCu80} , and R_{rCu40} biomasses during recovery phase compared to values of 73.40-77.03 for reactor R_{sCu40} , R_{sCu80} , and R_{rCu40} biomasses during stressed phase II operation. However, there is little improvement for SVI for reactor R_{rCu80} biomass during recovery phase compared to stressed phase II operation. Even average values of ISV have shown decreasing trend with values lying in the range of 3.10-3.36 m/h for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses hinting towards growth of filamentous fraction upon discontinuation of imposed stresses. The ASBA estimated towards the end of recovery phase operation for reactor R_{sCu40} ,

R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses has been 5.77, 2.82, 3.16 and 1.59 mg COD removed/mg VSS d, respectively, which is on a higher side compared to 2.89, 1.02, 1.65 and 0.86 mg COD removed/mg VSS d for respective reactor biomasses obtained towards the end of stressed phase II operation. It has indicated considerable improvement in bio-degradation capability of organics by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses under recovery phase operation. The COD removals have now increased to ~78%, 64%, 57% and 49% by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses under steady state of recovery phase from ~29%, 14%, 22% and 8% by respective reactors during steady state of stressed phase II operation — a huge improvement in organics degradation. Therefore, taking into consideration SVI, ISV and ASBA parameters together, the increasing trend in SVI and decreasing trend in ISV of biomasses may be taken as an indication for improvement in bio-degradation capability of biomasses as observed with increasing values of ASBA under recovery phase operation. However, complete recovery after discontinuation of imposed stresses might take much longer duration as improvements under steady-state conditions have been observed to be slow.

5.3.3.3 Biomass related microbiological parameters

Aerobic sludge biomass morphology and viability

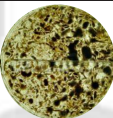
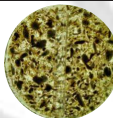
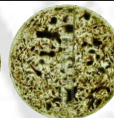
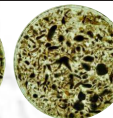
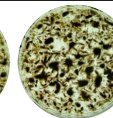
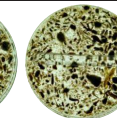
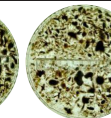
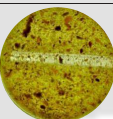
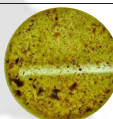
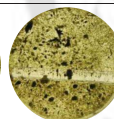
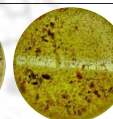
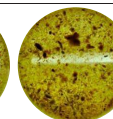
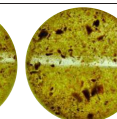
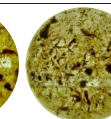
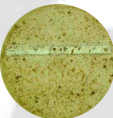
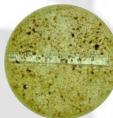
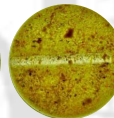
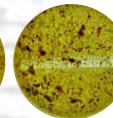
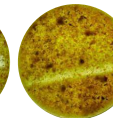
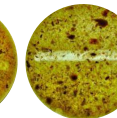
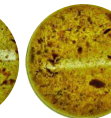
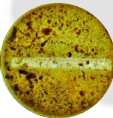
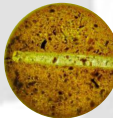
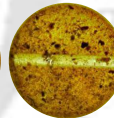
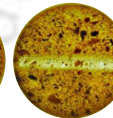
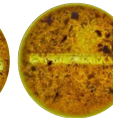
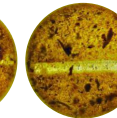
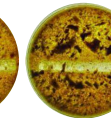
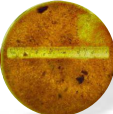
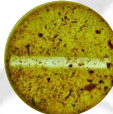
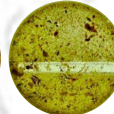
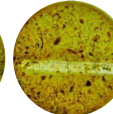
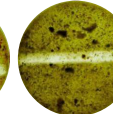
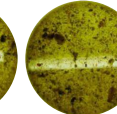
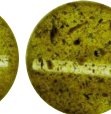
During recovery phase operation, microscopic examination after Gram's staining (at 5× and 100× magnifications), photographic observation, filament index, and cell viability test have been carried out to analyze flocs structure and morphology of aerobic sludge biomass. Microscopic images of aerobic biomass have been shown in Table 5.29. The reactor R_c biomass has shown uniformity in flocs structure (at 5× magnification) and almost similar filament density (at 100× magnification) from 78th d to 120th d. Similarly, reactor R_{sCu40} biomass has started to grow after 92nd d and increased flocs size has been observed after 99th d. The filament density has also been increasing over the time (from 78th d to 120th d) for reactor R_{sCu40} biomass. The increased flocs size has been observed for reactor R_{sCu80} biomass from 106th d and filament density has also started to increase. On the other hand, reactor R_{rCu40} and R_{rCu80} biomasses have started to increase in flocs size and number of filaments. Furthermore, for reactor R_{rCu40} and R_{rCu80} biomasses, the increase in flocs size and filaments has been observed slower than for reactor R_{sCu40} and R_{sCu80} biomasses (Table 5.29). The microscopic results have suggested that reactor R_{rCu40} and R_{rCu80} biomasses have been significantly affected in stressed phase II operation and slowly recovering during recovery phase operation.

The photographic observations have also been carried out to see the changes in flocs morphology during recovery phase operation, and the images have been presented in Table 5.30. The reactor R_c biomass has shown uniform floc size throughout the operation (78th to 120th d), with thick and denser floc structure. In addition, reactors R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have not shown changes from 78th d to 92nd d in terms of flocs morphology, whereas flocs size started to increase after 99th d onward. The reactor R_{sCu40} biomass have shown an increment in flocs size (1.0-11.0 mm, on 120th d) with reappearance of filaments. In contrast, reactor R_{sCu80} biomass has been fed with a high dose of Cu(II) (80

Table 5.29 Microscopic examination of Gram's stained aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.

Aerobic sludge biomass samples withdrawn on																						
Reactor	Mag.	Stressed phase I operation						Reactor	Mag.	Stressed phase II operation					Recovery phase operation							
		1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d			43 rd d	50 th d	57 th d	64 th d	71 st d	78 th d	85 th d	92 nd d	99 th d	106 th d	113 th d	120 th d	
R _c	5×							R _c	5×													
	100×								100×													
R _s	5×							R _{sCu40}	5×													
	100×								100×													
	R _r	5×							R _{rCu40}	5×												
		100×								100×												
R _r		5×							R _{rCu80}	5×												
		100×								100×												

Table 5.30 Photographic images of aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.

Aerobic sludge biomass samples withdrawn on																			
Reactor	Stressed phase I operation						Reactor	Stressed phase II operation					Recovery phase operation						
	1 st d	8 th d	15 th d	22 nd d	29 th d	36 th d		43 rd d	50 th d	57 th d	64 th d	71 st d	78 th d	85 th d	92 nd d	99 th d	106 th d	113 th d	120 th d
R _c							R _c												
R _s							R _{sCu40}												
	R _{sCu80}																		
R _r							R _{rCu40}												
	R _{rCu80}																		

mg/L); flocs morphology have failed to recover completely as compared to reactor R_c biomass; however slightly improved flocs size was observed on 120th d. The reactors R_{rCu40} and R_{rCu80} have been fed with raw PWW+Cu(II), which led to accumulation of small particles of solids and disintegrated flocs, hence small granular flocs have been observed in both the reactor R_{rCu40} and R_{rCu80} biomasses. The reactor R_{rCu80} biomass has not been able to recover in terms of flocs morphology.

The aerobic sludge biomass has been stained and observed under a fluorescence microscope at 20× magnification for cell viability, and results have been illustrated in Table 5.31. During recovery phase operation, for reactor R_c biomass, no change in the active part of aerobic sludge biomass has been observed as compared to stressed phase II operation images (as shown in Table 5.31). However, the discontinuation of PWW+Cu(II) in the feed has increased the active part of biomass in the stressed reactors towards the end of the recovery phase operation. On the 120th d of operation, live cells for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been estimated as 92.8%, 82.1%, 53.7%, 57.2%, and 45.6%, respectively. On the other hand, dead cells for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been estimated as 1.8%, 14.2%, 44.1%, 22.0, and 42.8% of total image area, respectively. The reactor R_{sCu40} and R_{rCu40} biomasses have a significantly higher active biomass than reactor R_{sCu80} and R_{rCu80} biomasses. In addition, the active part of reactor R_{sCu80} and R_{rCu80} biomasses have slightly increased during recovery phase operation. All the reactors have shown increased active biomass part during the recovery phase operation, while a small part of active biomass has been observed for reactor R_{sCu80} and R_{rCu80} biomasses (Table 5.31), which has suggested that Cu(II) at 80 mg/L along with PWW in the feed has adversely affected the aerobic sludge biomass.

FI have also been estimated during recovery phase operation, and data has been presented in Table 5.32. The reactor R_c biomass has shown FI of 5 indicating presence of filaments throughout the operations (from 78th to 120th d). In addition, FI for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been observed as 2, 1, 1, and 1, respectively, on 78th and 85th d of operation, which is similar to 71st d of stressed phase II operation. The FI value for reactor R_{sCu40} biomass has increased from 2 to 3 during 85th d to 113th d and FI of 4 has been observed towards the end of the recovery phase operation (120th d). Similarly, FI for reactor R_{sCu80} biomass has increased from 2 to 3 during 92nd d to 120th d. The reactor R_{rCu40} and R_{rCu80} biomasses have shown similarity in FI which have increased from 1 to 2 during 78th d to 120th d. The results suggested that the biomass exposed to lower doses of Cu(II) in reactor R_{sCu40} has shown reappearance of filaments during recovery phase operation. On the other hand, growth of filamentous biomass has been slow due to higher Cu(II) doses for reactor R_{sCu80} biomass and raw PWW+Cu(II) for reactor R_{rCu40} and R_{rCu80} biomasses. These results suggested that reactor R_{sCu40} and R_{rCu40} biomasses showed recovery in terms of active/inactive cells portion, whereas reactor R_{sCu80} and R_{rCu80} biomasses could not be able to recover completely towards the end of the recovery phase operation. The information related to the morphology of aerobic sludge biomass exposed to PWW and PWW+Cu(II) have not

Table 5.31 Fluorescence microscopic images of aerobic sludge biomasses withdrawn from reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} in recovery phase operation along with images of biomass withdrawn during stressed phase I and II operations.

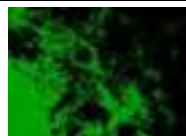
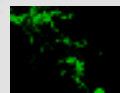
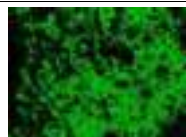
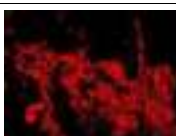
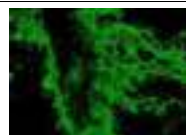
Aerobic sludge biomass samples withdrawn on													
Reactors	Stressed phase I operation						Reactors	Stressed phase II operation			Recovery phase operation		
	1 st d			41 st d				72 nd d			120 th d		
	Bright field (20×)	Live cells (20×)	Dead cells (20×)	Bright field (20×)	Live cells (20×)	Dead cells (20×)		Bright field (20×)	Live cells (20×)	Dead cells (20×)	Bright field (20×)	Live cells (20×)	Dead cells (20×)
R _c							R _c						
	R _s							R _{sCu40}					
							R _{sCu80}						
R _r							R _{rCu40}						
							R _{rCu80}						

Table 5.32 Filament index of aerobic sludge biomass estimated during stressed phase I, II and recovery phase operations.

Biomass withdrawn on		Stressed phase I operation				
Day	R _c	R _s			R _r	
1 st d	5	5			5	
8 th d	5	5			4	
15 th d	5	4			4	
22 nd d	5	4			3	
29 th d	5	4			3	
36 th d	5	3			2	
Stressed phase II operation						
	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	
43 rd d	5	3	3	2	2	
50 th d	5	3	2	2	2	
57 th d	5	3	2	2	2	
64 th d	5	3	2	1	1	
71 st d	5	2	1	1	1	
Recovery phase operation						
	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	
78 th d	5	2	1	1	1	
85 th d	5	2	1	1	1	
92 nd d	5	3	2	1	1	
99 th d	5	3	2	1	1	
106 th d	5	3	3	1	1	
113 th d	5	3	3	1	1	
120 th d	5	4	3	2	2	

been reported in the literature. The available literature have focused on reactor performance and nutrients removal (Obaja et al., 2003; Sombatsompop et al., 2011; Zhou et al., 2016) and therefore comparison of the results could not be carried out.

Polysaccharide, protein and extracellular polymeric substance

The variations in PS, PN, and EPS contents of aerobic sludge biomass during recovery phase operation have been shown in Fig. 5.33 and data has been summarized in Table 5.33. PS, PN, and EPS contents in reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses have appeared to be stabilized during recovery phase operation. On the 120th d of operation, PS content in reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses have been observed as 16.42, 17.64, 17.80, 19.22, and 19.12 mg/g VSS, respectively [Fig. 5.33(a)], while PN content has been observed as 40.46, 43.92, 44.52, 47.34, and 47.00 mg/g VSS, respectively [Fig. 5.33(b)]. Similarly, EPS content in reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses have been observed as 55.72, 61.03, 62.12, 64.92, and 65.02 mg/g VSS, respectively, towards the end of the recovery phase operation [Fig. 5.33(c)]. The discontinuation of PWW+Cu(II) in the feed to the reactors during recovery phase operation has relieved the stress to aerobic sludge biomass. In response to relived stress, secretion of EPS has been stabilized towards the end of the operation.

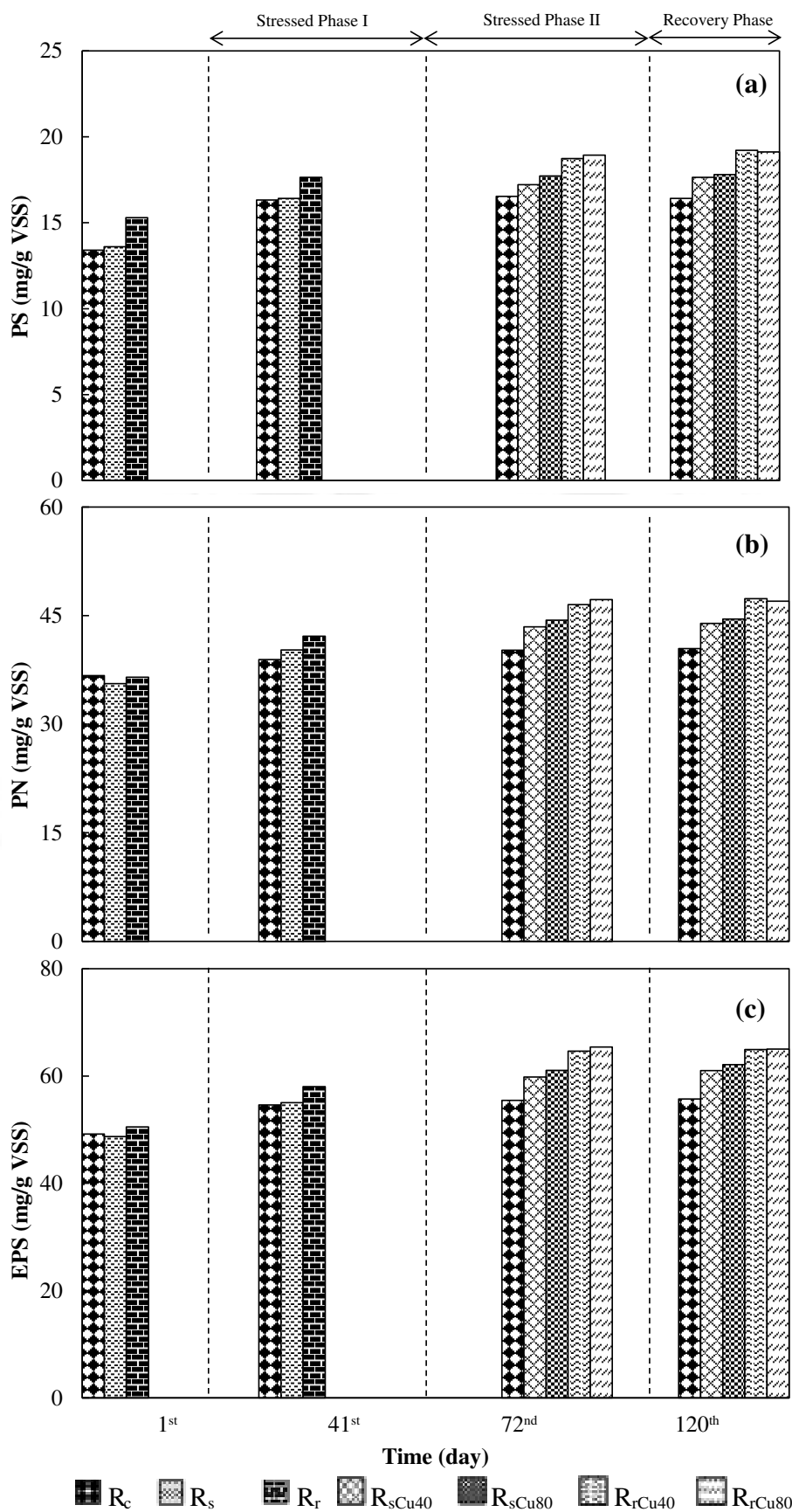


Fig. 5.33 Aerobic sludge biomass assessed for (a) PS, (b) PN, and (c) EPS contents during recovery phase operation along with data for stressed phase I and II operations.

Table 5.33 PS, PN and EPS contents in aerobic sludge biomass estimated during recovery phase operation along with data for stressed phase I and II operations.

Biomass withdrawn on	Reactor biomass	PS	PN	EPS
(mg/g VSS)				
Stressed phase I operation				
1st d	R_c	13.42±1.51	36.71±3.04	49.22±4.18
	R_s	13.60±1.22	35.62±2.31	48.71±3.27
	R_r	15.31±1.10	36.50±3.21	50.51±2.88
41st d	R_c	16.32±1.42	38.92±2.40	54.62±4.64
	R_s	16.42±1.62	40.26±1.42	55.02±3.42
	R_r	17.64±1.32	42.14±2.32	58.02±3.20
Stressed phase II operation				
72nd d	R_c	16.54±1.02	40.22±1.42	55.44±4.04
	R_{sCu40}	17.22±0.80	43.46±1.82	59.82±4.62
	R_{sCu80}	17.72±1.02	44.36±1.60	61.06±4.06
	R_{rCu40}	18.73±1.44	46.52±1.32	64.62±4.54
	R_{rCu80}	18.94±1.02	47.22±1.92	65.42±2.02
Recovery phase operation				
120th d	R_c	16.42±1.20	40.46±1.82	55.72±4.20
	R_{sCu40}	17.64±1.26	43.92±1.12	61.00±3.52
	R_{sCu80}	17.80±1.20	44.52±2.12	62.12±4.52
	R_{rCu40}	19.22±1.32	47.34±1.90	64.92±3.52
	R_{rCu80}	19.12±1.52	47.00±1.42	65.02±2.52

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

Enzyme activity

Four enzymes namely catalase, dehydrogenase, protease and urease activities have been estimated on 120th d of operation under recovery phase operation as shown in Fig. 5.34 and summary is presented in Table 5.34. E_{Catalase} for all the reactor biomasses on 120th d of operation has been shown in Fig. 5.34(a). When PWW+Cu(II) has been discontinued in the feed, E_{Catalase} for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses has been observed as 0.44, 0.31, 0.15, 0.29, and 0.13 mg/g MLSS h, respectively, towards the end of recovery phase operation. During stressed phase operation, E_{Catalase} for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses has been observed as 0.42, 0.20, 0.10, 0.18 and 0.08 mg/g MLSS h, respectively, which is lower than the values obtained during recovery phase. These results have suggested partial recovery of aerobic biomass in terms of E_{Catalase} .

$E_{\text{Dehydrogenase}}$ for all reactor biomasses has been shown in Fig. 5.34(b). On 120th d of operation, $E_{\text{Dehydrogenase}}$ for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses has been observed as 29.74, 25.41, 18.90, 23.11, and 12.11 µg/g MLSS h, respectively. Whereas, $E_{\text{Dehydrogenase}}$ for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses has been obtained as 30.12, 19.23, 12.33, 16.53, and 8.01 µg/g MLSS h, respectively, during stressed phase II operation. Discontinuation of PWW+Cu(II) in the feed have resulted in recovery of $E_{\text{Dehydrogenase}}$. The reactor R_{sCu40} and R_{rCu40} biomasses have significantly recovered as compared

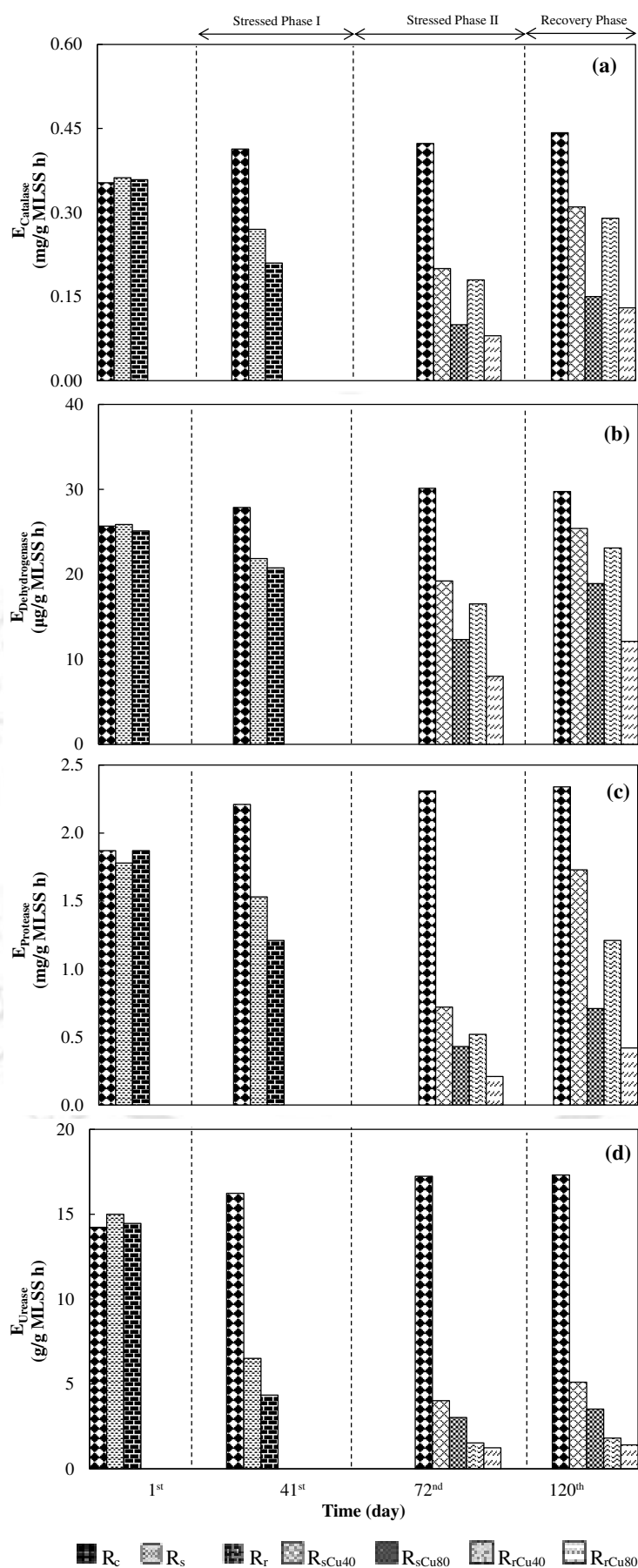


Fig. 5.34 Enzyme activities of aerobic sludge biomass in recovery phase operation (a) $E_{Catalase}$, (b) $E_{Dehydrogenase}$, (c) $E_{Protease}$, and (d) E_{Urease} along with data for stressed phase I and II operations.

Table 5.34 Summary of enzyme activities of aerobic sludge biomass assessed during recovery phase along with data for stressed phase I and II operations.

Biomass withdrawn on	Reactor biomass	E _{Catalase} (mg/g MLSS h)	E _{Dehydrogenase} (μg/g MLSS h)	E _{Protease} (mg/g MLSS h)	E _{Urease} (g/g MLSS h)
Stressed phase I operation					
1st d	R_c	0.35±0.03	25.67±1.88	1.87±0.17	14.23±1.10
	R_s	0.36±0.03	25.87±1.57	1.78±0.19	15.04±1.28
	R_r	0.36±0.02	25.11±2.14	1.87±0.12	14.46±1.17
41st d	R_c	0.41±0.02	27.87±2.23	2.21±0.12	16.23±0.92
	R_s	0.27±0.03	21.87±1.81	1.53±0.10	6.51±0.92
	R_r	0.21±0.03	20.76±1.72	1.21±0.12	4.34±1.11
Stressed phase II operation					
72nd d	R_c	0.42±0.03	30.12±1.81	2.31±0.11	17.23±0.80
	R_{sCu40}	0.20±0.01	19.23±1.73	0.72±0.07	4.02±0.81
	R_{sCu80}	0.10±0.01	12.33±2.12	0.43±0.10	3.02±1.04
	R_{rCu40}	0.18±0.01	16.53±2.05	0.52±0.08	1.52±0.64
	R_{rCu80}	0.08±0.03	8.01±2.04	0.21±0.05	1.24±0.51
Recovery phase operation					
120th d	R_c	0.44±0.04	29.74±1.94	2.34±0.15	17.31±1.22
	R_{sCu40}	0.31±0.02	25.40±2.12	1.73±0.12	5.10±0.63
	R_{sCu80}	0.15±0.03	18.90±1.36	0.71±0.08	3.52±0.90
	R_{rCu40}	0.29±0.03	23.11±1.95	1.21±0.10	1.81±0.62
	R_{rCu80}	0.13±0.02	12.11±1.12	0.42±0.13	1.41±0.61

[The data is presented in xx±yy format where xx is the average value, yy is the standard deviation.]

to reactor R_c biomass, while reactor R_{sCu80} and R_{rCu80} biomasses have shown partial recovery.

E_{Protease} for all reactor biomasses during recovery phase operation has been shown in Fig. 5.34(c). On 120th d of operation, 2.34, 1.73, 0.71, 1.21, and 0.42 mg/g MLSS h of E_{Protease} has been observed for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses, respectively, which has increased from 2.31, 0.72, 0.43, 0.52, and 0.21 mg/g MLSS h, respectively, of the stressed phase II operation (on 72nd d). The reactor R_{sCu40} and R_{rCu40} biomasses have shown recovery; however, reactor R_{sCu80} and R_{rCu80} biomasses have failed to recover during recovery phase operation.

E_{Urease} for all reactor biomasses have also been significantly affected in the presence of PWW+Cu(II) in the feed and have slightly recovered during recovery phase operation as shown in Fig. 5.34(d). On 120th d of operation, E_{Urease} has been observed as 17.31, 5.10, 3.52, 1.81, and 1.41 g/g MLSS h for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses, respectively. E_{Urease} values have been 17.23, 4.02, 3.02, 1.52, and 1.24 g/g MLSS h for reactor R_c, R_{sCu40}, R_{sCu80}, R_{rCu40}, and R_{rCu80} biomasses, respectively, on 72th d of stressed phase II operation. E_{Urease} has been significantly affected during stressed phase II operation and post-exposure impacts of PWW+Cu(II) has lasted in recovery phase operation. The reactor biomasses exposed to 40 mg/L of Cu(II) along with screened PWW has tried to recover and biomass exposed to 80 mg/L of Cu(II) with raw PWW has shown slight recovery.

The results suggested that E_{Catalase} , $E_{\text{Dehydrogenase}}$, E_{Protease} , and E_{Urease} have been severely affected during stressed phase II operation (as shown in Fig. 5.34 and Table 5.34). Furthermore, E_{Catalase} , $E_{\text{Dehydrogenase}}$, and E_{Protease} has shown significant recovery for reactor R_{Scu40} and R_{rCu40} biomasses, while slight improvement has been observed for the reactor R_{Scu80} and R_{rCu80} biomasses. This shows that biomasses exposed to lower doses of Cu(II) (40 mg/L) have shown recovery in terms of enzyme activities, whereas biomasses exposed to higher Cu(II) (80 mg/L) could not recover from Cu(II) stress. E_{Urease} has been drastically affected during stressed phase II operation, and it has not recovered during recovery phase operation.

Amplified ribosomal DNA restriction analysis (ARDRA)

The aerobic sludge biomass samples have been withdrawn on 120th d of the operation and examined through ARDRA to determine the possible changes in microbial communities. Fig. 5.35 shows the gel run after the digestion of three different restriction enzymes (*AluI*, *HhaI*, and *MspI*) and dendrogram has been shown in Fig. 5.36, which has been prepared based on the similarity matrix presented in Table 5.35.

The gel image has shown different banding pattern when digested with *AluI*, *HhaI*, and *MspI* restriction enzymes. The restriction enzyme *AluI* has produced 14, 10, 9, 10, and 7 bands at different positions for reactor R_c , R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Fig. 5.36(g)) has shown that reactor R_{Scu40} biomass has been closer to the control reactor R_c biomass as compared to reactor R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses. On the other hand, *HhaI* has produced 12, 12, 1, 9, and 8 bands at different positions for reactor R_c , R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Fig. 5.36(h)) has shown that reactor R_{Scu40} and R_{rCu40} biomasses have been closer to the control reactor R_c biomass as compared to reactor R_{Scu80} , and R_{rCu80} biomasses. Similarly, *MspI* restriction enzyme has produced 10, 10, 8, 8 and 7 bands at different positions for reactor R_c , R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, and dendrogram (Fig. 5.36(i)) has shown that reactor R_{rCu40} biomass has been closer to the control reactor R_c biomass as compared to reactor R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses.

The restriction enzyme *AluI* has shown 85.7%, 59.8%, 78.3%, and 47.3% similarity during recovery phase operation for reactor R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, which has improved from 52.1%, 43.4%, 33.8%, and 29.4%, respectively, of stressed phase II operation. Similarly, *HhaI* has exhibited 80.7%, 61.3%, 83.2%, and 59.4% of similarity for reactor R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, which has also improved from 51.4%, 41.2%, 39.3%, and 31.0%, respectively, when compared to stressed phase II operation. Similarity of 78.4%, 54.7%, 81.3%, and 61.3% has been observed for reactor R_{Scu40} , R_{Scu80} , R_{rCu40} , and R_{rCu80} biomasses withdrawn during recovery phase operation, respectively, when digested with *MspI*, which has shown improvement from 62.5%, 58.5%, 54.8%, and 48.3%, respectively, of stressed phase II operation. When PWW+Cu(II) addition has been discontinued in the feed to reactors during recovery phase operation,

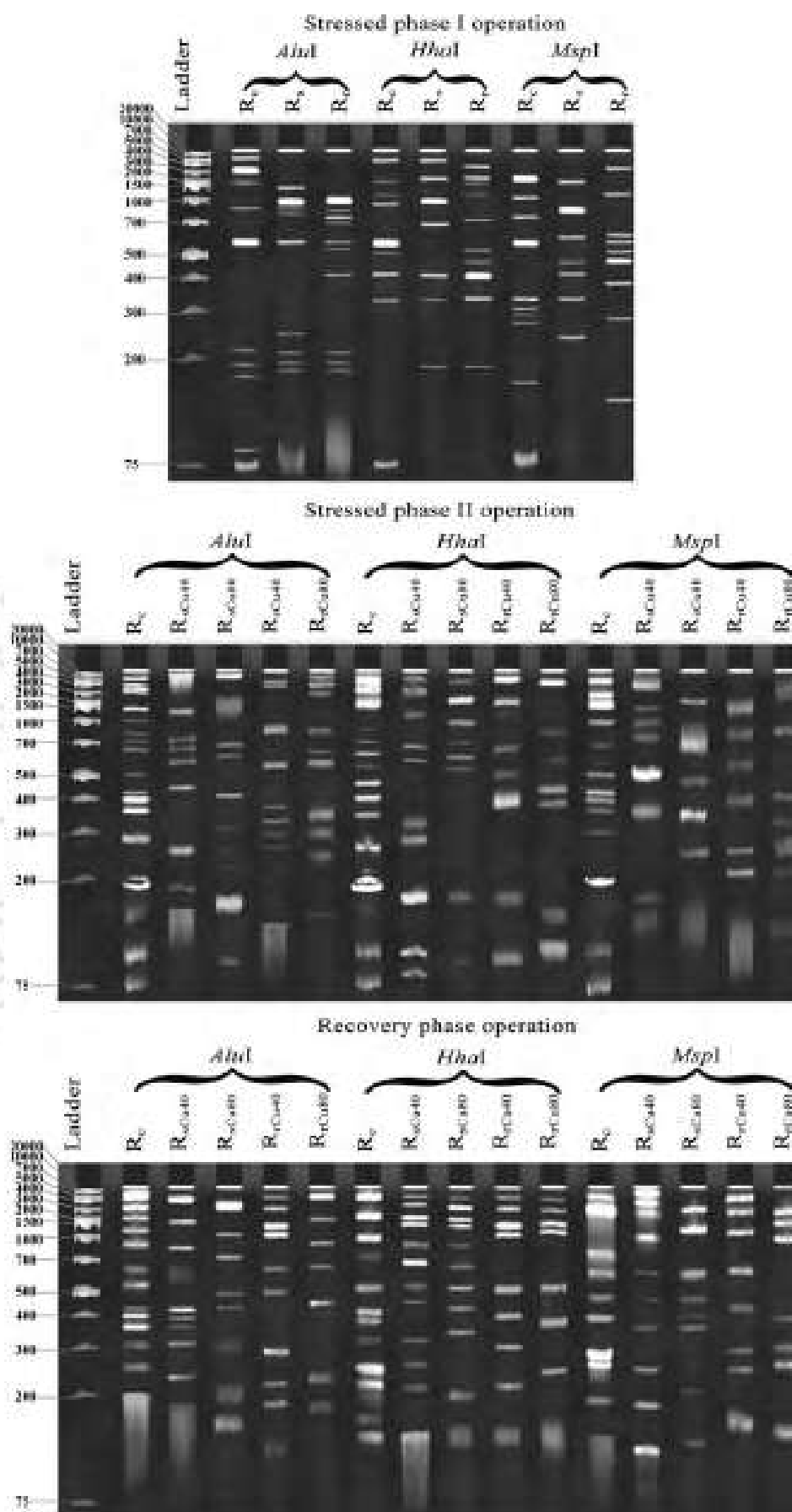


Fig. 5.35 Digestion pattern of aerobic sludge biomass treated with restriction enzymes *AluI*, *HhaI*, and *MspI* during stressed phase I, II and recovery phase operations.

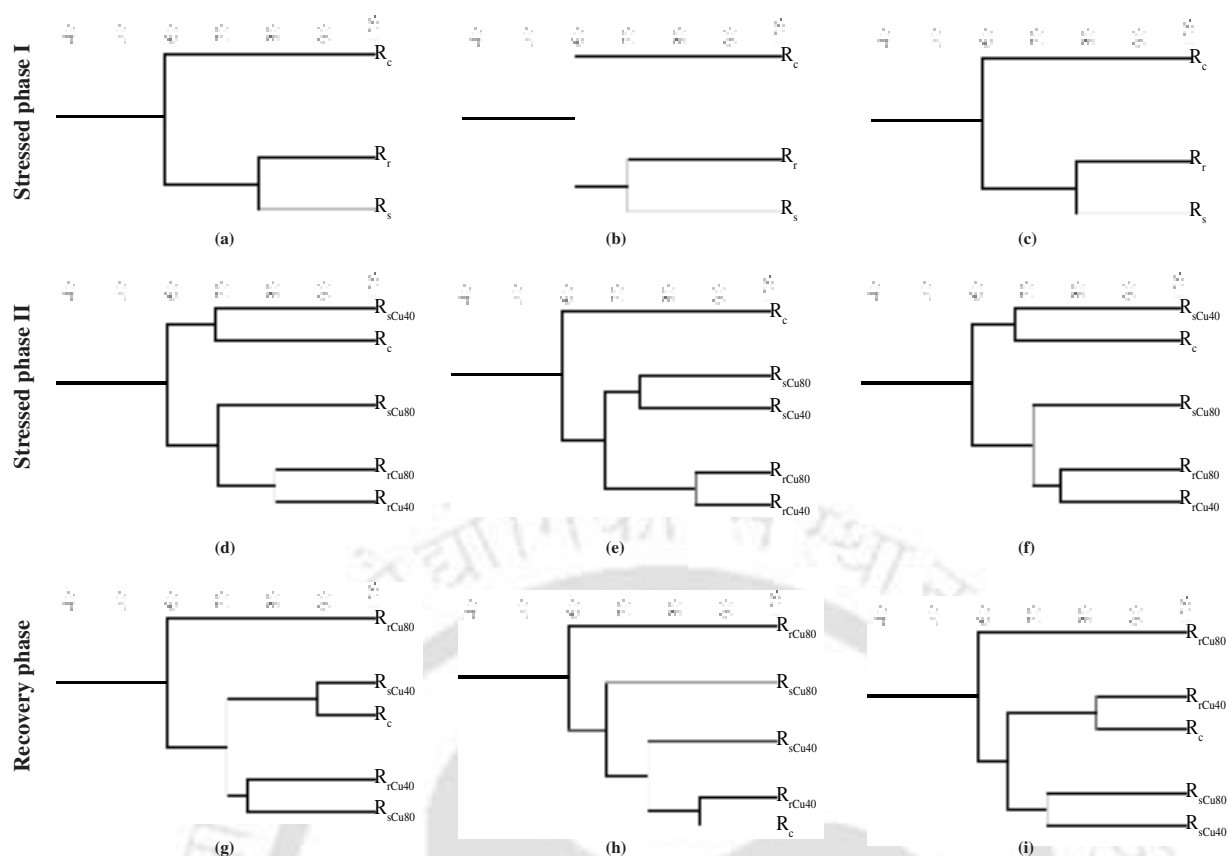


Fig. 5.36 Dendrogram of aerobic sludge biomass treated with restriction enzymes during stressed phase I operation (a) *AluI*, (b) *HhaI*, and (c) *MspI*; stressed phase II operation (d) *AluI*, (e) *HhaI*, and (f) *MspI*; and recovery phase operation (g) *AluI*, (h) *HhaI*, and (i) *MspI*.

aerobic sludge biomass has tried to regain its microbial community structure. The recovery in terms of microbial community structure has not been completely achieved as compared to reactor R_c biomass. The reactor R_{sCu40} and R_{rCu40} biomasses have exhibited significant recovery, whereas slow recovery has been observed for reactor R_{sCu80} and R_{rCu80} biomasses.

The microscopic examinations of biomasses have indicated growth and subsequent increase in floc sizes after 27 and 34 days of recovery phase operation for reactor R_{sCu40} and R_{sCu80} biomasses, respectively. On the other hand, regrowth and increase in floc sizes for reactor R_{rCu40} and R_{rCu80} biomasses have yet to take place even after 3-4 weeks of operation under recovery phase indicating very slow recovery for biomasses as these biomasses have been severely impacted due to Cu(II) and raw PWW. The photographic examinations have started to show improvement in floc morphology with filamentous growth and increase in size only from 27th d and 48th d of recovery phase operation for reactor R_{sCu40} and R_{sCu80} biomasses, respectively. However, no visible improvement in floc morphology has been observed in the case of reactor R_{rCu40} and R_{rCu80} biomasses during recovery phase operation. The fluorescence microscopic examination conducted towards the end of recovery phase operation for reactor biomasses have indicated increased fraction of active cells in reactor R_{sCu40} and R_{rCu40} biomasses, while reactor R_{sCu80} and R_{rCu80} biomasses contained higher fraction of inactive cells indicating very slow recovery. The FI has increased to 4, 3, 2, and 2

Table 5.35 Similarity matrix resulting from digestion of aerobic sludge biomass samples by restriction enzymes withdrawn during recovery phase operation along with data for stressed phase I and II operations.

Stressed phase I operation															
AluI						HhaI					MspI				
Reactor	R _c	R _s		R _r		R _c	R _s		R _r		R _c	R _s		R _r	
R _c	1	0.9223		0.8845		1	0.9003		0.8410		1	0.8912		0.8712	
R _s	0.9223	1		0.9461		0.9003	1		0.9543		0.8912	1		0.9105	
R _r	0.8845	0.9461		1		0.8410	0.9543		1		0.8712	0.9105		1	
Stressed phase II operation															
AluI						HhaI					MspI				
Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
R _c	1	0.5213	0.4338	0.3384	0.2938	1	0.5143	0.4123	0.3927	0.3102	1	0.6249	0.5845	0.5476	0.4834
R _{sCu40}	0.5213	1	0.4831	0.4294	0.3217	0.5143	1	0.6215	0.4923	0.3793	0.6249	1	0.6471	0.4723	0.4367
R _{sCu80}	0.4338	0.4831	1	0.5653	0.4922	0.4123	0.6215	1	0.7343	0.5043	0.5845	0.6471	1	0.6875	0.6372
R _{rCu40}	0.3384	0.4294	0.5653	1	0.6967	0.3927	0.4923	0.7343	1	0.7756	0.5476	0.4723	0.6875	1	0.7217
R _{rCu80}	0.2938	0.3217	0.4922	0.6967	1	0.3102	0.3793	0.5043	0.7756	1	0.4834	0.4367	0.6372	0.7217	1
Recovery phase operation															
AluI						HhaI					MspI				
R _c	1	0.8571	0.5983	0.7834	0.4734	1	0.8072	0.6128	0.8319	0.5941	1	0.7838	0.5471	0.8127	0.6128
R _{sCu40}	0.8571	1	0.6649	0.5467	0.4918	0.8072	1	0.5851	0.6415	0.4736	0.7838	1	0.7134	0.5642	0.5186
R _{sCu80}	0.5983	0.6649	1	0.6953	0.4563	0.6128	0.5851	1	0.7239	0.5318	0.5471	0.7134	1	0.6545	0.5788
R _{rCu40}	0.7834	0.5467	0.6953	1	0.6173	0.8319	0.6415	0.7239	1	0.6473	0.8127	0.5642	0.6545	1	0.5963
R _{rCu80}	0.4734	0.4918	0.4563	0.6173	1	0.5941	0.4736	0.5318	0.6473	1	0.6128	0.5186	0.5788	0.5963	1

for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, respectively, towards the end of recovery phase operation from FI of 2, 1, 1, and 1 in the beginning of this phase for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, respectively. The polysaccharide (PS), protein (PN) and extracellular polymeric substance (EPS) contents of reactor biomasses (including control reactor) estimated towards the end of recovery phase operation vary within the range of 16.42-19.12 mg/g VSS for PS, 40.46-47.00 mg/g VSS for PN and 55.72-65.02 mg/g VSS for EPS which fails to distinguish the impact of discontinuation of imposed stresses as the contents have been in the range of 16.54-18.94 mg/g VSS for PS, 40.22-47.22 mg/g VSS for PN and 55.44-65.42 mg/g VSS for EPS. This might be due to incomplete recovery of reactor biomasses after discontinuation of imposed stresses. The enzyme activities have shown variations: $E_{Catalase}$ of 0.3-0.1 mg/g MLSS h for stressed reactor biomasses compared to 0.4 mg/g MLSS h for reactor R_c biomass; $E_{Dehydrogenase}$ of 25-12 μ g/g MLSS h for stressed reactor biomasses compared to 30 μ g/g MLSS h for reactor R_c biomass; $E_{Protease}$ of 1.7-0.4 mg/g MLSS h for stressed reactor biomasses compared to 2.3 mg/g MLSS h for reactor R_c biomass; and E_{Urease} of 5.1-1.4 g/g MLSS h for stressed reactor biomasses compared to 17.3 g/g MLSS h for reactor R_c biomass. These values have slightly improved over values obtained during stressed phase II operation. Again, it indicated failure of complete recovery by reactor biomasses. The gel run of ARDRA carried out with three different restriction enzymes towards the end of recovery phase operation has shown similar banding patterns for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses with *HhaI* restriction enzymes while the banding patterns with restriction enzymes *AluI* and *MspI* have been significantly different. But none of the banding patterns have been closer to banding patterns of control reactor R_c biomass. The dendrogram of reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses have shown improved similarity (in the range of 47% to 86%) during recovery phase operation compared with lower similarity (in the range of 29% to 63%) obtained during stressed phase II operation when digested with the three restriction enzymes, but these have been definitely different from the control reactor R_c biomass indicating incomplete recovery in microbial community structures during 48 days of operation in recovery phase.

5.4 Summary

This study evaluates the impact of PWW and PWW+Cu(II) on aerobic sludge biomass, when fed to the reactors for a long-terms operation. The findings show that PWW has a little influence on performance-related parameters, while biomass related parameters have been significantly affected, during stressed phase I operation. During steady-state condition, the COD removal for control reactor R_c has been observed as ~95%, while COD removal for PWW fed reactors R_s and R_r has been observed as ~76% and 65%, respectively. Alkalinity for the reactors R_s and R_r have drastically increased due to addition of PWW and has been observed in the range of 780-807 mg/L as $CaCO_3$ during steady-state condition of stressed phase I operation. The reactor R_c has exhibited continuous growth of biomass, while screened PWW fed reactor (R_s) has shown inhibited biomass growth. However, it is difficult to observe the biomass growth for reactor fed with raw PWW (R_r) due to higher fraction of suspended

solids in PWW. A minor change in MLVSS/MLSS ratio (0.86-0.88) has been observed for reactor R_s and R_r biomasses, when compared to reactor R_c biomass (0.92). A significant change has been observed in SVI and ISV. It can be seen that reactor R_s and R_r biomasses have lower SVI (93 mL/g and 89 mL/g) as compared to reactor R_c biomass (127 mL/g). On the other hand, reactor R_c biomass (2.43 m/h) has shown reduced ISV as compared to PWW fed reactors R_s and R_r (3.05 m/h for R_s and 3.16 m/h for R_r). This result concludes that addition of PWW has improved the settling behavior of aerobic sludge biomass. However, ASBA has shown the deterioration in bio-degradation of organics. The ASBA of reactor R_s and R_r biomasses (5.87 and 5.05 mg COD removed/mg VSS d) appears to be much lower than reactor R_c biomass (7.03 mg COD removed/mg VSS d). The decreased ASBA values for reactor R_s and R_r biomasses have shown the resemblance with reduced COD removal for both the reactors i.e., ~76% and 65% by reactors R_s and R_r , respectively. The PWW has severely affected the biomass at microscopic level. When PWW (screened and raw) has been added to reactors as feed, the morphology of the flocs has transformed from dense to pinpoint. Even filamentous biomass has started to disappear that has lowered the SVI and increased the ISV. FI have been reduced from 5 and 5 to 3 and 2 for reactor R_s and R_r biomasses, respectively, while FI remained 5 for control reactor. The fluorescence images have indicated that active portion of biomass has also decreased in the reactor biomasses exposed to PWW. PS, PN, and ESP contents have shown minor changes for PWW exposed reactors as compared to control reactor. The addition of PWW in feed has inhibited the enzymes in the order $E_{\text{Urease}} > E_{\text{Catalase}} > E_{\text{Dehydrogenase}} > E_{\text{Protease}}$. The ARDRA result has indicated minor changes in the microbial community of reactor R_s and R_r biomasses (84-92% of similarity) when compared to reactor R_c biomass.

The addition of Cu(II) (40 and 80 mg/L) along with PWW during stressed phase II operation has a significant impact on performance-related parameters, although biomass-related parameters have already been adversely affected by PWW during stressed phase I operation. During the steady-state condition of stressed phase II operation, control reactor has exhibited ~94% of COD removal, while reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ have shown a drastic reduction in COD removal (~8-29%), when compared to reactor R_c . In addition, very low Cu(II) retention has been observed for reactor $R_{s\text{Cu}40}$ and $R_{s\text{Cu}80}$ (14.9% and 11.3%), whereas reactor $R_{r\text{Cu}40}$ and $R_{r\text{Cu}80}$ has shown 40.6% and 25.2% of Cu(II) retention, respectively. The biomass growth has further inhibited due to addition of Cu(II) and MLVSS/MLSS ratio has been observed ~0.80 for reactor $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, and $R_{r\text{Cu}40}$ biomasses, while a drastic reduction in MLVSS/MLSS ratio has been observed for reactor $R_{r\text{Cu}80}$ biomass (0.65). The PWW+Cu(II) fed reactor biomasses have exhibited minimum variation in SVI (73.4-76.0 mL/g), when compared to reactor R_c biomass (111.6 mL/g). In addition, higher ISV has been observed for PWW+Cu(II) fed reactor biomasses (3.36-3.46 m/h) than reactor R_c biomass (2.22 m/h). The COD removal by reactors R_c , $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ can be correlated with ASBA. A drastic difference in ASBA have been observed between control and PWW+Cu(II) fed reactors. The ASBA for reactor R_c biomass has been 7.75 mg COD removed/mg VSS d, which has

been much higher than ASBA obtained for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses i.e. 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, respectively. The microscopic examination has revealed the disintegration of flocs and disappearance of filaments that has started from 43rd d and continued till 71st d for PWW+Cu(II) fed reactor biomasses, whereas reactor R_c biomass has exhibited uniform increase in floc size and abundance of filamentous biomass. The photographic observation has revealed the floc size that has been in the range of 1.0-5.0 mm for PWW+Cu(II) fed reactor biomasses, while the floc size for reactor R_c biomass has been in the range of 1.0-14.0 mm. FI and active biomass portions have further decreased due to Cu(II) exposure. In addition, PS, PN and EPS secretion has enhanced in response to Cu(II) exposure. The addition of PWW+Cu(II) in feed has inhibited the enzymes in the order $E_{Urease} > E_{Protease} > E_{Catalase} > E_{Dehydrogenase}$. The microbial community have also significantly changed with exposure of PWW+Cu(II). ARDRA has revealed a drastic shift in microbial community structure due to addition of Cu(II). The similarity between control and PWW+Cu(II) fed reactor biomasses have been observed in the range of 29%-63%.

When PWW+Cu(II) has been discontinued in the feed, the reactors have been operated for 48 d (73-120 d) during recovery phase operation. The steady-state COD removal for reactors R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} have been obtained as 95.0%, 78.0%, 63.7%, 57.1%, and 49.2%, respectively, which has significantly improved from 94.2%, 29.0%, 14.5%, 22.3%, and 7.6%, respectively, of the stressed phase II steady-state COD removal. The reactor R_{sCu40} and R_{rCu40} biomasses have exhibited the increased biomass growth as compared to reactor R_{sCu80} and R_{rCu80} biomasses. MLVSS/MLSS ratio has been observed in the range of 0.81-0.92 for reactor R_{sCu40} , R_{sCu80} , and R_{rCu40} biomasses, while a drastic reduction in MLVSS/MLSS ratio has been observed for reactor R_{rCu80} biomass (0.52). The lower SVI have been observed in the range of 77-90 mL/g for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, when compared to reactor R_c biomass (132 mL/g). In addition, lower ISV has been obtained for reactor R_c biomass (1.86 m/h) due to abundance of filaments in reactor. The reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been exhibited higher ISV in the range of 3.10-3.36 m/h. The ASBA has revealed that reactors have partially recovered. The ASBA for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been obtained as 7.80, 5.77, 2.82, 3.16, and 1.59 mg COD removed/mg VSS d, respectively, during recovery phase operation, which has improved from 7.75, 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, of stressed phase II operation. The microscopic results have suggested that reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have been significantly affected in stressed phase II operation and has slowly recovered during recovery phase operation. Increased floc size and reappearance of filaments has shown the recovery of aerobic sludge biomass for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} . The flocs size for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses have started to increase after 99th d of operation, whereas control reactor has exhibited uniform flocs size. The biomass has not been able to completely recover to its original level (as compared to reactor R_c) considering the morphology. The PS, PN, and EPS contents in aerobic sludge biomass have been observed to be stabilized during recovery phase

operation. E_{Catalase} , $E_{\text{Dehydrogenase}}$, and E_{Protease} has shown significant recovery for the reactor biomasses exposed to lower doses of Cu(II), while slight improvement has been observed for the reactor biomasses exposed to higher doses of Cu(II). E_{Urease} could not be able to recover during recovery phase operation. ARDRA results have suggested 47-85% of similarity between control reactor and other reactor biomasses after discontinuation of PWW+Cu(II) in the feed. Overall, complete recovery can not be achieved for the stressed aerobic sludge biomass.

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6

Tools and Techniques to Assess Health of Aerobic Sludge Biomass at Treatment Plant Site

The domestic sewage produced in a city is generally treated using aerobic biological processes. The active aerobic biomass (or sludge biomass) maintained in the biological treatment system helps in reducing the contents of colloidal and soluble organics present in domestic sewage. These organics are converted to carbon dioxide and water by the active aerobic biomass, thereby making the treated sewage free from organics to a large extent. While converting organics during the course of the treatment, a significant fraction of organics is also utilized to produce more active biomass in the treatment system. Depending upon the influent concentration of organics (represented in terms of BOD or COD) and the desired level of treatment to be imparted, a specified level of active aerobic biomass has to be maintained in the treatment system throughout, and the excess biomass needs to be taken out of the treatment system at regular intervals. In the entire treatment system, the active aerobic biomass is considered to be the heart of the system. Therefore, the efficacy of the treatment plant very much depends on the proportion of active biomass present in the system to interact with the sewage for its stabilization.

In general, the design of a biological process-based sewage treatment plant considers the usual characteristics of sewage produced by domestic and allied activities. The sewage coming out from the residential area, hotels and restaurants, railway and bus stations, cinema halls, stadium/playgrounds, schools/colleges, hospitals (domestic sewage part only), offices and commercial establishments, etc. do contain a large fraction of biodegradable organics in the form of suspended, colloidal and dissolved solids. The sewage from these sources is very much amenable for biological treatment as it does not cause any significant adverse impact on the sludge biomass. However, city area of developing countries such as India is also

teeming with other activities involving, for example, metal plating works (probably a small scale industrial activity); storage and distribution points for pharmaceutical drugs, pesticides, insecticides, and herbicides; animal rearing using feed with growth factors; automobile service and repair workshops; discarded electronic items (dumped at intermediate solid waste collection sites spread across the city); painting works; etc. It is likely that effluents or residues or spillages at higher concentrations levels from these activities (unwanted components) may find its way to nearby sewer lines intentionally or otherwise and thereby may enter into the biological process-based sewage treatment plant. These effluents or residues, or spillages may have the potential to interfere with active aerobic biomass altering its activities and morphological characteristics either temporarily or permanently, leading to adversely impacting the treatment efficacy of the plant. Therefore, there appears to be a need to find tool(s) to monitor the health of the active aerobic biomass in the sewage treatment plant on a regular basis and to act as an alert if unwanted components are entering the treatment plant and interfering adversely with the active biomass.

The biological treatment system of sewage is critically dependent on the presence of an adequate quantity of active aerobic sludge biomass in the system throughout. If the incoming sewage to the treatment plant contains unwanted components detrimental to the biomass, it is likely to impact the sludge biomass adversely, which in turn shall impact the treatment efficacy. In the present studies, investigations have been carried out to assess the impact of unwanted components present in the influent (or feed) such as a heavy metal ([Chapter 2](#)), an antibiotic ([Chapter 3](#)), a pesticide ([Chapter 4](#)) and an actual piggery wastewater (spiked with a heavy metal) ([Chapter 5](#)) on active aerobic sludge biomass. Essentially, the active aerobic sludge biomasses have been subjected to stresses caused by these unwanted components, and its impact on the health of the aerobic sludge biomass has been observed. The impact on aerobic sludge biomass has been observed through solids analysis (MLSS and MLVSS), solids settle-ability tests (SVI and ISV), microscopic and photographic comparisons, cell viability test, filament indexing, biomass activity (ASBA) test, enzymatic tests (E_{Catalase} , $E_{\text{Dehydrogenase}}$, E_{Protease} , and E_{Urease}), biochemical content analysis (polysaccharide (PS), protein (PN) and extracellular polymeric substance (EPS)) and changes in microbial community structure through amplified ribosomal DNA restriction analysis (ARDRA). In addition, studies have also been carried out to assess the recovery potential of the stressed sludge biomass when the stresses of unwanted components have been removed.

In this Chapter, attempts have been made to critically review each of the sludge biomass-related tests/observations/data with an aim to identify a few which has potential for field application in Indian conditions. These tests/observations/data may be used to assess the health of the sludge biomass and to get an alert if unwanted components are present in the incoming sewage to the treatment plant. While selecting tests/observations/data for monitoring the health of sludge biomass at the treatment plant site, attention has also to be paid to educational and skill set available with the plant operators as well as the status of the existing laboratory infrastructure at the plant site.

6.1 Critical review of tests/observations/data obtained for stressed aerobic sludge biomass

In the present studies, aerobic sludge biomasses have been subjected to stresses caused by (i) addition of Cu(II) (10, 20, 40, and 80 mg/L) in the feed and operated for 54 days (details available in [Chapter 2](#)), (ii) addition of amoxicillin (500, 1000 and 2000 mg/L) in the feed and operated for 78 days (details available in [Chapter 3](#)), (iii) addition of chlorpyrifos (50, 100 and 200 mg/L) in the feed and operated for 76 days (details available in [Chapter 4](#)), and (iv) addition of piggyery wastewater (PWW) (details available in [Chapter 5](#)). The reactor sludge biomasses have been operated under the stressed conditions to obtain steady-state reactor performance-related data, as well as tests/observations/data, which have been carried out or generated to monitor the health of the stressed sludge biomass. Thereafter, imposed stresses have been removed for all the reactor sludge biomasses and have been operated under a steady state to assess the recovery potential and health of the stressed sludge biomasses. The steady-state reactor performance-related data (initial soluble COD levels, effluent soluble COD levels, and percentage COD removal) and biomass health-related tests/observations/data (MLSS, MLVSS, SVI, ISV, microscopic and photographic visualizations, filament indexing, cell viability tests, ASBA tests, and enzymatic tests (E_{Catalase} , $E_{\text{Dehydrogenase}}$, E_{Protease} , and E_{Urease}) have been summarized in [Table 6.1](#) for stress caused by Cu(II), [Table 6.2](#) for stress caused by amoxicillin, [Table 6.3](#) for stress caused by chlorpyrifos and [Table 6.4](#) for stress caused by PWW+Cu(II). In the subsequent subsections, efforts are being made to critically analyze each of the biomass health-related tests/observations/data to identify the potential ones for field application.

6.1.1 MLSS and MLVSS tests

The MLSS and MLVSS levels of sludge biomasses stressed with Cu(II) have varied within a narrow range of 1313 to 1335 mg/L and 1202 to 1294 mg/L, respectively, whereas the same for control reactor (without stress) have yielded 4021 and 3735 mg/L, respectively ([Table 6.1](#)). However, sludge biomasses stressed with Cu(II) have shown COD removal of only 38% with 80 mg/L of Cu(II) stress and 75% with 10 mg/L of Cu(II) stress in comparison of 90% without Cu(II) stress ([Table 6.1](#)). On the other hand, MLSS and MLVSS levels under recovery phase [no Cu(II) in feed] have varied for 40, and 80 mg/L Cu(II) stressed sludge biomasses within a narrow range of 1380 to 1413 mg/L and 1263 to 1280 mg/L, respectively; for 10 and 20 mg/L Cu(II) stressed sludge biomasses within a range of 1739 to 1975 mg/L and 1609 to 1858 mg/L respectively; but yet to achieve the level of the control reactor (without stress) having 4857 and 4639 mg/L respectively ([Table 6.1](#)). It is apparent that only 10 and 20 mg/L Cu(II) stressed sludge biomasses have shown recovery potential for MLSS and MLVSS levels. However, Cu(II) stressed sludge biomasses have shown increased COD removal of 72-82% for 10 and 20 mg/L Cu(II) stressed reactors and 41-58% for 40 and 80 mg/L Cu(II) stressed reactors during the recovery phase but yet to achieve the COD removal levels of the control reactor (without stress) of 91% ([Table 6.1](#)). The MLSS and MLVSS

Table 6.1 Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with Cu(II) in feed.

Parameters	Reactor	R _{Cu0}	R _{Cu10}	R _{Cu20}	R _{Cu40}	R _{Cu80}
	Lactogen 3 (g/L)	0.50	0.50	0.50	0.50	0.50
	Cu(II) (mg/L)	0	10	20	40	80
Stressed phase operation	Initial soluble COD (mg/L)	528.57±21.10 (14)	508.57±19.00 (14)	522.86±22.42 (14)	508.57±27.28 (14)	505.71±25.10 (14)
	Effluent soluble COD (mg/L)	48.57±14.23 (14)	127.14±13.14 (14)	198.57±18.11 (14)	258.57±20.68 (14)	310.00±16.18 (14)
	COD removal (%)	90.78±2.78 (14)	74.94±2.77 (14)	61.92±4.21 (14)	49.17±3.21 (14)	38.52±5.16 (14)
	MLSS (mg/L)	4021.50±184.55 (4)	1326.22±60.81 (4)	1334.50±31.82 (4)	1335.08±27.07 (4)	1313.20±24.04 (4)
	MLVSS (mg/L)	3734.73±179.08(4)	1202.52±61.55 (4)	1276.50±47.38 (4)	1294.12±18.38 (4)	1291.50±33.23 (4)
	SVI (mL/g)	224.72±12.95 (4)	90.72±9.49 (4)	86.55±4.28 (4)	87.55±3.78 (4)	87.35±0.07 (4)
	ISV (m/h)	0.06±0.00 (4)	17.22±0.00 (4)	18.42±0.42 (4)	18.66±0.23 (4)	18.990.13 (4)
	Microscopic examination	Filamentous biomass	Less filamentous biomass	Loose filamentous biomass	No filamentous biomass	No filamentous biomass
	Photographic observation (floc size in mm)	Loose flocs 2.0-16.0	Dense flocs 1.0-6.0	Disintegrated flocs 1.0-4.0	Disintegrated flocs 1.0-3.0	Disintegrated flocs 1.0-2.0
	Filament index	5	2	1	1	1
	ASBA (mg COD removed/mg VSS d)	7.00	2.28	1.89	1.55	1.43
	E _{Catalase} (mg/g MLSS h)	0.33±0.12	0.09±0.01	0.05±0.01	0.03±0.01	0.01±0.01
	E _{Dehydrogenase} (µg/g MLSS h)	27.52±1.85	17.34±2.54	14.05±1.41	8.75±1.28	4.02±0.87
	E _{protease} (mg/g MLSS h)	1.85±0.05	0.93±0.07	0.82±0.04	0.56±0.07	0.29±0.05
	E _{urease} (g/g MLSS h)	15.59±2.15	5.71±0.83	5.19±0.97	3.72±0.68	2.58±0.74
	ARDRA (% similarity)	100	66 to 71	52 to 65	43 to 57	38 to 48
Recovery phase operation	Initial soluble COD (mg/L)	515.71±21.32 (14)	517.14±26.79 (14)	511.43±25.31 (14)	514.29±21.31 (14)	520.00±24.14 (14)
	Effluent soluble COD (mg/L)	45.71±14.96 (14)	92.86±24.12 (14)	142.86±17.08 (14)	212.86±21.21 (14)	304.29±16.24 (14)
	COD removal (%)	91.10±2.93 (14)	82.10±4.46 (14)	72.00±3.63 (14)	58.59±4.38 (14)	41.39±3.70 (14)
	MLSS (mg/L)	4857.50±51.62 (4)	1975.50±408.02 (4)	1739.62±205.06 (4)	1413.28±83.44 (4)	1380.38±12.02 (4)
	MLVSS (mg/L)	4639.10±83.64 (4)	1858.50±458.91 (4)	1609.54±185.26 (4)	1280.50±119.50 (4)	1263.24±60.81 (4)
	SVI (mL/g)	192.47±2.32 (4)	68.34±12.33 (4)	70.82±6.32 (4)	83.23±2.41 (4)	77.89±1.92 (4)
	ISV (m/h)	0.06±0.00 (4)	15.81±0.38 (4)	16.35±0.30 (4)	17.04±0.17 (4)	17.88±0.25 (4)
	Microscopic examination	Dense filamentous biomass	Reappearance of filamentous biomass	Less filamentous biomass	Weak filamentous biomass	Loose filamentous biomass
	Photographic observation (floc size in mm)	Loose flocs 2.0-6.0	Granular flocs 1.0-4.0	Granular flocs 1.0-4.0	Granular flocs 1.0-2.0	Disintegrated flocs 1.0-2.0
	Filament index	5	5	4	4	3
	ASBA (mg COD removed/mg VSS d)	8.34	3.10	2.40	1.91	1.78
	E _{Catalase} (mg/g MLSS h)	0.34±0.08	0.13±0.05	0.08±0.02	0.03±0.01	0.02±0.01
	E _{Dehydrogenase} (µg/g MLSS h)	32.87±2.85	23.08±3.58	19.18±1.86	13.78±2.14	4.85±1.07
	E _{protease} (mg/g MLSS h)	2.04±0.04	1.51±0.07	1.18±0.06	0.99±0.03	0.46±0.01
	E _{urease} (g/g MLSS h)	18.52±3.14	9.38±1.57	7.65±1.61	5.80±0.83	3.30±0.97
	ARDRA (% similarity)	100	84 to 86	80 to 82	75 to 76	61 to 64

Table 6.2 Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with AMX in feed.

Parameters	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	4.25	3.00	0.50
	AMX (mg/L)	0	500	1000	2000
Stressed phase operation	Initial soluble COD (mg/L)	5556.10±108.64 (14)	5556.10±131.19 (14)	5535.71±178.07 (14)	5604.05±145.60 (14)
	Effluent soluble COD (mg/L)	393.19±117.52 (14)	1473.67±139.15 (14)	3449.57±217.01 (14)	4295.38±192.60 (14)
	COD removal (%)	92.95±1.99 (14)	73.49±2.33 (14)	37.68±3.45 (14)	23.35±2.84 (14)
	MLSS (mg/L)	3126.32±101.82 (4)	2834.50±362.75 (4)	2728.50±557.91 (4)	2536.56±568.51 (4)
	MLVSS (mg/L)	2659.98±263.38 (4)	2321.73±257.37 (4)	2420.81±683.89 (4)	2134.26±513.42 (4)
	SVI (mL/g)	151.65±31.24 (4)	182.15±33.12 (4)	129.88±22.16 (4)	132.75±6.32 (4)
	ISV (m/h)	1.36±0.04(4)	1.10±0.10 (4)	1.78±0.06 (4)	1.87±0.01 (4)
	Microscopic examination	Dense filamentous biomass	Dense filamentous biomass	Weak filamentous biomass	Loose filamentous biomass
	Photographic observation (floc size in mm)	Flocculant flocs 1.0-7.0	Thick flocs 1.0-7.0	Thick flocs 1.0-5.0	Loose flocs 1.0-5.0
	Cell viability	Live cells (%)	97.7	87.2	39.2
		Dead cells (%)	0.4	30.8	58.9
	Filament index	5	5	5	4
	ASBA (mg COD removed/mg VSS d)	9.43	7.65	3.84	2.22
	E _{Catalase} (mg/g MLSS h)	0.37±0.02	0.18±0.02	0.14±0.02	0.05±0.02
	E _{Dehydrogenase} (µg/g MLSS h)	27.34±2.98	20.34±1.50	15.23±1.70	13.43±1.10
	E _{protease} (mg/g MLSS h)	1.65±0.09	1.32±0.06	0.85±0.13	0.76±0.17
	E _{Urease} (g/g MLSS h)	9.43±2.32	6.34±0.50	5.76±0.60	4.43±1.10
	ARDRA (% similarity)	100	72 to 80	59 to 75	52 to 74
Parameters	Reactor	R _{amx0}	R _{amx500}	R _{amx1000}	R _{amx2000}
	Lactogen 3 (g/L)	5.50	5.50	5.50	5.50
	AMX (mg/L)	0	0	0	0
Recovery phase operation	Initial soluble COD (mg/L)	5540.00±122.36 (14)	5540.00±143.54 (14)	5570.14±175.24(14))	5433.38±143.98 (14)
	Effluent soluble COD (mg/L)	384.05±106.86 (14)	433.86±122.91 (14)	399.29±149.23 (14)	448.00±147.79 (14)
	COD removal (%)	93.07±1.95 (14)	92.18±2.19 (14)	92.88±2.47 (14)	91.73±2.80 (14)
	MLSS (mg/L)	2979.12±35.36 (4)	2925.08±70.71 (4)	3039.50±86.97 (4)	3052.50±99.70 (4)
	MLVSS (mg/L)	2710.39±52.09 (4)	2662.75±147.08 (4)	2766.48±120.07 (4)	2776.97±107.20 (4)
	SVI (mL/g)	169.04±43.42 (4)	181.97±28.61 (4)	168.16±38.41 (4)	128.23±6.38 (4)
	ISV (m/h)	1.11±0.13 (4)	0.84±0.06 (4)	1.39±0.31 (4)	1.85±0.07 (4)
	Microscopic examination	Dense filamentous biomass	Dense filamentous biomass	Dense filamentous biomass	Dense filamentous biomass
	Photographic observation (floc size in mm)	Thick flocs 2.0-16.0	Thick flocs 2.0-16.0	Thick flocs 2.0-20.0	Thick flocs 2.0-20.0
	Cell viability	Live cells (%)	98.1	88.2	81.3
		Dead cells (%)	0.8	7.4	11.0
	Filament index	5	5	5	5
	ASBA (mg COD removed/mg VSS d)	11.58	9.22	8.96	7.96
	E _{Catalase} (mg/g MLSS h)	0.41±0.03	0.43±0.04	0.44±0.03	0.43±0.02
	E _{Dehydrogenase} (µg/g MLSS h)	28.54±0.82	26.54±1.71	26.43±2.23	25.34±1.95
	E _{protease} (mg/g MLSS h)	1.70±0.11	1.63±0.09	1.53±0.09	1.54±0.05
	E _{Urease} (g/g MLSS h)	11.23±0.82	11.75±0.30	11.02±1.20	9.89±0.70
	ARDRA (% similarity)	100	80 to 94	73 to 85	68 to 85

Table 6.3 Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with CPS in feed.

Parameters	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.14	1.58	0.47
	CPS (mg/L)	0	50	100	200
Stressed phase operation	Initial soluble COD (mg/L)	2778.08±93.60 (20)	2789.48±104.62 (20)	2781.27±146.74 (20)	2700.00±127.08 (20)
	Effluent soluble COD (mg/L)	259.83±48.11 (20)	765.00±93.29 (20)	1687.00±106.75 (20)	2220.20±86.59 (20)
	COD removal (%)	90.65±1.68 (20)	72.48±3.97 (20)	39.21±4.53 (20)	17.70±2.53 (20)
	MLSS (mg/L)	3115.12±131.52 (4)	2660.64±692.96 (4)	3009.60±73.54 (4)	3037.60±95.97 (4)
	MLVSS (mg/L)	2912.99±145.22 (4)	2415.70±592.98 (4)	2690.84±86.72 (4)	2673.04±106.68 (4)
	SVI (mL/g)	164.06±16.01 (4)	122.45±9.30 (4)	108.67±5.48 (4)	104.46±5.25 (4)
	ISV (m/h)	1.72±0.10 (4)	2.49±0.17 (4)	2.61±0.17 (4)	2.66±0.18 (4)
	Microscopic examination	Dense filamentous biomass	Weak filamentous biomass	Loose filamentous biomass	Damaged filamentous biomass
	Photographic observation (floc size in mm)	Thick flocs 2.0-10.0	Disintegrated flocs 2.0-8.0	Loose flocs 2.0-8.0	Flocculant flocs 1.0-4.0
	Cell viability	Live cells (%)	98.2	48.3	33.7
		Dead cells (%)	0.7	44.9	58.4
	Filament index	5	5	4	4
	ASBA (mg COD removed/mg VSS d)	7.42	6.57	4.30	2.04
	E _{Catalase} (mg/g MLSS h)	0.33±0.02	0.26±0.02	0.12±0.02	0.10±0.02
	E _{Dehydrogenase} (µg/g MLSS h)	23.23±1.64	16.43±1.96	6.54±2.01	2.23±0.81
	E _{Protease} (mg/g MLSS h)	1.92±0.14	1.54±0.06	0.85±0.13	0.62±0.17
	E _{Urease} (g/g MLSS h)	9.21±1.60	4.23±0.51	2.02±0.61	1.20±0.32
	ARDRA (% similarity)	100	69 to 79	58 to 68	42 to 55
Parameters	Reactor	R _{cps0}	R _{cps50}	R _{cps100}	R _{cps200}
	Lactogen 3 (g/L)	2.70	2.70	2.70	2.70
	CPS (mg/L)	0	0	0	0
Recovery phase operation	Initial soluble COD (mg/L)	2881.67±127.72 (10)	2746.67±76.56 (10)	2776.00±150.68 (10)	2770.12±108.94 (10)
	Effluent soluble COD (mg/L)	357.40±69.94 (10)	256.00±53.01 (10)	382.00±75.23 (10)	305.23±54.30 (10)
	COD removal (%)	87.58±2.50 (10)	90.61±2.18 (10)	86.25±2.48 (10)	88.94±2.25 (10)
	MLSS (mg/L)	3051.22±55.15 (4)	3033.12±24.04 (4)	2872.33±395.62 (4)	2913.43±412.66 (4)
	MLVSS (mg/L)	2837.43±51.29 (4)	2759.86±21.02 (4)	2691.34±376.77 (4)	2729.75±395.22 (4)
	SVI (mL/g)	188.39±8.18 (4)	168.16±2.20 (4)	144.86±19.15 (4)	135.42±17.07 (4)
	ISV (m/h)	1.40±0.14 (4)	1.85±0.02 (4)	1.70±0.06 (4)	2.09±0.07 (4)
	Microscopic examination	Dense filamentous biomass	Dense filamentous biomass	Dense filamentous biomass	Weak filamentous biomass
	Photographic observation (floc size in mm)	Thick flocs 2.0-12.0	Loose flocs 2.0-12.0	Loose flocs 1.0-10.0	Granular flocs 2.0-6.0
	Cell viability	Live cells (%)	94.8	85.7	67.6
		Dead cells (%)	3.2	9.6	27.2
	Filament index	5	5	5	5
	ASBA (mg COD removed/mg VSS d)	8.13	7.45	7.13	7.10
	E _{Catalase} (mg/g MLSS h)	0.38±0.03	0.36±0.01	0.37±0.02	0.36±0.01
	E _{Dehydrogenase} (µg/g MLSS h)	27.23±0.83	26.52±2.11	23.23±1.30	22.32±0.91
	E _{Protease} (mg/g MLSS h)	2.21±0.22	2.12±0.09	1.90±0.09	1.76±0.05
	E _{Urease} (g/g MLSS h)	10.78±1.20	9.23±0.31	8.23±1.21	7.88±0.71
	ARDRA (% similarity)	100	86 to 93	81 to 84	78 to 81

Table 6.4 Variation in steady-state biomass-related general and microbiological parameters under stressed and recovery phase operation with PWW in feed.

Parameters	Reactor	R _c	R _s	R _r
	Lactogen 3 (g/L)	0.90	0	0
	PWW (mL)	0	~500-750	~500-750
Initial soluble COD (mg/L)		885.71±48.50 (14)	995.71±49.01 (14)	1011.43±44.86 (14)
Effluent soluble COD (mg/L)		45.71±17.42 (14)	241.43±22.12 (14)	348.57±39.25 (14)
COD removal (%)		94.84±1.96 (14)	75.70±2.61 (14)	65.41±4.65 (14)
MLSS (mg/L)		3494.21±404.47 (4)	3334.44±444.06 (4)	2521.32±711.35 (4)
MLVSS (mg/L)		3252.28±425.57 (4)	2866.56±373.01 (4)	2082.34±604.69 (4)
SVI (mL/g)		132.12±2.83 (4)	88.00±0.00 (4)	81.50±2.12 (4)
ISV (m/h)		2.35±0.11 (4)	3.23±0.08 (4)	3.30±0.00 (4)
Microscopic examination		Dense filamentous biomass	Weak filamentous biomass	Damaged filamentous biomass
Photographic observation (floc size in mm)		Thick flocs 1.0-12.0	Grain-like flocs 1.0-5.0	Grain-like flocs 1.0-5.0
Cell viability	Live cells (%)	94.1	74.3	67.2
	Dead cells (%)	1.7	21.0	26.4
Filament index		5	3	2
ASBA (mg COD removed/mg VSS d)		7.03	5.87	5.05
E _{Catalase} (mg/g MLSS h)		0.41±0.02	0.27±0.03	0.21±0.03
E _{Dehydrogenase} (µg/g MLSS h)		27.87±2.23	21.87±1.81	20.76±1.72
E _{Protease} (mg/g MLSS h)		2.21±0.12	1.53±0.10	1.21±0.12
E _{Urease} (g/g MLSS h)		16.23±0.92	6.51±0.92	4.34±1.11
ARDRA (% similarity)		100	89 to 92	84 to 89

Parameters	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
	Lactogen 3 (g/L)	0.90	0	0	0	0
	PWW (mL)	0	~500-750	~500-750	~500-750	~500-750
	Cu(II) (mg/L)	0	40	80	40	80
Initial soluble COD (mg/L)		891.43±47.68 (14)	918.57±52.51 (14)	948.57±41.00 (14)	982.86±41.43 (14)	954.29±42.78 (14)
Effluent soluble COD (mg/L)		51.43±22.04 (14)	651.43±42.47 (14)	810.00±30.44 (14)	762.86±42.78 (14)	881.43±37.84 (14)
COD removal (%)		94.20±2.61 (14)	28.96±4.67 (14)	14.49±3.81 (14)	22.34±3.97 (14)	7.61±1.89 (14)
MLSS (mg/L)		2088.22±76.37 (4)	1795.32±91.92 (4)	1520.42±84.85 (4)	4715.12±91.92 (4)	4295.32±127.28 (4)
MLVSS (mg/L)		1921.50±99.79 (4)	1436.64±73.54 (4)	1171.58±86.83 (4)	3677.70±71.70 (4)	2264.07±63.39 (4)
SVI (mL/g)		110.50±6.36 (4)	75.50±2.12 (4)	71.50±0.71 (4)	75.00±0.71 (4)	74.00±2.83 (4)
ISV (m/h)		2.15±0.17 (4)	3.40±0.00 (4)	3.40±0.04 (4)	3.50±0.02 (4)	3.50±0.02 (4)
Microscopic examination		Dense filamentous biomass	Less filamentous biomass	No filamentous biomass	No filamentous biomass	No filamentous biomass
Photographic observation (floc size in mm)		Thick flocs 1.0-14.0	Disintegrated flocs 1.0-5.0	Disintegrated flocs 1.0-5.0	Disintegrated flocs 1.0-5.0	Disintegrated flocs 1.0-5.0
Cell viability	Live cells (%)	93.8	36.4	23.5	28.7	16.8
	Dead cells (%)	1.4	46.8	62.0	61.2	86.4
Filament index		5	2	1	1	1
ASBA (mg COD removed/mg VSS d)		7.75	2.89	1.02	1.65	0.86
E _{Catalase} (mg/g MLSS h)		0.42±0.03	0.20±0.01	0.10±0.01	0.18±0.01	0.08±0.03
E _{Dehydrogenase} (µg/g MLSS h)		30.12±1.81	19.23±1.73	12.33±2.12	16.53±2.05	8.01±2.04
E _{Protease} (mg/g MLSS h)		2.31±0.11	0.72±0.07	0.43±0.10	0.52±0.08	0.21±0.05
E _{Urease} (g/g MLSS h)		17.23±0.80	4.02±0.81	3.02±1.04	1.52±0.64	1.24±0.51
ARDRA (% similarity)		100	52 to 63	41 to 59	34 to 55	29 to 48

Parameters	Reactor	R _c	R _{sCu40}	R _{sCu80}	R _{rCu40}	R _{rCu80}
	Lactogen 3 (g/L)	0.90	0.90	0.90	0.90	0.90
	PWW (mL)	0	0	0	0	0
	Cu(II) (mg/L)	0	0	0	0	0
Initial soluble COD (mg/L)		898.57±50.94 (14)	861.43±25.80 (14)	860.00±41.58 (14)	871.43±43.36 (14)	848.57±33.09 (14)
Effluent soluble COD (mg/L)		45.13±12.95 (14)	190.00±29.92 (14)	311.43±22.73 (14)	372.86±44.64 (14)	430.00±26.15 (14)
COD removal (%)		94.99±1.36 (14)	77.98±3.17 (14)	63.66±3.83 (14)	57.11±5.53 (14)	49.25±3.10 (14)
MLSS (mg/L)		3056.76±67.88 (4)	2295.66±233.35 (4)	1820.12±197.99 (4)	3640.20±84.85 (4)	3470.32±98.99 (4)
MLVSS (mg/L)		2857.60±85.08 (4)	2113.05±247.13 (4)	1528.80±166.31 (4)	3421.60±79.76 (4)	2264.44±255.69 (4)
SVI (mL/g)		135.08±0.71 (4)	95.10±6.36 (4)	96.22±4.24 (4)	88.00±0.00 (4)	82.00±0.00 (4)
ISV (m/h)		1.80±0.01 (4)	3.00±0.03 (4)	3.10±0.03 (4)	3.20±0.03 (4)	3.30±0.00 (4)
Microscopic examination		Dense filamentous biomass	Less filamentous biomass	Less filamentous biomass	Less filamentous biomass	Less filamentous biomass
Photographic observation (floc size in mm)		Thick flocs 2.0-12.0	Weak flocs 1.0-10.0	Granular flocs 1.0-10.0	Grain-like flocs 1.0-10	Grain-like flocs 1.0-4.0
Cell viability	Live cells (%)	92.8	82.1	53.7	57.2	45.6
	Dead cells (%)	1.8	14.2	44.1	22.0	42.8
Filament index		5	4	3	2	2
ASBA (mg COD removed/mg VSS d)		7.80	5.77	2.82	3.16	1.59
E _{Catalase} (mg/g MLSS h)		0.44±0.04	0.31±0.02	0.15±0.03	0.29±0.03	0.13±0.02
E _{Dehydrogenase} (µg/g MLSS h)		29.74±1.94	25.40±2.12	18.90±1.36	23.11±1.95	12.11±1.12
E _{Protease} (mg/g MLSS h)		2.34±0.15	1.73±0.12	0.71±0.08	1.21±0.10	0.42±0.13
E _{Urease} (g/g MLSS h)		17.31±1.22	5.10±0.63	3.52±0.90	1.81±0.62	1.41±0.61
ARDRA (% similarity)		100	78 to 86	55 to 61	78 to 83	47 to 61

concentrations have decreased by two-thirds for the sludge biomasses stressed with Cu(II) compared to the control reactor. However, very little variations in MLSS and MLVSS levels have been observed for all the sludge biomasses stressed with Cu(II) from a lower level of 10 mg/L to higher level of 80 mg/L, which do not reflect the corresponding reduction in COD removals by sludge biomasses stressed with Cu(II). Whereas the levels of MLSS and MLVSS during the recovery phase appear to have increased marginally but still not in conformity with the corresponding increase in COD removals. Therefore, higher reduction of MLSS and MLVSS levels may just indicate as an alert for the ingress of Cu(II) in the influent to the biomass without giving the severity of Cu(II) ingress levels.

The sludge biomass MLSS and MLVSS levels stressed with amoxicillin have varied within a short range of 2536 to 2834 mg/L and 2134 to 2420 mg/L, respectively, whereas the same for the control reactor (without stress) have yielded 3126 and 2660 mg/L, respectively (Table 6.2). However, sludge biomasses stressed with amoxicillin have shown COD removal of only 23% with 2000 mg/L of amoxicillin stress and 73% with 500 mg/L of amoxicillin stress in comparison of 93% without amoxicillin stress (Table 6.2). In the case of recovery phase (no amoxicillin in feed), MLSS and MLVSS levels have varied in the narrow range of 2925 to 3052 mg/L and 2662 to 2777 mg/L respectively for amoxicillin stressed sludge biomasses; and appears to have closely achieved the levels of MLSS and MLVSS of the control reactor (without stress) of 2979 and 2710 mg/L respectively (Table 6.2). Even amoxicillin-stressed sludge biomasses have shown almost complete recovery in terms of COD removals in the range of 92-93% during the recovery phase, which appears to be almost similar to the COD removal levels of the control reactor (without stress) at 93% (Table 6.2). The amoxicillin-stressed sludge biomasses have shown complete recovery potential in terms of MLSS and MLVSS levels as well as COD removals. The MLSS and MLVSS concentrations have decreased marginally for the sludge biomasses stressed with amoxicillin compared to the control reactor. Also, it appears that variations within a short-range in MLSS and MLVSS levels during the stressed phase do not reflect the corresponding reduction in COD removals by sludge biomasses stressed with amoxicillin, while the levels of MLSS and MLVSS during the recovery phase appears to have increased a little bit compared to stressed phase but has yielded complete recovery in COD removals. Hence, MLSS and MLVSS tests may not be able to provide a meaningful alert in the deteriorating health status of sludge biomasses due to amoxicillin stresses.

The MLSS and MLVSS concentrations of sludge biomasses stressed with chlorpyrifos have varied within the range of 2660 to 3037 mg/L and 2415 to 2690 mg/L, respectively, whereas the same for the control reactor (without stress) have yielded 3115 and 2913 mg/L respectively (Table 6.3). However, sludge biomasses stressed with chlorpyrifos have yielded COD removal of only 17% with 200 mg/L of chlorpyrifos stress and 72% with 50 mg/L of chlorpyrifos stress in comparison to 90% without chlorpyrifos stress (Table 6.3). While considering the case of recovery phase (no chlorpyrifos in feed), MLSS and MLVSS levels have varied in the range of 2872 to 3033 mg/L and 2691 to 2760 mg/L, respectively, for chlorpyrifos stressed sludge biomasses; and appeared to have closely regained the levels of MLSS and MLVSS up to the level of the control reactor (without stress) of 3051 and 2837 mg/L respectively (Table 6.3). The chlorpyrifos stressed sludge biomasses have shown almost complete recovery in terms of COD removals in the range of 86-90% during the recovery phase, which has been very much similar to the COD removal levels of the control reactor (without stress) at 88% (Table 6.3). The chlorpyrifos stressed sludge biomasses have shown complete recovery in terms of MLSS and MLVSS levels as well as COD removals. The MLSS and MLVSS concentrations have decreased marginally for the sludge biomasses stressed with chlorpyrifos compared to the control reactor. It appears that MLSS and MLVSS levels of

sludge biomasses stressed with chlorpyrifos during the stressed phase as well as chlorpyrifos stressed sludge biomass during the recovery phase are more or less in the same range. Even though the stressed phase has recorded a very low level of COD removal at higher chlorpyrifos concentration, and the same has appeared to regain its COD removal level up to the level of control reactor (without chlorpyrifos in feed) but without any significant variations in solid contents. It directly indicates that MLSS and MLVSS tests may not be able to provide a meaningful alert in the deteriorating health status of sludge biomasses due to chlorpyrifos stresses.

The MLSS and MLVSS levels of sludge biomasses stressed with screened PWW+Cu(II) have varied within the range of 1520 to 1795 mg/L and 1171 to 1436 mg/L, respectively; with raw PWW+Cu(II) within the range of 4295 to 4715 mg/L and 2264 to 3677 mg/L, respectively, whereas the same for control reactor (without stress) have yielded 2088 and 1921 mg/L, respectively (Table 6.4). However, sludge biomasses stressed with PWW+Cu(II) have shown COD removal of only 14% with screen PWW+80 mg/L of Cu(II) stress and 29% with screen PWW+40 mg/L of Cu(II) stress; 7% with raw PWW+80 mg/L of Cu(II) stress and 22% with raw PWW+40 mg/L of Cu(II) stress in comparison to 94% without PWW+Cu(II) stress (Table 6.4). The recovery phase [no PWW+Cu(II) in feed] of screened PWW+Cu(II) stressed sludge biomasses have shown increased MLSS and MLVSS levels in the range of 1820 to 2295 mg/L and 1528 to 2113 mg/L, respectively, compared to the stressed phase, while raw PWW+Cu(II) stressed sludge biomasses have decreased MLSS levels in the range of 3470 to 3640 mg/L compared to stressed phase but have increased MLVSS levels in the range of 2264 to 3421 mg/L compared to stressed phase, whereas the same for control reactor (without stress) have yielded MLSS and MLVSS levels of 3056 and 2857 mg/L, respectively (Table 6.4). However, stressed sludge biomasses have shown increased COD removal of 63% with screened PWW+80 mg/L of Cu(II) stress and 78% with screened PWW+40 mg/L of Cu(II) stress; 49% with raw PWW+80 mg/L of Cu(II) stress and 57% with raw PWW+40 mg/L of Cu(II) stress in comparison to 95% without PWW+Cu(II) stress (Table 6.4). The recovery phase has indicated an increase in solids contents with improvement in COD removals. In the case of stressed sludge biomasses with raw PWW+Cu(II) having decreased COD removal during stressed phase and increased COD removal during the recovery phase, the MLSS and MLVSS levels have not varied much, and therefore, these tests cannot serve as an alert as far as the condition of sludge biomass is concerned. Similarly, for the stressed sludge biomasses with screened PWW+Cu(II) having decreased COD removals indicate a marginal reduction in MLSS and MLVSS concentrations, while during the recovery phase with increased COD removals, the MLSS and MLVSS concentrations have shown a marginal increase, which again shows lack of confidence on depending upon these tests to use them as an alert for sludge biomass health.

The sewage coming to the treatment plant may have a mixture of unwanted components which shall interact with the solids or sludge biomass present in the treatment system. Generally, the solids content represented in terms of MLSS and MLVSS does not

provide any idea about the activeness of the microbial biomass, which gets impacted adversely by the imposed stresses. These stresses may cause the inactivation of a large fraction of microbial biomasses but still give very high MLSS and MLVSS, which lack the microbial activities required for organics removals. This is the major issue with these tests, and therefore, it may not serve as the alert for the health status of the sludge biomass if exposed to stresses caused by unwanted components in the incoming sewage in the field.

6.1.2 SVI and ISV tests

The sludge biomasses stressed with Cu(II) have shown SVI and ISV variations in the range of 87 to 91 mL/g and 17 to 19 m/h, respectively, while the same for the control reactor [no Cu(II) in feed] have been 225 mL/g and close to 0 m/h, respectively (Table 6.1). The sludge biomasses stressed with Cu(II) have yielded SVI of 87 mL/g and ISV of 19 m/h with 80 mg/L of Cu(II) stress; and SVI of 91 mL/g and ISV of 17 m/h with 10 mg/L of Cu(II) stress. Similarly, during the recovery phase [no Cu(II) in feed], the Cu(II) stressed sludge biomasses have shown SVI and ISV variations in the range of 68 to 83 mL/g and 16 to 18 m/h, respectively, with the control reactor giving SVI of 192 mL/g and ISV of close to 0 m/h. The 80 mg/L Cu(II) stressed sludge biomasses have yielded SVI of 78 mL/g and ISV of 18 m/h, while the 10 mg/L Cu(II) stressed sludge biomasses have shown SVI of 68 mL/g and ISV of 16 m/h (Table 6.1). The sludge biomasses stressed with Cu(II) have shown SVI of 91 mL/g and ISV of > 17 m/h, which may indicate the applicability of these tests as an alert for deteriorating health of sludge biomasses if ingressed with Cu(II). But when the Cu(II) stresses have been removed during the recovery phase, the Cu(II) stressed sludge biomasses have shown SVI of 83 mL/g and ISV of >15 m/h, still giving a false alert of Cu(II) ingress leading to the deterioration of sludge biomasses health, whereas the sludge biomasses have shown increased COD removals.

The sludge biomasses stressed with amoxicillin have shown SVI and ISV variations in the range of 130 to 182 mL/g and 1.10 to 1.87 m/h, respectively, while the same for the control reactor (no amoxicillin in feed) have been 152 mL/g and 1.36 m/h respectively. The sludge biomasses stressed with amoxicillin have yielded SVI of 133 mL/g and ISV of 1.87 m/h with 2000 mg/L of amoxicillin stress; and SVI of 182 mL/g and ISV of 1.10 m/h with 500 mg/L of amoxicillin stress (Table 6.2). During the recovery phase (no amoxicillin in feed), the amoxicillin-stressed sludge biomasses have shown SVI and ISV variations in the range of 128 to 182 mL/g and 0.84 to 1.85 m/h, respectively, with the control reactor giving SVI of 169 mL/g and ISV of 1.11 m/h. The 2000 mg/L amoxicillin stressed sludge biomasses have yielded SVI of 128 mL/g and ISV of 1.85 m/h, while the 500 mg/L amoxicillin stressed sludge biomasses have shown SVI of 182 mL/g and ISV of 0.84 m/h (Table 6.2). The sludge biomasses stressed with amoxicillin and amoxicillin stressed biomasses during the recovery phase have even shown higher SVI and ISV values compared to the control reactor. Therefore SVI and ISV may not be apt for use as an alert for monitoring the health of sludge biomasses if ingressed with amoxicillin.

The sludge biomasses stressed with chlorpyrifos have yielded SVI and ISV variations in the range of 104 to 122 mL/g and 2.49 to 2.66 m/h, respectively, while the same for the control reactor (no chlorpyrifos in feed) have been 164 mL/g and 1.72 m/h respectively. The sludge biomasses stressed with chlorpyrifos have yielded SVI of 104 mL/g and ISV of 2.66 m/h with 200 mg/L of chlorpyrifos stress; and SVI of 122 mL/g and ISV of 2.49 m/h with 50 mg/L of chlorpyrifos stress (Table 6.3). During the recovery phase (no chlorpyrifos in feed), the chlorpyrifos stressed sludge biomasses have shown SVI and ISV variations in the range of 135 to 168 mL/g and 1.70 to 2.09 m/h, respectively, with the control reactor giving SVI of 188 mL/g and ISV of 1.40 m/h. The 200 mg/L chlorpyrifos stressed sludge biomasses have yielded SVI of 135 mL/g and ISV of 2.09 m/h, while the 50 mg/L chlorpyrifos stressed sludge biomasses have shown SVI of 168 mL/g and ISV of 1.85 m/h (Table 6.3). The sludge biomasses stressed with chlorpyrifos and chlorpyrifos stressed biomasses during the recovery phase have shown lower SVI and higher ISV values compared to the control reactor, while COD removals have been drastically reduced during the stressed phase but have increased to the levels of COD removal by the control reactor; therefore SVI and ISV may not reflect the deterioration in the health of sludge biomasses if ingressed with chlorpyrifos.

The SVI and ISV of sludge biomasses stressed with screened PWW+Cu(II) have varied within the range of 71-75 mL/g and 3.40 m/h, respectively, with raw PWW+Cu(II) within the range of 74 to 75 mL/g and 3.50 m/h respectively whereas the same for control reactor (without stress) have yielded 110 mL/g and 2.15 m/h respectively (Table 6.4). The recovery phase [no PWW+Cu(II) in feed] of screened PWW+Cu(II) stressed sludge biomasses have shown increased levels of SVI in the range of 95 to 96 mL/g and decreased levels of ISV in the range of 3.00 to 3.10 m/h; while raw PWW+Cu(II) stressed sludge biomasses have shown increased levels of SVI in range of 82 to 88 mL/g and decreased levels of ISV in range of 3.20 to 3.30 m/h compared to stressed phases, whereas the same for control reactor (without stress) yielded SVI of 135 mL/g and ISV of 1.80 m/h (Table 6.4). The decreased SVI and increased ISV values during the stressed phase appear not to be in consonance with a drastic decrease in COD removals. Similarly, the slightly increased SVI and decreased ISV values also do not significantly improve COD removals. Therefore, the use of SVI and ISV values might not reflect the health of sludge biomasses if stressed with PWW+Cu(II) and, therefore, might not serve as an alert.

6.1.3 Microscopic and photographic observations

A microscopic examination has been performed at 5× and 100× magnifications, revealing floc shape and filament density. The reduction of filamentous biomass has been seen in the Cu(II) fed reactor, whereas the control reactor has shown continued filamentous growth. Lower doses of Cu(II) (10 and 20 mg/L) has a minor effect on filamentous growth, but higher doses (40 and 80 mg/L) have totally prevented filamentous growth and led to floc disintegration. The photographic observation has suggested significant changes in the physical properties of biomass (such as floc size and color) with increase in Cu(II) concentration from 10 to 80 mg/L, has also indicated disintegration of aerobic sludge biomass. The size of the flocs for

all reactors has varied from 2.0-16.0 mm on the 1st d, whereas on the 25th d, the size of the flocs for Cu(II) fed reactors has decreased to 1.0-4.0 mm during stressed phase operation. The FI began to drop once the biomass has been exposed to Cu(II). On the 1st d of operation, the FI for reactor R_{Cu10} biomass has been 5, but it has decreased to 2 when the stressed phase reached steady-state on the 20th d. Towards the end of the stressed phase operation, the FI for reactor R_{Cu40} , and R_{Cu80} biomasses have reduced to 1. During the recovery phase operation, filaments reappeared in reactor R_{Cu10} , R_{Cu20} , and R_{Cu80} biomasses starting on the 35th d and steadily grew over time. In comparison to other reactors, the reappearance of filaments has been delayed for reactor R_{Cu80} biomass, and the density of filaments has been much lower. During the recovery phase operation of Cu(II) stressed reactors, the flocs structure has looked to shift from pin-point to granular, and the flocs size appeared to rise in the range of 2.0-6.0 mm for reactors from the 35th to the 50th d of operation. Microscopic and photographic observations and FI values have revealed substantial changes in floc size, shape, and abundance of filaments due to Cu-induced stress and recovery phase operation in this study. These approaches might be used as an alert for a treatment facility to Cu(II)-contaminated influent.

According to the present study, AMX has no significant effect on the structure and morphology of aerobic sludge biomass. Throughout the experiment, steady development of filamentous microorganisms and denser flocs have been seen in reactor R_{amx0} , R_{amx500} , and $R_{amx1000}$ biomasses, and flocs size has also grown. In contrast, loose flocs structure has been observed for reactor $R_{amx2000}$ biomass. The microscopic results also indicated that AMX did not inhibit the filamentous biomass, implying that filamentous biomass has always been present in the reactor. During stressed phase operation, the flocs size has varied from 1.0 to 7.0 mm for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses (Table 6.2). FI for reactor R_{amx0} , R_{amx500} , and $R_{amx1000}$ biomasses have increased from 1 to 5, while FI for reactor $R_{amx2000}$ biomass has increased from 1 to 4. The fluorescence microscopic results have indicated 97.7%, 87.2%, 39.2%, and 17.3% of live cells and 0.4%, 30.8%, 58.9%, and 76.4% of dead cells for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, at steady-state condition of stressed phase operation. AMX dosage increase has led to a decrease in the proportion of live cells and an increase in the number of dead cells. The biomass flocs have increased in size and shape when AMX has been discontinued in the feed to the reactors. During the recovery period, all reactors have experienced uncontrolled filamentous growth. Images of the flocs taken during the stressed and recovery phases have indicated minor changes in shape and morphology. Towards the end of the recovery period, thick and denser floc structures have been found for all reactor biomasses with flocs sizes ranging from 2.0 to 20.0 mm. The photographic observation has clearly distinguished the morphology of stressed and recovery phase aerobic sludge biomass. Aerobic sludge biomass has appeared to be thicker and denser in the recovery phase than in the stressed phase. The FI has been constant at 5 during the recovery phase operation, suggesting that filamentous biomass has been abundance in all the reactors. Biomasses from reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$

have 98.1%, 88.2%, 81.3% and 64.2% live cell during recovery phase operation, up from 97.7%, 87.2%, 39.2%, and 17.3% during stressed phase operation. Similarly, dead cells have decreased during recovery phase operation to 0.8%, 7.4%, 11.0%, and 10.7% from 0.4%, 30.8%, 58.9%, and 76.4% of stressed phase operation for reactor R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively. Microscopic and photographic examinations do not act as an alert when biomass is being exposed to AMX. On the other hand, cell viability tests differentiate the stresses imposed by AMX on aerobic sludge biomass and can be utilized to warn a treatment plant to AMX-contaminated influent.

The microscopic examination has revealed that the control reactor has a high density of filaments, but the CPS-fed reactors have weak filaments. With a rise in the CPS concentration in the reactor, filaments have become weaker towards the end of stressed phase operation. The presence of thick and denser flocs in reactor R_{cps0} biomass has been reported; however, the addition of CPS to the reactors has resulted in floc disintegration. The floc size in CPS-fed reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has reduced progressively as the CPS doses in the feed increased. CPS stress to biomass has a minor impact on the FI values. The FI has been found to be 5 on the first day of operation for all reactors, and it stayed the same for reactor R_{cps0} and R_{cps50} biomasses towards the end of the stressed phase operation. In contrast, the FI for reactor R_{cps100} and R_{cps200} biomasses decreased from 5 to 4. At the steady-state condition of stressed phase operation, the proportion of live cells has decreased while the fraction of dead cells has increased. The fluorescence microscopic results have shown 98.2%, 48.3%, 33.7%, and 21.8% of live cells and 0.7%, 44.9%, 58.4%, and 72.2% dead cells for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. At the completion of the recovery phase operation, microscopic inspection revealed the existence of thick, denser, and abundant filaments in all reactor biomasses. In reactor R_{cps0} and R_{cps50} biomasses, the flocs size have varied from 2.0 to 12.0 mm, whereas it has varied from 1.0 to 10.0 mm for reactor R_{cps100} biomass and 1.0 to 6.0 mm for reactor R_{cps200} biomass towards the end of the recovery phase operation (Table 6.3). A similar rise in filamentous biomass has seen towards the end of the recovery phase operation, with a FI value of 5 for all reactor biomasses. According to the fluorescence microscopic findings for reactor R_{cps0} , R_{cps50} , R_{cps100} and R_{cps200} biomasses, there have been 94.8%, 85.7%, 67.6%, and 51.2% viable cells and 3.2%, 9.6%, 27.2, and 48.2% dead cells, respectively. As recovery phase operation has progressed to a steady-state condition, the percentage of live cells has grown, which has indicated the biomass recovery. When biomass is exposed to CPS, microscopic and photographic tests provide no warning. On the other hand, the cell viability test distinguishes between the stress imposed by CPS on aerobic sludge biomass and can be used to alert a treatment facility to CPS-contaminated influent.

The microscopic observations for reactor R_s and R_r biomasses have revealed a severe adverse impact of imposed stresses, resulting in complete floc disintegration and a significant reduction in filament density after the 8th d of stressed phase I operation, whereas the presence of filaments with abundance throughout has been observed for the control reactor

R_c biomass. The photographic observations have also revealed a significant negative impact with the disintegration of filamentous flocs into the grains-like structure for reactor R_s and R_r biomasses after 8th d of stressed phase I operation, whereas the floc sizes for the control reactor R_c biomass ranged between 1.0-12.0 mm throughout (Table 6.4). The FI for reactor R_s and R_r biomasses has constantly decreased from an initial FI value of 5 to 3 and 2 towards the end of stressed phase I operation due to the negative impact of the PWW. According to the fluorescence microscopic findings for reactor R_c , R_s , and R_r biomasses, there have been 94.1%, 74.3%, and 67.2% viable cells and 1.7%, 21.0%, and 26.4% dead cells, respectively. During stressed phase II operation, microscopic examination of biomasses has indicated complete disintegration of flocs with significant disappearance of filamentous growth at 40 mg/L of Cu(II) in feed for reactor R_{sCu40} and R_{rCu40} biomasses and complete disappearance of filaments at 80 mg/L of Cu(II) in feed for reactor R_{sCu80} and R_{rCu80} biomasses; However, in the control reactor R_c biomass, there has been continual filamentous growth. The photographic examination has also shown serious impact with complete disappearance of even grains like structure of flocs from reactor R_{sCu80} biomass while fewer grains in size range of 1.0-5.0 mm could be seen in reactor R_{sCu40} and R_{rCu40} biomasses, whereas reactor R_{rCu80} biomass has shown granular shaped particles which might be the retained suspended solids of the raw PWW feed. In contrast, the control reactor R_c biomass has shown flocs in the size range of 1.0-14.0 mm throughout the operation. The FI has decreased to 2, 1, 1, and 1 for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, towards the end of stressed phase II operation from FI of 3, 3, 2, and 2 at the beginning of this phase for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively. The fluorescence microscopic examination conducted towards the end of stressed phase II operation for reactor biomasses has indicated a much higher fraction of inactive cells in reactors fed with 80 mg/L dose of Cu(II), while the reactors fed with 40 mg/L of Cu(II) have shown relatively lower inactive cells. According to the fluorescence microscopic findings for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, there have been 93.8%, 36.4%, 23.5%, 28.7%, and 16.8% viable cells and 1.4%, 46.8%, 62.0%, 61.2%, and 86.4% dead cells, respectively. After 27 and 34 days of recovery phase operation, microscopic studies of biomasses have revealed growth and subsequent increase in floc diameters for reactor R_{sCu40} and R_{sCu80} biomasses, respectively. However, even after 3-4 weeks of operation under the recovery phase, regrowth and increase in floc sizes for reactor R_{rCu40} and R_{rCu80} biomasses have yet to occur, suggesting very slow recovery for biomasses as these biomasses have been significantly damaged by Cu(II) and raw PWW. The photographic examinations have started to show improvement in floc morphology with filamentous growth and increase in size only from 27th d and 48th d of recovery phase operation for reactor R_{sCu40} and R_{sCu80} biomasses, respectively. However, during the recovery phase operation, no discernible change in floc shape has been seen in reactor R_{rCu40} and R_{rCu80} biomasses. The FI for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has increased to 4, 3, 2, and 2 towards the end of the recovery phase operation, up from 2, 1, 1, and 1 at the start of this phase, respectively. The fluorescence microscopic analysis of reactor biomasses towards the end of the recovery phase operation has revealed increased proportion of active cells in

reactor R_{sCu40} and R_{rCu40} biomasses. While reactor R_{sCu80} and R_{rCu80} biomasses have a larger percentage of inactive cells, suggesting a very slow recovery. According to the fluorescence microscopic findings for reactor R_c , R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses, there have been 92.8%, 82.1%, 53.7%, 57.2%, and 45.6% viable cells and 1.8%, 14.2%, 44.1%, 22.0, and 42.8% dead cells, respectively. All three methods (microscopic, photographic, and cell viability test) are appropriate for distinguishing the changes in aerobic sludge biomass when exposed to PWW and PWW+Cu(II).

Microscopic and photographic observations, as well as cell viability tests have performed admirably in the study of Cu(II) and PWW in the feed, revealing the stress-response of aerobic sludge biomass. However, microscopic and photographic techniques have failed to distinguish the stress response when aerobic sludge biomass has been exposed to AMX and CPS. When aerobic sludge biomass has been exposed to various stress agents, a cell viability assay has effectively indicated the stress-response in terms of active and dead cells. Hence, it may be used to estimate the active and dead cells when a biological process-based treatment plant is infiltrated with stressing agents. Undoubtedly, microscopic and photographic techniques are low-cost approaches that can be readily conducted using microscopes and digital cameras. Compared to microscopic and photographic approaches, cell viability testing is slightly more expensive due to the chemicals required for staining. Also, an expansive fluorescence microscope is required to observe stained samples.

6.1.4 Aerobic sludge biomass activity (ASBA) tests

The ASBA test indicates that aerobic sludge biomass has the capacity to degrade organic matter. The Cu(II) stress to the aerobic sludge biomass has affected ASBA values, that has decreased with increased Cu(II) concentration in feed. A reduction in ASBA has been seen with an increase in Cu(II) concentration in feed. The ASBA for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has also been declined due to the addition of Cu(II) in feed, which has been observed as 7.00, 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d (Table 6.1). The reduction in ASBA has led to a drop in COD removal. Similarly, COD removal for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} has been 75%, 62%, 49%, and 39%, respectively, whereas reactor R_{Cu0} has shown 91% of COD removal during the steady-state condition of stressed phase operation. Moreover, ASBA for reactor R_{Cu0} , R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has been 8.34, 3.10, 2.40, 1.91, and 1.78 mg COD removed/mg VSS d, respectively, during the recovery phase. For reactor R_{Cu10} and R_{Cu20} biomasses, this indicates that organic degrading ability has increased to a certain degree, whereas organics degradation capability for reactor R_{Cu40} and R_{Cu80} biomasses has improved slightly. ASBA has been considerably different in the control reactor and Cu(II) fed reactor biomasses, indicating that ASBA may be use as an indicator for active aerobic sludge biomass in a biological-based treatment plant.

A detrimental impact of AMX has been observed on aerobic sludge biomass. The ASBA for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses has been 7.65, 3.84, and 2.22 mg COD removed/mg VSS d, which is on a lower side compared to 9.43 mg COD removed/mg VSS

d for control reactor R_{amx0} biomass (Table 6.2). Similarly, COD removal for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ have been 74%, 38%, and 23%, whereas reactor R_{amx0} has shown 93% of COD removal during the steady-state condition of stressed phase operation. The ASBA test has shown the organic degradation capability has been affected due to the presence of AMX in the reactor. The ASBA for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses has been 9.22, 8.96, and 7.96 mg COD removed/mg VSS d, which is on a lower side compared to 11.58 mg COD removed/mg VSS d for control reactor R_{amx0} biomass. Similarly, the COD removal for reactors R_{amx0} , R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ has been observed as $\sim 93\%$, which has indicated almost complete recovery.

ASBA has also been substantially impacted due to CPS stress. ASBA for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has been 6.57, 4.30, and 2.04 mg COD removed/mg VSS d, which is on lower side as compared to control reactor biomass (7.42 mg COD removed/mg VSS d) (Table 6.3). The ASBA has suggested that CPS stress has affected the organic degrading capability of aerobic sludge biomass. The adverse impact of CPS on ASBA can be directly correlated to COD removal. During the steady-state condition of stressed phase operation, the COD removal for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} has been 91%, 72%, 39%, and 18%, respectively, which has also shown a declining trend like ASBA. ASBA has suggested a significant recovery in organic degradation capability of biomass during recovery phase operation. The ASBA has improved from 7.42, 6.57, 4.30, and 2.04 mg COD removed/mg VSS d during stressed phase operation to 8.13, 7.45, 7.13, and 7.10 mg COD removed/mg VSS d during recovery phase operation for reactor R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. It's no coincidence that ASBA has improved along with COD removal in the reactors. The COD removal for reactors R_{cps0} , R_{cps50} , R_{cps100} , and R_{cps200} has ranged between 86-91% during the steady-state condition of recovery phase operation.

The ASBA for reactor R_s and R_r biomasses have been 5.05-5.87 mg COD removed/mg VSS d, respectively, which is slightly lower than reactor R_c biomass (7.03 mg COD removed/mg VSS d) (Table 6.4). Similarly, COD removal by reactors R_s and R_r have been found to be 76% and 65%, respectively, whereas control reactor R_c biomass has shown 95% COD removal during steady-state condition under stressed phase I operation. ASBA estimated towards the end of stressed phase II operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has been 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, respectively, which is on a much lower side compared to 7.75 mg COD removed/mg VSS d for control reactor R_c biomass. It has indicated a severe deterioration in bio-degradation capability of organics by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses under stressed phase II operation. The reduced COD removals of 29%, 14%, 22%, and 8% have been observed by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively in comparison to 94% by control reactor R_c biomass. PWW+Cu(II)-fed reactors have shown a decrease in the bio-degradation capability of biomasses during stressed phase II operation. On the other hand, ASBA estimated towards the end of recovery phase operation for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has been 5.77, 2.82, 3.16, and 1.59 mg COD removed/mg VSS d, respectively, which is

on a higher side compared to 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d for respective reactor biomasses obtained towards the end of stressed phase II operation. The bio-degradation of organics by reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses during recovery phase operation has been improved significantly. In the steady-state recovery phase of operation, the COD removals of reactor R_{sCu40} , R_{sCu80} , R_{rCu40} and R_{rCu80} biomasses have improved to 78 %, 64 %, 57 % and 49%, respectively, from 29%, 14%, 22%, and 8%, respectively, of stressed phase II operation.

These results show that ASBA has reduced when reactor biomasses have been exposed to stressing agents. On the other hand, the concentration of stress factors determines the influence of stress agents on ASBA. Under the stressed and recovery phase, Cu(II), AMX, CPS, and PWW+Cu(II) have shown to have detrimental effects on biomasses as indicated by ASBA. Hence, it can serve as a warning system, as well as a tool for estimating the influence of stressing agents on aerobic sludge biomass. The main benefit of ASBA testing is that it can be carried out in a laboratory with minimal facilities (such as a COD digester and a furnace), and the plant staffs can be trained suitably with the minimum efforts.

6.1.5 Enzymatic tests

During the present study, the four cellular enzyme activities viz. $E_{Catalase}$, $E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease} have been estimated towards the end of the stressed and recovery phases of operation (Table 6.1). Increased Cu(II) dosages have a significant impact on enzyme activity. $E_{Catalase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 0.01-0.09 mg/g MLSS h, while 0.33 mg/g MLSS h for reactor R_{Cu0} biomass. $E_{Dehydrogenase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 4.02-17.34 μ g/g MLSS h, while 27.52 μ g/g MLSS h for reactor R_{Cu0} biomass. $E_{Protease}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 0.29-0.93 mg/g MLSS h, while 1.85 mg/g MLSS h for reactor R_{Cu0} biomass. Similarly, the E_{Urease} for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 2.58-5.71 g/g MLSS h, while 15.59 g/g MLSS h for reactor R_{Cu0} biomass. The percent inhibition of $E_{Catalase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has been 71.9%, 84.0%, 91.5%, and 95.9%, respectively. Similarly, percent inhibition of $E_{Dehydrogenase}$ of 36.9%, 48.9%, 68.2%, and 85.4%; $E_{Protease}$ of 49.9%, 55.7%, 69.9%, and 84.2%; and E_{Urease} of 63.4%, 66.6%, 76.1%, and 83.4% has been obtained for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. The enzyme activity test has indicated that $E_{Catalase}$ has been severely inhibited (72-96%) by Cu(II) stress. The recovery phase results have suggested that enzyme activity may be recovered, but it may take a longer time. During recovery phase operation, $E_{Catalase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 0.02-0.13 mg/g MLSS h, while 0.34 mg/g MLSS h for reactor R_{Cu0} biomass. $E_{Dehydrogenase}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 4.85-23.08 μ g/g MLSS h, while 32.87 μ g/g MLSS h for reactor R_{Cu0} biomass. $E_{Protease}$ for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 0.46-1.51 mg/g MLSS h, while 2.04 mg/g MLSS h for reactor R_{Cu0} biomass. Similarly, the E_{Urease} for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses has ranged 3.30-9.38 g/g MLSS h, while 18.52 g/g MLSS h for reactor R_{Cu0} biomass. These findings have shown that $E_{Catalase}$ has been highly suppressed

during the stress phase and has been unable to fully recover during the recovery phase. However, the activity of the other three enzymes ($E_{\text{Dehydrogenase}}$, E_{Protease} , and E_{Urease}) have improved slightly. In this situation, E_{Catalase} test may be suitable for assessing the quality of aerobic sludge biomass and therefore could be beneficial for evaluating the quality of aerobic sludge biomass of a treatment facility that receives Cu(II) as an influent.

In the case of AMX in feed during the stressed phase operation, E_{Catalase} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 0.05-0.18 mg/g MLSS h, while 0.37 mg/g MLSS h for reactor R_{amx0} biomass (Table 6.2). $E_{\text{Dehydrogenase}}$ for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 13.43-20.34 $\mu\text{g/g MLSS h}$, while 27.34 $\mu\text{g/g MLSS h}$ for reactor R_{amx0} biomass. E_{Protease} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 0.76-1.32 mg/g MLSS h, while 1.65 mg/g MLSS h for reactor R_{amx0} biomass. Similarly, the E_{Urease} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 4.43-6.34 g/g MLSS h, while 9.43 g/g MLSS h for reactor R_{amx0} biomass. The percent inhibition of E_{Catalase} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has been 51.4%, 62.0%, and 84.6%, respectively. Similarly, percent inhibition of $E_{\text{Dehydrogenase}}$ of 25.6%, 44.3%, and 50.9%; E_{Protease} of 20.1%, 48.3%, and 53.8%; and E_{Urease} of 32.8%, 38.9%, and 53.0% has been obtained for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses, respectively. The AMX stress to aerobic sludge biomass has indicated that E_{Catalase} has been severely affected (51-85%) as compared to other enzyme activities. During the recovery phase operation, all four enzymes have recovered and showed pretty much identical values between AMX fed reactors and control reactor. E_{Catalase} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 0.43-0.44 mg/g MLSS h, while 0.41 mg/g MLSS h for reactor R_{amx0} biomass. $E_{\text{Dehydrogenase}}$ for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 25.34-26.54 $\mu\text{g/g MLSS h}$, while 28.54 $\mu\text{g/g MLSS h}$ for reactor R_{amx0} biomass. E_{Protease} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 1.54-1.63 mg/g MLSS h, while 1.70 mg/g MLSS h for reactor R_{amx0} biomass. Similarly, the E_{Urease} for reactor R_{amx500} , R_{amx1000} , and R_{amx2000} biomasses has ranged 9.89-11.75 g/g MLSS h, while 11.23 g/g MLSS h for reactor R_{amx0} biomass. When comparing the control and AMX fed reactors towards the end of the recovery phase operation, all enzyme activity have exhibited nearly identical values. However, $E_{\text{Dehydrogenase}}$, E_{Protease} , and E_{Urease} appear to be less effective than E_{Catalase} in evaluating the stresses induced by AMX on aerobic sludge biomass.

When aerobic sludge biomass has been exposed to CPS, $E_{\text{Dehydrogenase}}$ and E_{Urease} activities have been the most severely affected among the four enzymes tested (Table 6.3). E_{Catalase} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 0.10-0.26 mg/g MLSS h, while 0.33 mg/g MLSS h for reactor R_{cps0} biomass. $E_{\text{Dehydrogenase}}$ for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 2.23-16.43 $\mu\text{g/g MLSS h}$, while 23.23 $\mu\text{g/g MLSS h}$ for reactor R_{cps0} biomass. E_{Protease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 0.62-1.54 mg/g MLSS h, while 1.92 mg/g MLSS h for reactor R_{cps0} biomass. Similarly, the E_{Urease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 1.20-4.23 g/g MLSS h, while 9.21 g/g MLSS h for reactor R_{cps0} biomass. The percent inhibition of E_{Catalase} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has been 21.6%, 64.0%, and 70.0%, respectively. Similarly,

percent inhibition of $E_{\text{Dehydrogenase}}$ of 29.3%, 71.8%, and 90.4%; E_{Protease} of 19.8%, 55.5%, and 67.8%; and E_{Urease} of 54.1%, 78.3%, and 86.9% has been obtained for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively. The CPS stress to aerobic sludge biomass has indicated that $E_{\text{Dehydrogenase}}$ and E_{Urease} have been severely affected in the range of 29-90% and 54-86%, respectively, as compared to other enzyme activities. It appears that $E_{\text{Dehydrogenase}}$ and E_{Urease} have been found more effective than E_{Catalase} and E_{Protease} in measuring the stress imposed by CPS. Similarly, enzyme activity values have also indicated the recovery of biomass during recovery phase operation, as compared to stressed phase values. E_{Catalase} and $E_{\text{Dehydrogenase}}$ for all reactor biomasses have ranged 0.36-0.38 mg/g and 22.32-27.23 $\mu\text{g/g MLSS h}$, respectively. E_{Protease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 1.76-2.21 mg/g MLSS h. Similarly, the E_{Urease} for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses has ranged 7.88-4.23 g/g MLSS h, while 10.78 g/g MLSS h for reactor R_{cps0} biomass. As soon as CPS has been discontinued from the feed during the recovery phase operation, the enzyme activity of aerobic sludge biomass has returned to initial levels. The cellular machinery of aerobic sludge biomass microorganisms appear has to been recovered, and enzyme activity levels have improved.

Towards the end of stressed phase I operation, E_{Catalase} for reactor R_s and R_r biomasses has ranged 0.21-0.27 mg/g MLSS h, while 0.41 mg/g MLSS h for reactor R_c biomass. $E_{\text{Dehydrogenase}}$ for reactor R_s and R_r biomasses has ranged 20.76-21.87 $\mu\text{g/g MLSS h}$, while 27.87 $\mu\text{g/g MLSS h}$ for reactor R_c biomass (Table 6.4). E_{Protease} for reactor R_s and R_r biomasses has ranged 1.21-1.53 mg/g MLSS h, while 2.21 mg/g MLSS h for reactor R_c biomass. Similarly, the E_{Urease} for reactor R_s and R_r biomasses has ranged from 4.34-6.51 g/g MLSS h, while 16.23 g/g MLSS h for reactor R_c biomass. The percent inhibition of E_{Catalase} for reactor R_s and R_r biomasses has been 34.6% and 49.1%, respectively. Similarly, the percent inhibition of $E_{\text{Dehydrogenase}}$ of 21.5% and 25.5%; E_{Protease} of 30.8% and 45.2%; and E_{Urease} of 10.9% and 73.3% has been obtained for reactor R_s and R_r biomasses, respectively. The PWW stress to aerobic sludge biomass has indicated that E_{Urease} has been severely affected (11-73%) as compared to other enzyme activities, which has reflected the changes in biomass characteristics due to imposed stresses. When aerobic sludge biomass has been exposed to PWW+Cu(II) in the feed during stressed phase II operation, E_{Catalase} for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has ranged 0.10-0.20 mg/g MLSS h, while 0.42 mg/g MLSS h for reactor R_c biomass. $E_{\text{Dehydrogenase}}$ for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has ranged 8.01-19.23 $\mu\text{g/g MLSS h}$, while 30.12 $\mu\text{g/g MLSS h}$ for reactor R_c biomass. E_{Protease} for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has ranged 0.21-0.72 mg/g MLSS h, while 2.31 mg/g MLSS h for reactor R_c biomass. Similarly, the E_{Urease} for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has ranged 1.24-4.02 g/g MLSS h, while 17.23 g/g MLSS h for reactor R_c biomass. The percent inhibition of E_{Catalase} for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses has been 52.7%, 76.7%, 57.8%, and 81.1%, respectively. Similarly, percent inhibition of $E_{\text{Dehydrogenase}}$ of 36.2%, 59.1%, 45.1%, and 73.4%; E_{Protease} of 68.8%, 81.4%, 77.5%, and 90.9%; and E_{Urease} of 76.7%, 82.5%, 91.2%, and 92.8% has been obtained for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} ,

and R_{Cu80} biomasses, respectively. The PWW+Cu(II) stress to aerobic sludge biomass has indicated that $E_{Protease}$ and E_{Urease} have been severely affected in the range of 69-91% and 77-93%, respectively, compared to other enzyme activities, which has reflected the changes in biomass characteristics due to imposed stresses. When PWW+Cu(II) has been discontinued in the feed during recovery phase operation, enzyme activities variations have been: $E_{Catalase}$ of 0.13-0.31 mg/g MLSS h for stressed reactor biomasses compared to 0.44 mg/g MLSS h for reactor R_c biomass; $E_{Dehydrogenase}$ of 12.11-25.40 μ g/g MLSS h for stressed reactor biomasses compared to 29.74 μ g/g MLSS h for reactor R_c biomass; $E_{Protease}$ of 0.42-1.73 mg/g MLSS h for stressed reactor biomasses compared to 2.34 mg/g MLSS h for reactor R_c biomass; and E_{Urease} of 1.41-5.10 g/g MLSS h for stressed reactor biomasses compared to 17.31 g/g MLSS h for reactor R_c biomass. These values have shown slight improvements over values obtained during stressed phase II operation. Again, it has indicated its failure of showing complete recovery by reactor biomasses.

It appears that Cu(II) and AMX have inhibited $E_{Catalase}$ by 72-96% and 51-85%, respectively. CPS has inhibited $E_{Dehydrogenase}$ and E_{Urease} by 29-90% and 54-86%, respectively. Furthermore, $E_{Protease}$ and E_{Urease} have been inhibited by PWW+Cu(II) in the range of 69-91% and 77-93%, respectively. As a result, all four enzyme activities have appeared to be useful in predicting the activeness of aerobic sludge biomass dependent on the type of stressing agent used and therefore its suitability as a general tool is questionable.

6.1.6 Amplified ribosomal DNA restriction analysis (ARDRA)

Microorganisms are sensitive to toxic agents. The microbial community of aerobic sludge biomass has shifted when exposed to stressing agents – Cu(II)/AMX/CPS/PWW+Cu(II). The degree of change has been dependent on the toxicity of the stressing agents. The ARDRA technique is commonly used to detect changes in the microbial community structure. ARDRA has revealed the changes in microbial community structure during stressed phase operation. *AluI* has shown 71.4%, 64.7%, 56.8%, and 48.1% of similarity, while *MspI* has shown 65.8%, 51.5%, 42.8% and 37.9% of similarity for reactor biomasses fed with Cu(II) concentration of 10, 20 40, and 80 mg/L, respectively, as compared to reactor R_{Cu0} biomass. Cu(II) stress may be responsible for alterations in microbial populations in reactors. However, recovery phase microbial community changes have also been reflected by ARDRA, which has shown increased similarity compared to stressed phase data. *AluI* has shown 85.9%, 79.8%, 76.2%, and 61.4% of similarity, while *MspI* has shown 83.5%, 82.1%, 74.8% and 64.2% similarity for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively, compared to control reactor R_{Cu0} biomass.

When aerobic sludge biomass has been exposed to AMX during stressed phase operation, ARDRA has revealed the microbial community structure changes. *AluI* has shown 78.6%, 68.7%, and 57.1% of similarity; *HhaI* has shown 80.0%, 75.0%, and 74.0% of similarity; and *MspI* has shown 72.2%, 59.3%, and 51.9% of similarity for reactors biomasses fed with AMX concentration of 500, 1000, and 2000 mg/L, respectively, as compared to

reactor R_{amx0} biomass. ARDRA has shown a significant change in microbial community structure with increased doses of AMX. When AMX has been discontinued in the feed during recovery phase operation, ARDRA has revealed regain in microbial community structure of aerobic sludge biomass during recovery phase operation. *AluI* has shown 91.2%, 72.3%, and 68.0% of similarity; *HhaI* has shown 94.3%, 84.5%, and 84.5% of similarity; and *MspI* has shown 80.0%, 73.3%, and 83.9% of similarity for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses, respectively, as compared to reactor R_{amx0} biomass.

When aerobic sludge biomass has been exposed to CPS during stressed phase operation, ARDRA has revealed the microbial community structure changes. *AluI* has shown 69.3%, 57.5%, and 42.3% of similarity; *HhaI* has shown 78.5%, 68.4%, and 54.6% of similarity; and *MspI* has shown 72.3%, 63.2%, and 51.9% of similarity for reactor biomasses fed with CPS concentration of 50, 100, and 200 mg/L, respectively, as compared to reactor R_{cps0} biomass. When CPS has been discontinued in the feed during the recovery phase operation, ARDRA has revealed the recovery in microbial community structure. *AluI* has shown 93.4%, 81.2%, and 77.5% of similarity; *HhaI* has shown 86.0%, 82.3%, and 80.4% of similarity; and *MspI* has shown 90.1%, 84.2%, and 81.2% of similarity for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, as compared to reactor R_{cps0} biomass.

Towards the end of stressed phase I operation, *AluI* has shown 92.2%, and 88.4% of similarity; *HhaI* has shown 90.0%, and 84.1% of similarity; and *MspI* has shown 89.1%, and 87.1% of similarity for reactor R_s and R_r biomasses, respectively, as compared to reactor R_c biomass. Towards the end of stressed phase II operation, *AluI* has shown 52.1%, 43.3%, 33.8%, and 29.3% of similarity; *HhaI* has shown 51.4%, 41.2%, 39.2%, and 31.0% of similarity; and *MspI* has shown 62.4%, 58.4%, 54.7% and 48.3% of similarity for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, as compared to reactor R_c biomass. Towards the end of recovery phase operation, *AluI* has shown 85.7%, 59.8%, 78.3%, and 47.3% of similarity; *HhaI* has shown 80.7%, 61.2%, 83.1% and 59.4% of similarity; and *MspI* has shown 78.3%, 54.2%, 81.2%, and 61.2% of similarity for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, as compared to reactor R_c biomass.

ARDRA has revealed that aerobic sludge biomass has shown 38-71% of similarity in the presence of Cu(II), 52-80% of similarity in the presence of AMX, 42-79% of similarity in the presence of CPS, and 29-62% of similarity in the presence of PWW+Cu(II). ARDRA has identified a significant shift in microbial community structure. As a result, this approach may be employed; however, the chemicals, apparatus, and facilities required to execute this method are prohibitively expensive and cannot be performed in a treatment plant laboratory, and also trained manpower is needed. The ARDRA test may be available in the nearby research and higher academic institutions and therefore it has to be kept in mind while considering this test for biomass assessment.

6.2 Summary

Considering biomass related general and microbiological parameters for assessment of biomass, it has been observed that MLSS and MLVSS do not provide a meaningful alert in the deteriorating health status of sludge biomasses. Furthermore, SVI and ISV have been impacted in the presence of Cu(II) and PWW+Cu(II) instances, and only marginally in the presence of AMX and CPS. However, based on the overall performance, it can be said that SVI and ISV also do not provide the warning that biomass is being affected. The microscopic examination has revealed the floc structure and shape of biomass, which have shown significant changes in the presence of Cu(II) and PWW+Cu(II). On the other hand, AMX and CPS stresses have a minor impact on floc morphology. However, microscopic examination is required to have a thorough understanding of the flocs physiology of aerobic sludge biomass. Cell viability has also revealed significant variations in aerobic sludge biomass in response to stresses imposed by Cu(II)/AMX/CPS/PWW+Cu(II). Cell viability has decreased when Cu(II)/AMX/CPS/PWW+Cu(II) fed in reactors, but has increased when Cu(II)/AMX/CPS/PWW+Cu(II) discontinued in the feed. As a result, cell viability test might be utilized as an alerting tool for treatment plant biomass. The enzyme activity values have also been inhibited due to presence of Cu(II)/AMX/CPS/PWW+Cu(II) in the feed. The higher doses of Cu(II)/AMX/CPS/PWW+Cu(II) have inhibited E_{Catalase} (70-96%), $E_{\text{Dehydrogenase}}$ (73-90%), E_{Protease} (54-91%), and E_{Urease} (53-91%). Based on the findings, it appears that E_{Catalase} and $E_{\text{Dehydrogenase}}$ have been considerably affected in the presence of stressing agents, and these two enzyme activities may be utilized as a warning indicator for biomass quality degradation. The microbial community structure has also been changed in aerobic sludge biomass, when exposed to Cu(II)/AMX/CPS/PWW+Cu(II). ARDRA can be utilized as a warning system, but not as a preliminary test for assessing aerobic biomass. ARDRA must be utilized once the above-mentioned techniques confirm the deterioration of biomass. Therefore, major techniques such as microscopic examination and the ASBA test may be used as preliminary tests, while cell viability tests, enzyme activity assays (E_{Catalase} and $E_{\text{Dehydrogenase}}$), and ARDRA may be used as confirmation tests during the evaluation of the aerobic sludge biomass quality. A minimum laboratory equipment needed for tests are listed in [Table A-V.1](#). It appears that enzyme activity testing can be carried out at a laboratory located at a treatment facility with a treatment capacity of 5 MLD or more, whereas cell viability test and the ARDRA must be outsourced as treatment plants with a capacity of more than 5 MLD do not have a laboratory where cell viability tests and ARDRA may be performed.

Summary and Limitations

This chapter summarizes the major findings of the research work presented along with the limitations associated with the current studies. This chapter also provides scope for future research.

7.1 Impacts of copper(II) stress on aerobic sludge biomass

The impacts of 10, 20, 40, and 80 mg/L of Cu(II) have been investigated on aerobic sludge biomass in four different reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} . These reactors have been operated for 25 d with Cu(II) in the feed and this operation has been termed as stressed phase operation. After completion of the stressed phase operation, Cu(II) has been stopped in the feed of the reactors and have then been operated for another 29 d, which has been termed as recovery phase operation. The key findings of both the phases have been highlighted below:

7.1.1 Stressed phase operation

- * The COD removal for reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have been observed as 75%, 62%, 49%, and 39% at steady-state condition, respectively, whereas control reactor R_{Cu0} has yielded uniform COD removal of 91% throughout. It clearly indicates the adverse impact of Cu(II) on organics removal of aerobic sludge biomass.
- * Addition of Cu(II) has inhibited growth of aerobic sludge biomass.
- * The reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown SVI of 86, 87, 98, and 84 mL/g; and ISV of 17.22, 17.89, 17.64, and 18.63 m/h, respectively, indicating very good settling properties. However, it is at the cost of organics removal due to adverse impact of Cu(II) on sludge biomass.
- * ASBA values for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been observed as 2.28, 1.89, 1.55, and 1.43 mg COD removed/mg VSS d, respectively, which reflects deterioration in organic degradation capacity of biomasses due to adverse impact of Cu(II) in the feed.

- * The lower Cu(II) (10 and 20 mg/L) doses have inhibited filamentous growth and have transformed filamentous biomass to granular shaped biomass, whereas high concentration (80 mg/L) of Cu(II) has transformed the biomass into pinpoint flocs resulting in complete floc disintegration.
- * Cu(II) stress has a substantial impact on filament index (FI). FI values have dropped for reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses to 1 compared to FI value 5 for the control reactor biomass, indicating absence of filamentous biomass growth.
- * Cu(II) doses have significantly influenced enzyme activities of aerobic sludge biomass. The enzyme activity of Cu(II) fed reactors have ranged between 0.01-0.09 mg/g MLSS h for $E_{Catalase}$; 4.02-17.34 μ g/g MLSS h for $E_{Dehydrogenase}$; 0.29-0.93 mg/g MLSS h for $E_{Protease}$; and 2.58-5.71 g/g MLSS h for E_{Urease} , towards the steady-state stressed phase operation, which have been very low compared to 0.33 mg/g MLSS h for $E_{Catalase}$; 27.52 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.85 mg/g MLSS h for $E_{Protease}$; and 15.59 g/g MLSS h for E_{Urease} of control reactor R_{Cu0} biomass. The results have indicated that all four enzymes have severely been inhibited by Cu(II).
- * ARDRA test has shown changes in microbial community due to Cu(II) stress to sludge biomasses. The restriction enzyme *AluI* has shown 71.4%, 64.7%, 56.8%, and 48.1% of similarity, while *MspI* has shown 65.8%, 51.5%, 42.8%, and 37.9%, of similarity for reactors biomasses fed with Cu(II) concentration of 10, 20 40, and 80 mg/L, respectively, as compared to reactor R_{Cu0} biomass. The microbial community structure have changed drastically (38-71% of similarity) in response to 10-80 mg/L of Cu(II) stress as compared to control reactor biomass.

7.1.2 Recovery phase operation

- * Reactors R_{Cu10} and R_{Cu20} have shown improvement in COD removals; however, no recovery symptoms have been observed for reactors R_{Cu40} and R_{Cu80} . The reactors R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} have shown 82%, 72%, 59%, and 41% of COD removal, respectively, at steady-state condition of recovery phase operation. It has indicated marginal improvement in COD removal during recovery phase operation.
- * The reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown SVI of 75, 76, 88, and 82 mL/g; and ISV of 16.44, 17.31, 17.71, and 18.31 m/h, respectively, indicating no significant changes compared to stressed phase operation even with marginal improvement in COD removal.
- * The FI has increased to 4, 4, and 3 for reactor R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses, respectively. It has indicated reappearance of filamentous biomass in the reactors during recovery phase operation.
- * ASBA for reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have been observed as 3.10, 2.40, 1.91, and 1.78 mg COD removed/mg VSS d, respectively, during recovery phase operation. It has indicated marginal improvement in bio-degradation capability of

biomasses.

- * All four enzymes have shown higher recovery for reactor R_{Cu10} and R_{Cu20} biomasses in the range of 0.08-0.13 mg/g MLSS h for $E_{Catalase}$; 19.18-23.08 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.18-1.51 mg/g MLSS h for $E_{Protease}$; and 7.65-9.38 g/g MLSS h for E_{Urease} . However, $E_{Catalase}$ has not recovered for reactor R_{Cu40} and R_{Cu80} biomasses, while the activity of other three enzymes ($E_{Dehydrogenase}$, $E_{Protease}$, and E_{Urease}) have shown recovery trends.
- * The reactor R_{Cu10} , R_{Cu20} , R_{Cu40} , and R_{Cu80} biomasses have shown similarity of 85.9%, 79.8%, 76.2%, and 61.4%, respectively, when digested with *AluI*; and 83.5%, 82.1%, 74.8%, and 64.2%, respectively, when digested with *MspI*, under recovery phase operation, as compared to reactor R_{Cu0} biomass, which has been significantly higher than the similarity values obtained in stressed phase operation. The microbial community structure has considerably recovered (61-86% similarity with control biomass) towards the end of the recovery phase.

The Cu(II) stressed reactors have recovered partially in terms of COD removal, with marginally improved floc morphology. In addition, ASBA has also shown increasing trend which has correlated well with COD removal. Moreover, enzyme activity and microbial community of aerobic sludge biomass could recover partially. Hence, it has appeared that addition of Cu(II) in the feed has hindered reactor performance, biomass behavior and characteristics, and Cu(II) discontinuation in the feed has only allowed partial recovery of aerobic sludge biomass.

7.2 Effects of amoxicillin IV stress on aerobic sludge biomass

The effects of 500, 1000, and 2000 mg/L of amoxicillin IV (AMX) have been investigated on aerobic sludge biomass in three different reactors R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$. These reactors have been operated for 43 d with AMX in the feed and this operation has been termed as stressed phase operation. After completion of stressed phase operation, AMX has been stopped in the feed of reactors and have then been operated for another 35 d, which has been termed as recovery phase operation. The following key observations can be made for both the phases:

7.2.1 Stressed phase operation

- * COD removal for reactors R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ has been observed as 74%, 38%, and 23%, respectively, whereas control reactor R_{amx0} has shown 93% of COD removal during the steady-state condition. It has indicated adverse impact of AMX on organic removal capability of aerobic sludge biomass.
- * The reactor R_{amx500} biomass has shown SVI of 145 mL/g, while reactor $R_{amx1000}$ and $R_{amx2000}$ biomasses have exhibited SVI in the range of 121-126 mL/g compared to 140 mg/L for control reactor biomass. ISV has been observed in the range of 1.49-1.93 m/h during stressed phase operation for all the reactor biomasses compared to 1.50

m/h for control reactor biomass. It has indicated slight impact of AMX on SVI and ISV of aerobic sludge biomasses.

- * ASBA has been observed as 7.65, 3.84, and 2.22 mg COD removed/mg VSS d for reactor $R_{\text{amx}500}$, $R_{\text{amx}1000}$, and $R_{\text{amx}2000}$ biomasses, respectively, which has been on a lower side compared to 9.43 mg COD removed/mg VSS d for control reactor $R_{\text{amx}0}$ biomass. It has indicated deterioration in organic degradation capability of aerobic sludge biomass due to AMX in the feed.
- * The microscopic results have indicated that AMX has enhanced filamentous biomass, which implied that filamentous biomass has been always present in the reactors fed with AMX.
- * The photographic observation has suggested that floc size of aerobic sludge biomasses have continuously increased due to filamentous growth outside flocs. FI for aerobic sludge biomasses have increased from 1 (lower filament counts) to 5 (higher filament counts), which has indicated abundance of filamentous biomass in AMX stressed reactors.
- * The fluorescence microscopic observation has shown decrease in active portion of aerobic sludge biomass. The live cell of 87% has been observed in reactor $R_{\text{amx}500}$ biomass, while it has been only 17% in reactor $R_{\text{amx}2000}$ biomass indicating dead cell portion in the range of 13-83%. It has indicated adverse impact of AMX on active portion of aerobic sludge biomasses.
- * The polysaccharides (PS), proteins (PN), and extracellular polymeric substance (EPS) contents for all reactor biomasses have increased towards the end of the stressed phase operation. Since PS, PN, and EPS contents in AMX fed reactor biomasses and the control reactor biomass have shown close similarity, which has indicated absence of any significant impact of AMX on PS, PN, and EPS contents of aerobic sludge biomass.
- * The enzyme activities of AMX fed reactors have ranged between 0.05-0.18 mg/g MLSS h for E_{Catalase} ; 13.43-20.34 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 0.76-1.32 mg/g MLSS h for E_{Protease} ; and 4.43-6.34 g/g MLSS h for E_{Urease} compared to 0.37 mg/g MLSS h for E_{Catalase} ; 27.34 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 1.65 mg/g MLSS h for E_{Protease} ; and 9.43 g/g MLSS h for E_{Urease} of control reactor $R_{\text{amx}0}$ biomass. Among all the four enzymes, E_{Catalase} has severely been affected due to AMX stress.
- * *AluI* has shown 78.6%, 68.7%, and 57.1% of similarity; *HhaI* has shown 80.0%, 75.0%, and 74.0% of similarity; and *MspI* has shown 72.2%, 59.3%, and 51.9% of similarity for reactor biomasses fed with AMX concentration of 500, 1000, and 2000 mg/L, respectively, as compared to reactor $R_{\text{amx}0}$ biomass. The microbial community structure have changed (52-80% of similarity with control reactor biomass) in response to 500-2000 mg/L of AMX in feed. It has indicated adverse impact of AMX on microbial community structure of aerobic sludge biomass.

7.2.2 Recovery phase operation

- * Reactors R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ have shown almost 93% of COD removal at the steady-state condition of recovery phase operation. It has indicated that discontinuation of AMX in the feed has resulted in almost complete recovery of aerobic sludge biomass for COD removals.
- * The average SVI for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged between 130-188 mL/g, whereas ISV have been observed in the range of 0.89-1.84 m/h during recovery phase operation compared to SVI of 167 mL/g and ISV of 1.24 m/h for control reactor biomass. No significant changes have been observed in SVI and ISV, while comparing both stressed and recovery phases operations.
- * ASBA has shown very high recovery during recovery phase operation in the range of 7.96-9.22 mg COD removed/mg VSS d for AMX stressed reactor biomasses. It has indicated almost complete recovery of aerobic sludge biomass for its organic degradation capability after discontinuation of AMX in feed.
- * The discontinuation of AMX has further enhanced filamentous growth suggested by microscopic examination; hence thick and denser filamentous structure have been observed in photographic observation for all the reactors. The floc size with filaments for reactor biomasses have increased from 1-7 mm (in stressed phase operation) to 2-20 mm (in recovery phase operation).
- * The FI has been observed as 5 (higher filaments) for all reactor biomasses during recovery phase operation. It has indicated no reduction in filamentous growth throughout the operation.
- * During recovery phase, live cell portion has increased to 64-88% in aerobic sludge biomasses – higher in reactor R_{amx500} and lower in reactor $R_{amx2000}$ biomasses. It has indicated increase in live cells after discontinuation of AMX in the feed.
- * PS, PN, and EPS contents have slightly increased towards the end of the recovery phase operation, indicating no significant impact of AMX on PS, PN, and EPS contents of the aerobic sludge biomass, after its discontinuation in the feed.
- * The enzyme activity of reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses have ranged between 0.43-0.44 mg/g MLSS h for $E_{Catalase}$; 25.34-26.54 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.54-1.63 mg/g MLSS h for $E_{Protease}$; and 9.89-11.75 g/g MLSS h for E_{Urease} in comparison to 0.41 mg/g MLSS h for $E_{Catalase}$; 28.54 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.70 mg/g MLSS h for $E_{Protease}$; and 11.23 g/g MLSS h for E_{Urease} of control reactor R_{amx0} biomass. All four enzyme activities have shown almost complete recovery comparable to reactor R_{amx0} biomass.
- * The restriction enzyme *AluI* has shown 91.2%, 74.3%, and 68.0% similarity; *HhaI* has exhibited 94.3%, 84.5%, and 84.5% similarity; and *MspI* has shown 80.0%, 73.3% and 83.9% similarity for reactor R_{amx500} , $R_{amx1000}$, and $R_{amx2000}$ biomasses,

respectively, during recovery phase operation as compared to control reactor. Stressed reactor biomasses has regained its microbial community structure (68-94% similarity) compared to control reactor biomass.

The addition of AMX during stressed phase operation has drastically impacted COD removal along with reduction in ASBA, live cell fractions and microbial community structure, while enhanced number of filamentous biomass observed in this phase with FI of 4-5. However, during recovery phase operation, all the reactors have regained its COD removal capabilities to more than 93% with corresponding increase in ASBA, live cell fraction and microbial community structure indicating complete recovery of aerobic sludge biomasses. However, enhanced number of filamentous biomass during stressed phase operation needs further in-depth investigation.

7.3 Effects of chlorpyrifos stress on aerobic sludge biomass

The effects of 50, 100, and 200 mg/L of chlorpyrifos (CPS) have been investigated on aerobic sludge biomass in three different reactors R_{cps50} , R_{cps100} , and R_{cps200} . These reactors have been operated for 34 d with CPS in the feed and this phase has been termed as stressed phase operation. After completion of stressed phase operation, CPS has been discontinued in the feed of reactors and have been further operated for 42 d, which has been termed as recovery phase operation. The major findings of this study are as follows:

7.3.1 Stressed phase operation

- * The COD removal for reactors R_{cps50} , R_{cps100} , and R_{cps200} have been observed as 72%, 39%, and 18%, respectively, during the steady-state condition, whereas control reactor R_{cps0} has shown 91% of COD removal. It has indicated that CPS in the feed has an adverse impact on the organic removal capability of reactors.
- * The addition of CPS has slightly lowered SVI in the range of 104-114 mL/g for CPS fed reactor biomasses, while control reactor R_{cps0} biomass has shown SVI of 136 mL/g. ISV has been observed in the range of 2.42-2.52 m/h during stressed phase operation for CPS fed reactor biomasses, while reactor R_{cps0} biomass has shown ISV of 2.13 m/h. It has indicated slight impact of CPS on SVI and ISV, which has resulted in marginal improvement in settling properties of aerobic sludge biomass, but at the cost of organic removal capability.
- * ASBA has been observed as 6.57, 4.30, and 2.04 mg COD removed/mg VSS d for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, which has been on a lower side compared to 7.42 mg COD removed/mg VSS d for control reactor R_{cps0} biomass, indicating decreased organic degradation capability of aerobic sludge biomass due to CPS stress.
- * The microscopic examination has shown slightly decreased number of filamentous for CPS fed reactor biomasses. With a increase in CPS concentration, filaments have become lower in counts towards the end of stressed phase operation, indicating slight inhibition in filamentous biomass. The floc size for CPS fed reactor biomasses have

reduced progressively with increase in CPS doses resulting in disintegration of floc. Reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have been observed to have FI of 5, 4, and 4, respectively, indicating higher proportion of loose filaments in the reactor, thereby showing very marginal impact of CPS on filamentous biomass.

- * Active portion of aerobic biomass has decreased in the range of 22-48% with increased CPS concentration towards the end stressed phase operation. Reactor R_{cps200} biomass has shown higher decrease of 48% in active portion. It has indicated negative impact of CPS on active portion of aerobic sludge biomass.
- * PS, PN, and EPS contents in reactor biomasses fed with CPS have increased slightly, indicating not much impact of CPS.
- * The enzyme activity of CPS fed reactors have ranged between 0.10-0.26 mg/g MLSS h for $E_{Catalase}$; 2.23-16.43 μ g/g MLSS h for $E_{Dehydrogenase}$; 0.62-1.54 mg/g MLSS h for $E_{Protease}$; and 1.20-4.23 g/g MLSS h for E_{Urease} in comparison to 0.33 mg/g MLSS h for $E_{Catalase}$; 23.23 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.92 mg/g MLSS h for $E_{Protease}$; and 9.21 g/g MLSS h for E_{Urease} of control reactor R_{cps0} biomass. It has indicated that $E_{Dehydrogenase}$ and E_{Urease} activities have been most severely affected amongst the four enzymes tested.
- * *AluI* has shown 69.3%, 57.5%, and 42.3% of similarity; *HhaI* has shown 78.5%, 68.4%, and 54.6% of similarity; and *MspI* has shown 72.3%, 63.2%, and 51.9% of similarity for reactors biomasses fed with CPS concentration of 50, 100, and 200 mg/L, respectively, as compared to reactor R_{cps0} biomass. ARDRA has established the relation between control and CPS fed reactors indicating microbial community changes (42-79% similarity with control reactor biomass) during stressed phase operation.

7.3.2 Recovery phase operation

- * Reactors R_{cps50} , R_{cps100} , and R_{cps200} have shown COD removal of more than 88% during steady-state condition of recovery phase operation, indicating that discontinuation of CPS in the feed has resulted in high recovery of organic removal capability of sludge biomasses.
- * Reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses have shown SVI of 152, 145, and 135 mL/g; and ISV of 2.06, 2.08, and 2.28 m/h, respectively, while control reactor biomass has SVI and ISV of 185 mL/h and 1.48 m/h, respectively. SVI has increased for all the reactors due to abundance of filamentous biomass resulting in decreased ISV, indicating poor setting properties of aerobic sludge biomass.
- * ASBA have been observed as 7.45, 7.13, and 7.10 mg COD removed/mg VSS d for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, compared to 8.13 mg COD removed/mg VSS d for control reactor biomass. It has indicated that aerobic sludge biomass has regained almost completely its organic degradation capability after discontinuation of CPS.
- * The loose and weak flocs of CPS fed reactors (during stressed phase) has transformed

into extremely dense and thick flocs and filaments (during recovery phase), indicating biomass growth after stoppage of CPS in the feed. FI has been observed as 5 (higher filament counts) for CPS fed reactor biomasses, towards the end of recovery phase operation. It has indicated filamentous biomass growth.

- * Active portion of biomass has increased in the range of 51-85% after discontinuation of CPS. Reactor R_{cps200} biomass has active portion of 51%. It has indicated recovery of aerobic sludge biomass.
- * Enzyme activity values have also improved close to the control reactor values. The enzyme activity values of reactors R_{cps50} , R_{cps100} , and R_{cps200} have ranged between 0.36-0.37 mg/g MLSS h for $E_{Catalase}$; 22.32-26.52 μ g/g MLSS h for $E_{Dehydrogenase}$; 1.76-2.12 mg/g MLSS h for $E_{Protease}$; and 7.88-9.23 g/g MLSS h for E_{Urease} with 0.38 mg/g MLSS h for $E_{Catalase}$; 27.23 μ g/g MLSS h for $E_{Dehydrogenase}$; 2.21 mg/g MLSS h for $E_{Protease}$; and 9.43 g/g MLSS h for E_{Urease} for control reactor biomass.
- * The restriction enzyme *AluI* has shown 93.4%, 81.2%, and 77.5% similarity; *HhaI* has exhibited 86.0%, 82.3%, and 80.4% similarity; and *MspI* has shown 90.1%, 84.2%, and 81.2% similarity for reactor R_{cps50} , R_{cps100} , and R_{cps200} biomasses, respectively, during recovery phase operation as compared to control reactor. ARDRA has shown that CPS fed reactor biomasses has regained microbial community structure (78-93% similarity compared to reactor R_{cps0} biomass).

During stressed phase operation, reactor R_{cps200} has shown very little COD removal, while there has been slight reduction in FI values, while no changes have been observed in PS, PN, and EPS contents; $E_{Catalase}$ and $E_{Protease}$ activities; despite significant changes in cell viability, ASBA, $E_{Dehydrogenase}$, E_{Urease} and microbial community structure. This aspect needs further investigation. The discontinuation of CPS in feed has shown recovery in performance and morphology related parameters. The COD removal for CPS fed reactors have improved to its initial level. Thick and strong filamentous biomass has started to grow again and resulted in higher SVI and lower ISV. ASBA has also significantly improved showing recovery in organic degradation capability of the sludge biomasses. All four enzymes have regain their initial level after discontinuation of CPS in the feed. In addition, ARDRA has revealed increase in similarity between control and CPS fed reactor biomasses. Overall, it has appeared that complete recovery of aerobic sludge biomass has been possible after discontinuation of CPS in the feed.

7.4 Impacts of piggery wastewater stress on aerobic sludge biomass

The impacts of piggery wastewater (PWW) on aerobic sludge biomass have been studied with three reactors named as R_c , R_s , and R_r fed with Lactogen 3 (synthetic wastewater), screened PWW and raw PWW, respectively. The reactors have been operated with PWW feed for 41 d under stressed phase I operation. After completion of stressed phase I operation, reactor R_s and R_r biomasses have been evenly divided into reactors named as R_{sCu40} , and R_{sCu80} ; R_{rCu40} ,

and R_{Cu80} , respectively, and fed with 40 and 80 mg/L of Cu(II), respectively, in addition to PWW. Thereafter reactors have been operated for 31 d with PWW+Cu(II) in the feed termed as stressed phase II operation. After completion of stressed phase II operation, addition of PWW+Cu(II) has been discontinued in the feed (after 72nd d) and thereafter reactors R_c , R_{Cu40} , R_{Cu80} , R_{Cu40} , and R_{Cu80} have been operated further for 48 d without PWW+Cu(II) but with Lactogen 3 in the feed under recovery phase operation. The following key points can be noted down from the study:

7.4.1 Stressed phase I operation

- * The COD removal for reactors R_s and R_r has been observed as 76% and 65%, respectively, during steady-state condition, while control reactor R_c has shown COD removal of 95%. It has indicated notable influence on COD removal capability of aerobic sludge biomass due to addition of PWW as feed.
- * The PWW fed reactor biomasses have shown lower SVI (93 mL/g for R_s and 89 mL/g for R_r) as compared to reactor R_c biomass (127 mL/g). On the other hand, reactor R_c biomass has shown reduced ISV (2.43 m/h) as compared to PWW fed reactor biomasses (3.05 m/h for R_s and 3.16 m/h for R_r). It has indicated that addition of PWW in the feed has improved the settling properties of aerobic sludge biomass, but at the cost of reduction in COD removal.
- * The ASBA for reactor R_s and R_r biomasses of 5.87 and 5.05 mg COD removed/mg VSS d, respectively, has been observed to be lower than reactor R_c biomass (7.03 mg COD removed/mg VSS d). It has indicated significant adverse impact on organic removal capability of PWW fed reactor biomasses.
- * Addition of PWW has transformed denser flocs to pinpoint type of biomasses. The flocs size for reactor R_s and R_r biomasses have ranged 1-5 mm, while reactor R_c biomass has revealed flocs size in the range of 1-12 mm. It has indicated that PWW in the feed has a negative impact on flocs morphology.
- * The reactor R_s and R_r biomasses have exhibited decreased FI values 3 and 2 (medium density of filaments), respectively, as compared to control reactor FI value of 5 (indicating higher filament counts). It has indicated that PWW has adverse impact on filamentous biomass growth and has resulted in decreased FI.
- * The active portion of aerobic sludge biomass has been observed as 74% and 67% for reactor R_s and R_r biomasses, respectively. The cell viability test has indicated reduction in active portion of aerobic sludge biomass due to PWW stress.
- * PS, PN, and ESP contents have shown minor changes for PWW stressed reactors as compared to control reactor, indicating no significant impacts of PWW on PS, PN, and ESP contents of aerobic sludge biomass.
- * The enzyme activity of PWW fed reactors have ranged between 0.21-0.27 mg/g MLSS h for E_{Catalase} ; 20.76-21.87 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 1.21-1.53 mg/g MLSS h for

E_{Protease} ; and 4.34-6.51 g/g MLSS h for E_{Urease} compared to 0.41 mg/g MLSS h for E_{Catalase} ; 27.87 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 2.21 mg/g MLSS h for E_{Protease} ; and 16.23 g/g MLSS h for E_{Urease} for control reactor R_c biomass. It has indicated that E_{Urease} has been severely affected due to PWW in the feed, as compared to other enzyme activities, which has reflected the changes in biomass characteristics due to imposed stresses.

- * *AluI* has shown 92.2% and 88.5% of similarity; *HhaI* has shown 90.0% and 84.1% of similarity; and *MspI* has shown 89.1% and 87.1% of similarity for reactor R_s , and R_r biomasses, respectively, as compared to reactor R_c biomass. The ARDRA result has indicated minor changes in the microbial community of reactor R_s and R_r biomasses (84-92% of similarity) when compared to reactor R_c biomass.

7.4.2 Stressed phase II operation

- * The reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ have shown drastic reduction in COD removal in the range of 8-29%, while control reactor R_c has exhibited 94% of COD removal. It has indicated that PWW+Cu(II) in the feed has severely affected the organic removal capability of aerobic sludge biomass.
- * The biomass growth has been reduced due to addition of Cu(II) and MLVSS/MLSS ratio has ranged 0.65-0.80 for PWW+Cu(II) fed reactor biomasses. It has indicated that PWW+Cu(II) in the feed has reduced biomass growth significantly.
- * The PWW+Cu(II) fed reactor biomasses have shown SVI of 73.4-76.0 mL/g, which has slightly decreased as compared to stressed phase I operation. While, ISV has marginally increased for PWW+Cu(II) fed reactor biomasses as compared to stressed phase I operation. The lower SVI and higher ISV have indicated good settling properties of aerobic sludge biomass; however, it is on the cost of lower COD removal by the PWW+Cu(II) fed reactors and therefore undesirable.
- * The ASBA for reactor $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ biomasses has been obtained as 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d, respectively, which are much lower than for the control reactor R_c biomass (7.75 mg COD removed/mg VSS d). It has suggested that PWW+Cu(II) has influenced ASBA, and the severity of the effect very much depended on the concentration of Cu(II) in PWW+Cu(II) feed.
- * The microscopic examination has revealed disintegration of flocs and disappearance of filaments for PWW+Cu(II) fed reactor biomasses. FI has significantly decreased in the range of 1-2 for PWW+Cu(II) fed reactor biomasses. It has indicated that filamentous biomass has been significantly affected due to PWW+Cu(II) stress.
- * The active portion in PWW+Cu(II) fed reactor biomasses have decreased significantly in the range of 17-36% – more decrease in reactor $R_{r\text{Cu}80}$ biomass. It has indicated PWW+Cu(II) stress has adversely impacted the active portion of aerobic sludge biomass.
- * PS, PN and EPS secretion of aerobic sludge biomass has slightly enhanced in response to Cu(II) stress, indicating marginal impact of PWW+Cu(II) stress.

- * The enzyme activity of PWW+Cu(II) fed reactors have ranged between 0.08-0.20 mg/g MLSS h for E_{Catalase} ; 8.01-19.23 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 0.21-0.72 mg/g MLSS h for E_{Protease} ; and 1.24-4.02 g/g MLSS h for E_{Urease} in comparison to 0.42 mg/g MLSS h for E_{Catalase} ; 30.12 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 2.31 mg/g MLSS h for E_{Protease} ; and 17.23 g/g MLSS h for E_{Urease} in control reactor R_c biomass. PWW+Cu(II) stress to aerobic sludge biomass has indicated severe impact on E_{Protease} and E_{Urease} compared to other enzyme activities.
- * *AluI* has shown 52.1%, 43.4%, 33.8%, and 29.4% of similarity; *HhaI* has shown 51.4%, 41.2%, 39.3%, and 31.0% of similarity; and *MspI* has shown 62.5%, 58.5%, 54.8%, and 48.3% of similarity for reactor $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ biomasses, respectively, as compared to reactor R_c biomass. It has indicated that PWW+Cu(II) stress has caused major shift in microbial community structure (29-63% similarity compared to control reactor biomass).

7.4.3 Recovery phase operation

- * COD removal for reactors $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ have improved to 78.0%, 63.7%, 57.1%, and 49.2%, respectively, during the steady-state condition of recovery phase operation. It has indicated that organic removal efficiency has notably improved after discontinuation of PWW+Cu(II) in feed.
- * No significant changes have been observed in SVI and ISV of PWW+Cu(II) fed biomasses during recovery phase compared to stressed phases. However, lower SVI and higher ISV have indicated good settling properties of aerobic sludge biomass along with improved COD removal.
- * The ASBA for reactor $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ biomasses have recovered to 5.77, 2.82, 3.16, and 1.59 mg COD removed/mg VSS d, respectively, from ASBA of 2.89, 1.02, 1.65, and 0.86 mg COD removed/mg VSS d of stressed phase II operation. The increase in ASBA has indicated that reactors have partially recovered from stresses imparted by PWW+Cu(II).
- * FI has shown slow but gradual increase for PWW+Cu(II) stressed reactors after discontinuation of PWW+Cu(II) in the feed, indicating reappearance of filamentous biomass in reactors. Reactor $R_{s\text{Cu}40}$, $R_{s\text{Cu}80}$, $R_{r\text{Cu}40}$, and $R_{r\text{Cu}80}$ biomasses have not been able to recover completely to its original level as compared to morphology of reactor R_c biomass.
- * The enzyme activity of PWW+Cu(II) stressed reactors have ranged between 0.13-0.31 mg/g MLSS h for E_{Catalase} ; 12.11-25.40 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 0.42-1.73 mg/g MLSS h for E_{Protease} ; and 1.41-5.10 g/g MLSS h for E_{Urease} compared to 0.44 mg/g MLSS h for E_{Catalase} ; 29.74 $\mu\text{g/g MLSS h}$ for $E_{\text{Dehydrogenase}}$; 2.34 mg/g MLSS h for E_{Protease} ; and 17.31 g/g MLSS h for E_{Urease} in control reactor R_c biomass. E_{Catalase} , $E_{\text{Dehydrogenase}}$, and E_{Protease} have shown significant recovery for the reactor biomasses stressed with lower

doses of Cu(II), while slight improvement has been observed for the reactor biomasses stressed with higher doses of Cu(II). E_{Urease} could not recover during recovery phase operation.

- * *AluI* has shown 85.7%, 59.8%, 78.3%, and 47.3% of similarity; *HhaI* has shown 80.7%, 61.3%, 83.2%, and 59.4% of similarity; and *MspI* has shown 78.4%, 54.7%, 81.3%, and 61.3% of similarity for reactor R_{sCu40} , R_{sCu80} , R_{rCu40} , and R_{rCu80} biomasses, respectively, as compared to reactor R_{c} biomass. ARDRA results have suggested 47-85% of similarity between control reactor and other reactors after discontinuation of PWW+Cu(II), indicating partial recovery of aerobic sludge biomass.

During stressed phase I operation, screened and raw PWW has affected COD removal upto 35% with decreased levels of ASBA as well as live cell fractions. In stressed phase II operation, addition of Cu(II) along with PWW has impacted organic removal by reducing COD removal upto 8% in reactor R_{rCu80} . While, recovery phase operation has indicated improvement in organic removal efficiency. ASBA has also improved significantly showing correlation with organic removal efficiency along with increased fraction of live cells. In addition, ARDRA has indicated 47-85% of similarity between control reactor and PWW+Cu(II) fed reactor biomasses. Aerobic sludge biomass has appeared to recover partially from PWW+Cu(II) impacts.

7.5 Tools and techniques to assess health of aerobic sludge biomass at treatment plant site

This chapter has attempted to identify a few techniques capable of evaluating the quality of aerobic sludge biomass in the field when stressed with unwanted components particularly metal [Cu(II)], antibiotic (amoxicillin), pesticide (chlorpyrifos), and actual wastewater [piggery wastewater spiked with Cu(II)]. The following key points can be noteworthy:

- * MLSS and MLVSS has failed to provide an alert in deteriorating health status of sludge biomasses. In all the studies, MLSS and MLVSS levels have not shown enough variation to distinguish the presence of Cu(II)/AMX/CPS/PWW+Cu(II).
- * SVI and ISV levels have been significantly affected due to stresses caused by Cu(II) and PWW+Cu(II) in the feed, while presence of AMX and CPS have not impacted SVI and ISV levels. Hence, SVI and ISV levels might not be useful in providing warning for adverse impact on aerobic sludge biomass.
- * ASBA of aerobic sludge biomass has been significantly affected due to stresses of Cu(II)/AMX/CPS/PWW+Cu(II). Hence, ASBA has appeared to have potential to be used as an indicator for assessment of aerobic sludge biomass quality.
- * The microscopic examinations have revealed floc structure and shape of biomass, which have significantly changed due to stresses caused by Cu(II) and PWW+Cu(II). On the other hand, AMX and CPS stresses have minor impacts on floc morphology. Since,

microscopic examination is a basic technique and hence, could be use as preliminary test to understand the flocs structure.

- * Active portion of aerobic sludge biomass has shown significant variations in response to stresses caused by Cu(II)/AMX/CPS/PWW+Cu(II) in feed. The active portion has decreased when stressed with Cu(II)/AMX/CPS/ PWW+Cu(II), but have shown increase in active portion of aerobic sludge biomass when Cu(II)/AMX/CPS/PWW+Cu(II) stresses discontinued. As a result, cell viability test might be utilized as an alerting tool for assessing aerobic sludge biomass health status.
- * The enzyme activities have been inhibited due to stresses caused by Cu(II)/AMX/CPS/ PWW+Cu(II). It has appeared that E_{Catalase} and $E_{\text{Dehydrogenase}}$ have considerable affect in the presence of stressing agents, and these two enzyme activities might be utilized as a warning indicator for biomass quality degradation.
- * The microbial community structure has changed significantly in aerobic sludge biomass, when stressed with Cu(II)/AMX/CPS/PWW+Cu(II). Hence, ARDRA could be utilized as a final warning system for assessing aerobic sludge biomass health status.

Therefore, basic techniques such as microscopic examination and the ASBA test might be applied as preliminary testing. In addition, cell viability tests, enzyme activity assays (E_{Catalase} and $E_{\text{Dehydrogenase}}$), and ARDRA might be used as confirmation tests during the evaluation of the aerobic sludge biomass quality based on the availability of skilled manpower and laboratory facilities in the nearby research and higher institutions of learning.

7.6 Limitations

While carrying out this experimental investigations on the aerobic sludge biomass stressed with metal [Cu(II)], antibiotic (amoxicillin), pesticide (chlorpyrifos), and actual wastewater [piggery wastewater spiked with Cu(II)], and its recovery potential in the absence of stress-causing agents, a number of limitations have been identified. Some of the key limitations are described below:

- * The aerobic sludge biomass has been cultivated and maintained under laboratory conditions with synthetic feed. The biomass has been fed with Lactogen 3 giving initial COD around 550 mg/L. The biomass has successfully utilized ~88% of COD and has shown MLVSS/MLSS ratio of ~0.90-0.97. In an actual treatment plant in Indian situation, the influent wastewater is likely to have very low COD in the range of 100-150 mg/L and that may not have sludge characteristics as has been maintained under laboratory conditions. Therefore, aerobic sludge biomass from a working field unit might be used to validate the results as has been obtained in this study for its meaningful application.
- * The aerobic sludge biomass cultivated and maintained in the laboratory has ~98% of active portion. In practice, wastewater may contain a variety of organic and inorganic chemicals causing very adverse impact on biomass and therefore difficult to get 98%

active aerobic sludge biomass in the field treatment plant. Furthermore, aerobic sludge biomass might be retained in the secondary settling tank for 3-4 hours for settling and likely to develop anaerobic environment which might reduce active portion of aerobic sludge biomass.

- * When aerobic sludge biomass has been stressed with amoxicillin, it has encouraged increase in number of filamentous biomass even-though COD removal reduced significantly which is perplexing. Furthermore, CPS stress has also shown little effect on filamentous biomass population. However, AMX and CPS have considerable influences on ASBA and live portion of aerobic sludge biomasses. This aspect needs further investigation.
- * This investigation has shown that Cu(II) and PWW+Cu(II) stresses on aerobic sludge biomass have caused disappearance of filamentous biomass and therefore, stress biomass could recovery only partially. However, AMX and CPS stresses on aerobic sludge biomass have not impacted the filamentous biomass concentration and has shown almost complete recovery when stresses have been removed. It has indicated different mode of causing impact on the aerobic sludge biomass and it needs in-depth investigation.
- * ASBA has been estimated by feeding Lactogen 3. However, this substrate may not be the most appropriate feed for the aerobic sludge biomass present in actual treatment plant. Therefore appropriate feed for ASBA test is required to be ascertained.
- * The studies have been carried out by imposing stress by stress-causing agent compound at a time. However, the actual influent wastewater in the aerobic treatment plant may have more than one stress-causing agents. Hence, impacts of multiple stress-causing agents need to be studied.
- * The major constraint is lack of skilled personnel at the treatment plant to carry out its routine operation and maintenance under Indian condition. A skilled personnel may carryout all the experiments, reactor operation, and numerous testing, whereas lack of skilled personnel for operation and maintenance of treatment plant make it very challenging. Unless, the treatment plant has skilled operators, the tools/techniques identified in this investigation may not see the light of day. A minimum laboratory equipment needed for tests are listed in [Table A-V1](#). Tests, such as microscopic examination and the ASBA, can be performed at the treatment plant site if the skilled operators trained under proper guidance of an expert. While cell viability tests, enzyme activity assays, and ARDRA require a competent specialist and analysis can be outsourced to nearby research and educational institutions of higher learning, if specialist is not available.

The limitations as described here may serve as the scope for future studies.

Appendix-I

Table A-I.1 List of chemicals used.

Chemical used	Manufactured by	CAS/Catalogue No.	Chemical formula
1,3,5- triphenyltetrazolium formazan	HiMedia (India)	531-52-2	C ₁₉ H ₁₆ N ₄
2,3,5- triphenyltetrazolium chloride	HiMedia (India)	298-96-4	C ₁₉ H ₁₅ N ₄ Cl
Acetonitrile	HiMedia (India)	75-05-8	C ₂ H ₃ N
Ammonium hydroxide	Merck (India)	1336-21-6	NH ₄ OH
Amoxicillin	Zydus Cadila (India)	26787-78-0	C ₁₆ H ₁₉ N ₃ O ₅ S
Anthrone	HiMedia (India)	90-44-8	C ₁₄ H ₁₀ O
AluI	New England Biolabs	R0137S	
Bovine serum albumin (BSA)	Merck (India)	9048-46-8	
Bromocresol	HiMedia (India)	76-60-8	C ₂₁ H ₁₄ Br ₄ O ₅ S
Chlorpyrifos	Excel Crop Care Limited	2921-88-2	C ₉ H ₁₁ Cl ₃ NO ₃ PS
Copper sulphate	HiMedia (India)	7758-99-8	CuSO ₄ .5H ₂ O
Dipotassium hydrogen phosphate	HiMedia (India)	2139900	K ₂ HPO ₄
DNA Purification Kit	HiMedia (India)	MB543-50PR	
Ferroin indicator	Merck (India)	1091610100	
Ferrous ammonium sulphate	HiMedia (India)	7783-85-9	(NH ₄) ₂ Fe(SO ₄) ₂ .6H ₂ O
Folin-Giocalteu reagent	Merck (India)	1090010100	
Glucose	HiMedia (India)	50-99-7	C ₆ H ₁₂ O ₆
Glutaraldehyde	HiMedia (India)	111-30-8	C ₅ H ₈ O ₂
Gram staining kit	HiMedia (India)	K001-1KT	
HhaI	New England Biolabs	R0139S	
High-Fidelity DNA Polymerase	Thermofisher Scientific (USA)	F-530XL	
Hydrogen peroxide	HiMedia (India)	7722-84-1	H ₂ O ₂
Mercuric sulphate	Merck (India)	7783-35-9	HgSO ₄
Methyl red	HiMedia (India)	493-52-7	C ₁₅ H ₁₅ N ₃ O ₂
Methylbenzene	HiMedia (India)	108-88-3	C ₇ H ₈
MspI	New England Biolabs	R0106S	
Ninhydrin	HiMedia (India)	485-47-2	C ₉ H ₆ O ₄
Phenolphthalein	HiMedia (India)	77-09-8	C ₂₀ H ₁₄ O ₄
Phosphoric acid	Merck (India)	7664-38-2	H ₃ PO ₄
Potassium chloride	Merck (India)	7447-40-7	KCl
Potassium dichromate	Merck (India)	7778-50-9	K ₂ Cr ₂ O ₇
Potassium dihydrogen Phosphate	HiMedia (India)	7778-77-0	KH ₂ PO ₄
Potassium permanganate	HiMedia (India)	7722-64-7	KMnO ₄
Silver sulphate	Merck (India)	10294-26-5	Ag ₂ SO ₄
Sodium carbonate	Merck (India)	497-19-8	Na ₂ CO ₃
Sodium chloride	Merck (India)	7647-14-5	NaCl
Sodium hydroxide	Merck (India)	1310-73-2	NaOH
Sodium hypochlorite	Merck (India)	AS102-100ML	NaClO
Sodium nitrite	Merck (India)	7632-00-0	NaNO ₂

Sodium nitroprusside	Merck (India)	13755-38-9	$C_5FeN_6Na_2O \cdot 2H_2O$
Sodium potassium tartrate	Merck (India)	6381-59-5	$C_4H_4KNaO_6 \cdot 4H_2O$
Sodium sulphate	HiMedia (India)	7757-82-6	Na_2SO_4
Sulfuric acid	Merck (India)	7664-93-9	H_2SO_4
Tri-sodium citrate	Merck (India)	6132-04-3	$Na_3C_6H_5O_7 \cdot 2H_2O$
Urea	HiMedia (India)	57-13-6	N_2H_4CO

Table A-I.2 Characteristics of milli-Q water.

Parameter	Value/Concentration	Source
Resistivity	18.2 MΩcm at 25°C	Manufacturer's brochure
TOC	≤5 ppb (μg/L)	Manufacturer's brochure
Bacteria	<0.01 CFU/mL	Manufacturer's brochure
Pyrogens (endotoxins)	<0.001 EU/mL	Manufacturer's brochure
RNases	<1 pg/mL (RNase-free)	Manufacturer's brochure
DNases	<5 pg/mL (DNase-free)	Manufacturer's brochure

Table A-I.3 Characteristics of tap water.

Parameter	Value/Concentration	Source
pH	8.10±0.05	Tested in this study
Conductivity	0.22±0.01 mS/cm	Tested in this study
Turbidity	1.10±0.05 NTU	Tested in this study
Alkalinity (total)	50.00±10.00 mg $CaCO_3$ /L	Tested in this study
Chloride	4.55±0.05 mg Cl^- /L	Tested in this study
Sulfate	24.75±0.10 mg SO_4^{2-} /L	Tested in this study
Iron	0.10±0.01 mg Fe^{2+} /L	Tested in this study
Fluoride	0.33±0.05 mg F^- /L	Tested in this study
Nitrate	5.50±0.50 mg NO_3^- /L	Tested in this study
Ammonia	0.20±0.050 mg NH_4^+ /L	Tested in this study
Sodium	4.25±0.01 mg Na^+ /L	Tested in this study
Potassium	2.00±0.01 mg K^+ /L	Tested in this study
Calcium	10.75±0.05 mg Ca^{2+} /L	Tested in this study

Table A-I.4 Characteristics of distilled water.

Parameter	Value/Concentration	Source
pH	6.85	Tested in this study
Copper	0.003 mg/L	Tested in this study
Cadmium	0.085 mg/L	Tested in this study
Iron	0.102 mg/L	Tested in this study
Lead	0.21 mg/L	Tested in this study
Zinc	0.2956 mg/L	Tested in this study
Arsenic	1.0 μg/L	Tested in this study
Chloride	1.52 mg/L	Tested in this study
Flouride	0.102 mg/L	Tested in this study
Sulfate	18.5 mg/L	Tested in this study

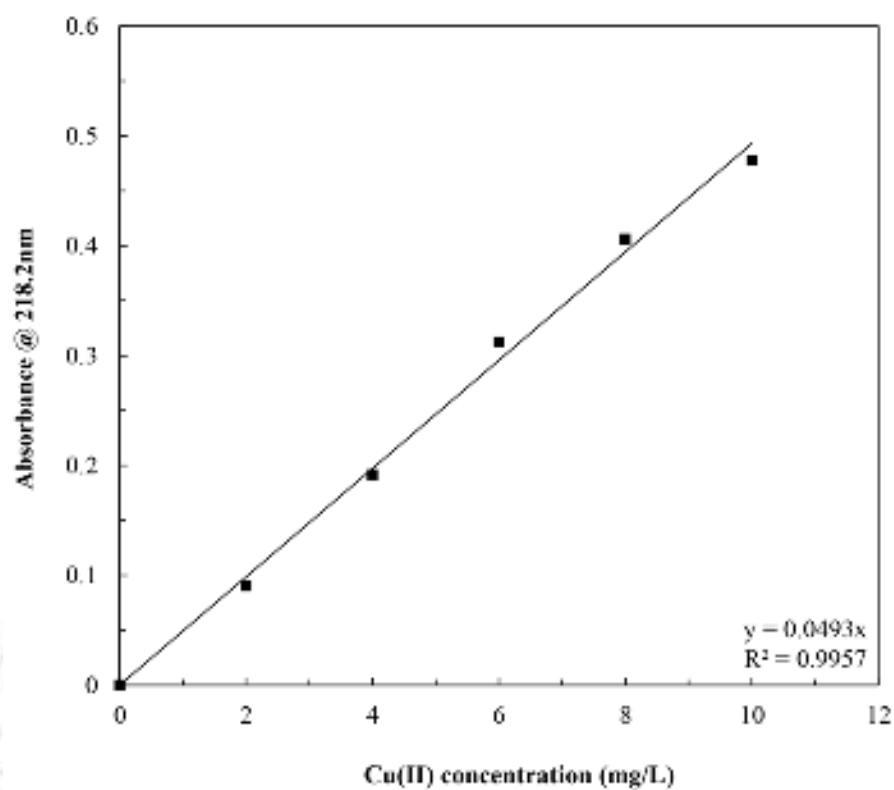


Fig. A-I.1 Calibration curve for the analysis of Cu(II).

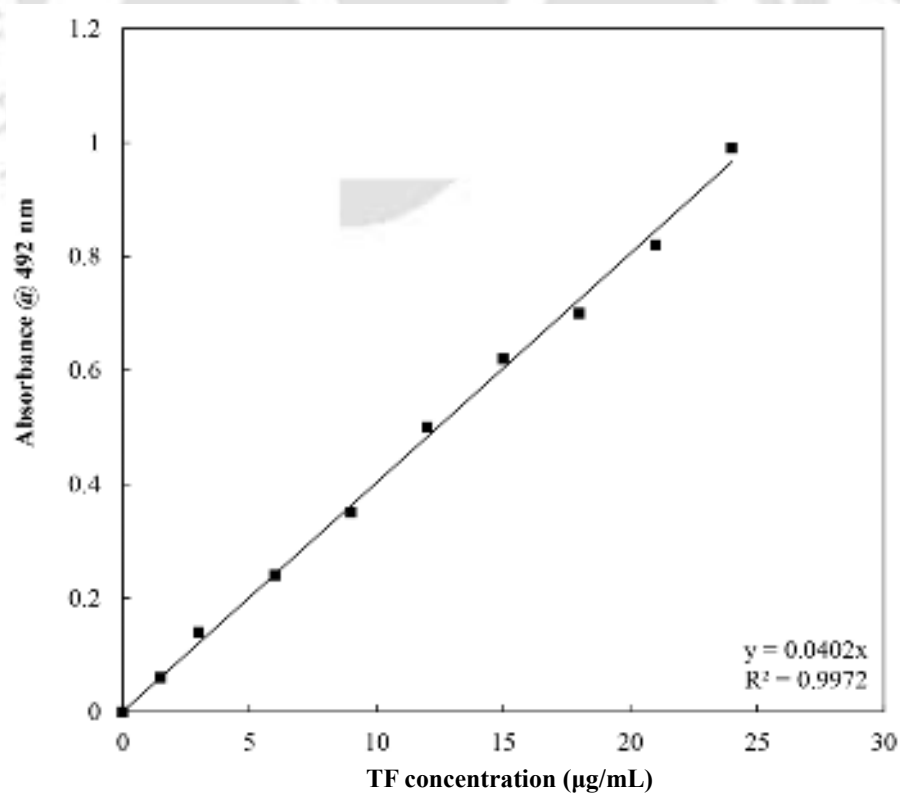


Fig. A-I.2 Calibration curve for the analysis of dehydrogenase activity.

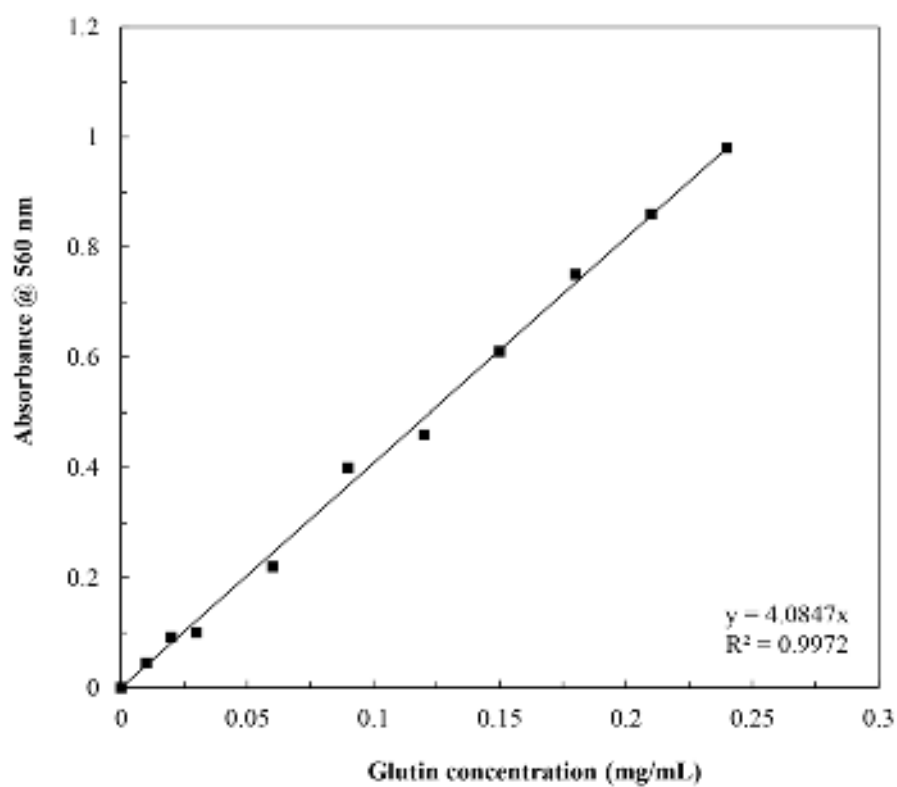


Fig. A-I.3 Calibration curve for the analysis of protease activity.

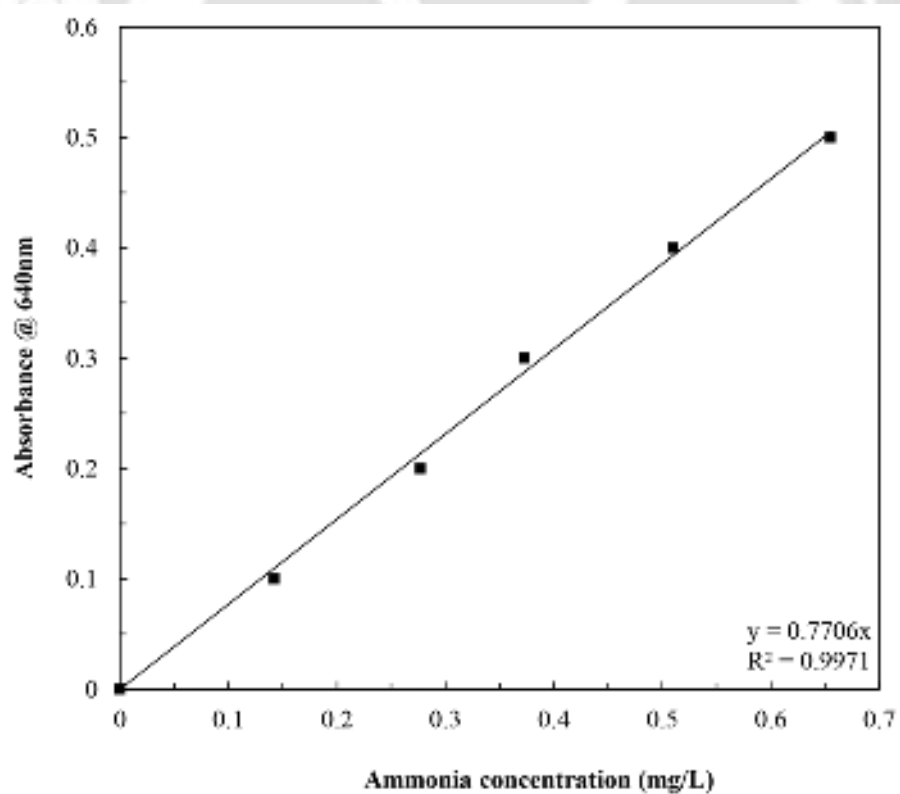


Fig. A-I.4 Calibration curve for the analysis of urease activity.

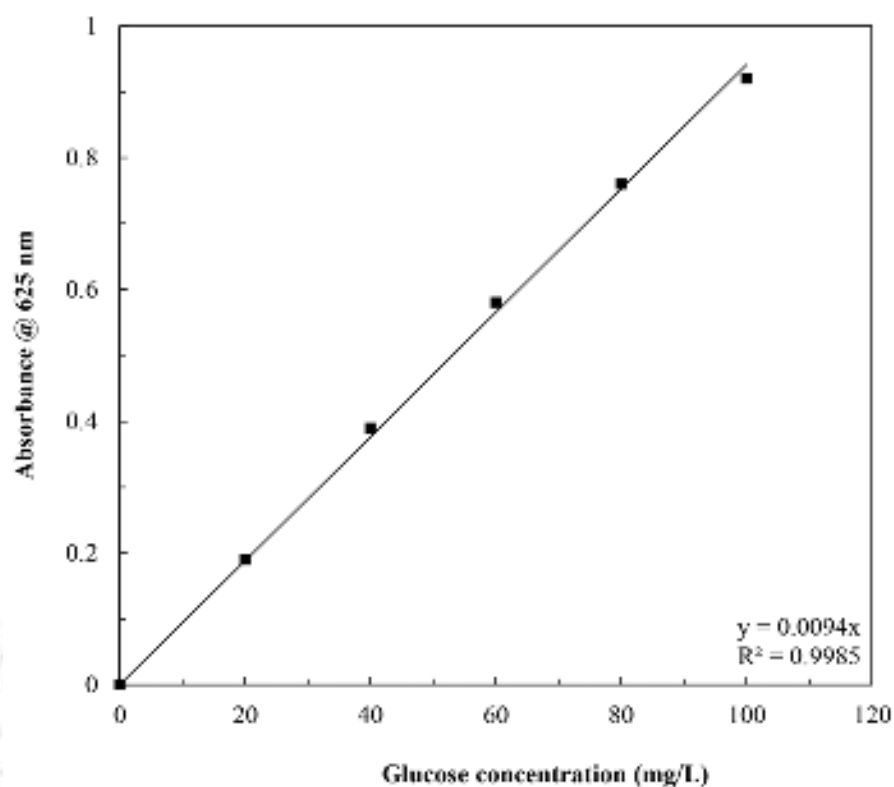


Fig. A-I.5 Calibration curve for the analysis of polysaccharides.

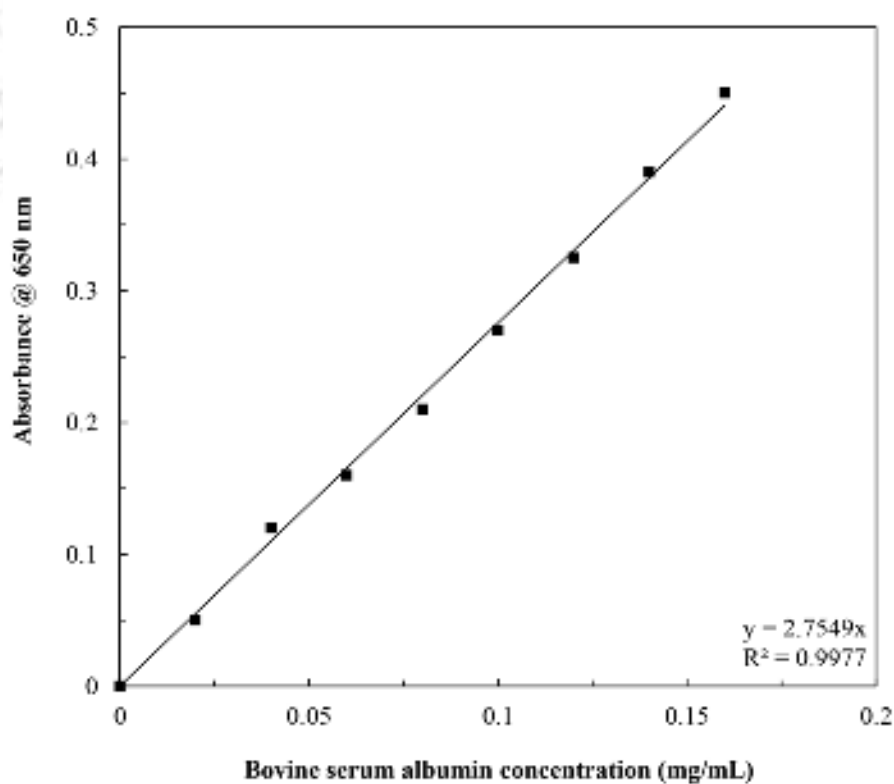


Fig. A-I.6 Calibration curve for the analysis of proteins.

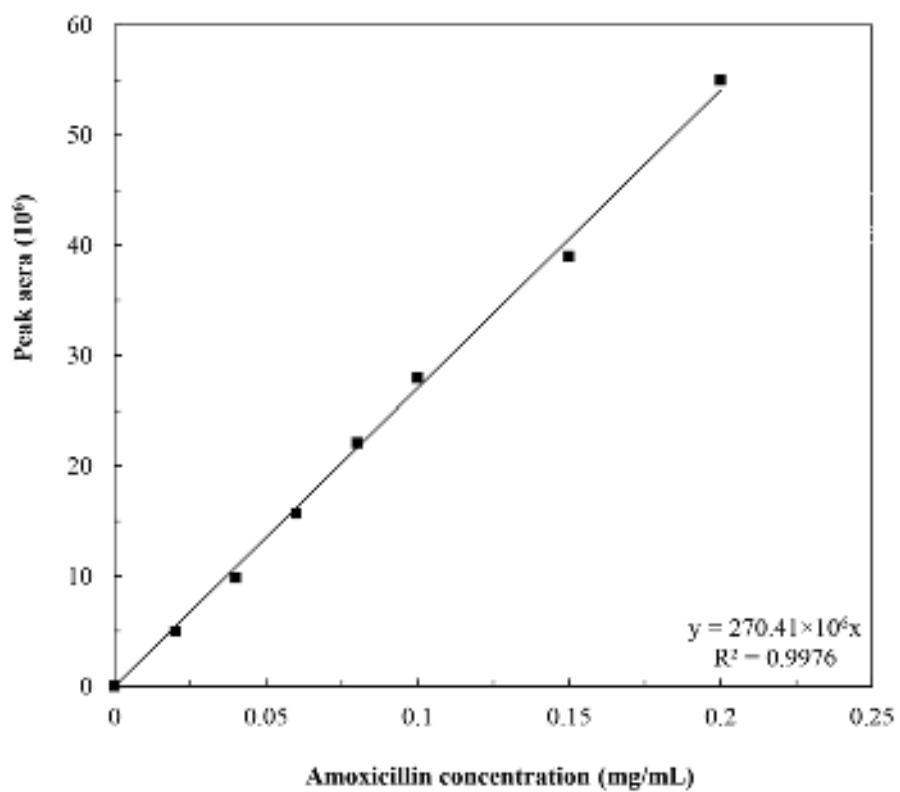


Fig. A-I.7 Calibration curve for amoxicillin analysis.

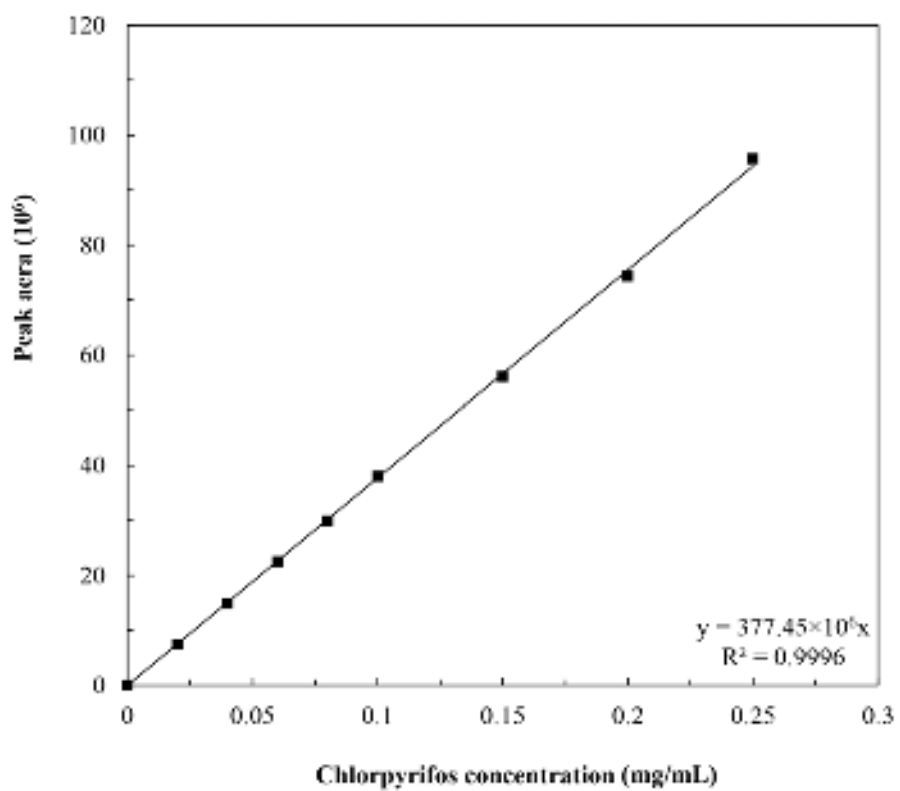


Fig. A-I.8 Calibration curve for chlorpyrifos analysis.

Appendix-II

Growth and Maintenance of Aerobic Sludge Biomass

A sufficient amount of active aerobic sludge biomass is required for the experimental investigation. The active aerobic sludge biomass has been cultivated in a laboratory-scale batch reactor operated under ambient conditions using synthetic wastewater. This appendix presents the experimental setup, sampling procedure, and methods for its analysis during growth and maintenance phases.

A-II.1 Background

Biological processes of wastewater treatment depend on the quantity and quality of aerobic sludge biomass present in the system. Hence, it is necessary to grow and maintain a sufficient amount of active aerobic sludge biomass in the reactor. The complex mixture containing bacteria, fungi, protozoans, and other microorganisms is collectively referred to as the aerobic sludge biomass. The aerobic sludge biomass has been cultivated in a batch reactor. The synthetic feed is added to a batch reactor containing active aerobic sludge biomass, allowed utilization of food for growth and maintenance for a duration of 680 min under aeration, then reactor content is settled and clear supernatant is discarded before next feeding. Aeration, mixing, and clarification—all the operations have been carried out in the same batch reactor.

A-II.2 Materials and Methods

A-II.2.1 Chemicals and reagents

All chemicals and reagents used in this study are analytical or HPLC grade, supplied by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals and reagents used in the study are given in *Appendix-I* ([Table A-I.1](#)). All the reagents and stock/standard solutions have been prepared by milli-Q water (Merck Millipore, Germany). The characteristics of milli-Q water used in this study are given in *Appendix-I* ([Table A-I.2](#)).

A-II.2.2 Collection of seed sludge

The seed sludge has been collected from a facultative lagoon (N26°11'22.77", E91°42'13.23") – the sewage treatment plant of IIT Guwahati, Assam, India and used as inoculum for the cultivation of aerobic sludge biomass in a batch reactor. The characteristics of seed sludge are presented in [Table A-II.1](#).

Table A-II.1 Characteristics of collected seed sludge.

Sl No.	Parameters	Unit	Values obtained
1	pH	—	7.1
2	Temperature	°C	24.5
3	COD (liquid fraction only)	mg/L	90.87±15.34
4	TDS (liquid fraction only)	mg/L	469
5	Conductivity (liquid fraction only)	mS/m	667
6	DO (liquid fraction only)	mg/L	1.38
7	MLSS	mg/L	176
8	MLVSS	mg/L	118

[COD: Chemical oxygen demand; TDS: Total dissolved solids; DO: Dissolved oxygen; MLSS: Mixed liquor suspended solids; MLVSS: Mixed liquor volatile suspended solids]

A-II.2.3 Feed compositions

The feed has been prepared using Lactogen 3, a baby feed manufactured by Nestle, India. The composition of Lactogen 3 is presented in [Table A-II.2](#). It contains carbohydrates, proteins, phosphorus, and traces amount of heavy metals required for the growth of the microorganisms. The feed has been prepared by adding a sufficient amount of Lactogen 3 in lukewarm tap water and mixed for 5 min using magnetic stirrer; the mixture has been allowed to cool down at room temperature (24°C-28°C). The 1000 mg/L of Lactogen 3 mixture gives COD 1042.64±15.90 mg/L. The characteristics of tap water used in this study are given in [Appendix-I \(Table A-I.3\)](#).

Table A-II.2 Composition of Lactogen 3 (Source: Nestle, 2014)

Nutrients	Composition per 100 g	Nutrients	Composition per 100 g
Energy (Kcal)	469	Iron (mg)	6.50
Protein (g)	14.20	Vitamin A (µg)	360
Carbohydrates (g)	59.30	Vitamin C (µg)	47
Sugar (g)	4.30	Vitamin D (µg)	5.25
Total Fat (g)	19.50	Vitamin E (mg)	4
Calcium (mg)	440	Thiamine (B1) (mg)	0.65
Phosphorus (mg)	310	Riboflavin (B2) (mg)	1
Chloride (mg)	280	Niacin (mg)	3
Magnesium (mg)	44	Pantothenic acid (mg)	2.20
Copper (mg)	0.28	Folic Acid (µg)	100
Zinc (mg)	3	Vitamin B6 (mg)	0.35
Sodium (mg)	165	Biotin (µg)	12
Potassium (mg)	500	Vitamin B12 (µg)	0.70

A-II.2.3.1 Growth phase reactor (GPR)

Initially, sewage inoculum has been cultivated in 1 L working volume reactor designated as growth phase reactor (GPR) as shown in [Fig. A-II.1\(a\)](#). The GPR has been operated with fill, aerate, settle, and decant modes with feeding time of 5 min, aeration time of 23 h 20 min,

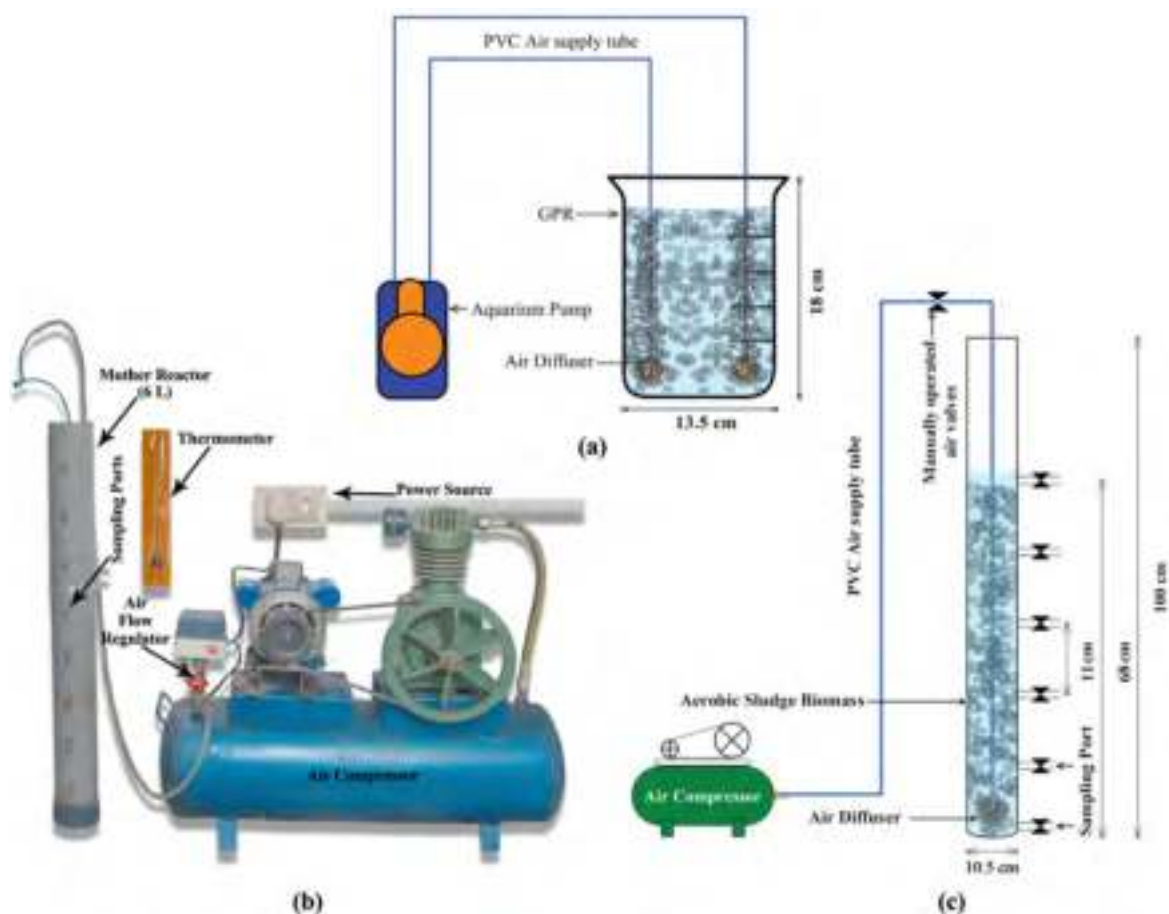


Fig. A-II.1 (a) Schematic diagram of Growth Phase Reactor (GPR) (b) Photograph of Mother Reactor (MR) setup (c) Schematic diagram of Mother Reactor (MR).

settling time of 30 min, and decant time of 5 min as shown in Fig. A-II.2. The reactor has been aerated using an aquarium pump with an airflow rate of 2.5 L/min. The dimensions of the reactor setup are presented in Table A-II.3. During the growth phase operation, feed COD has been gradually increased from 250 mg/L to 1000 mg/L throughout 30 d. When sufficient active biomass (approx. 3000 mg/L) has been grown in GPR, then whole mixed liquor has been transferred to the mother reactor as discussed in the subsequent subsection.

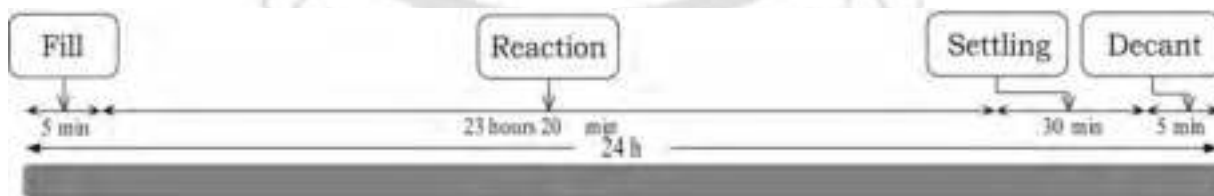


Fig. A-II.2 Schematic representation of cycle time for Growth Phase Reactor.

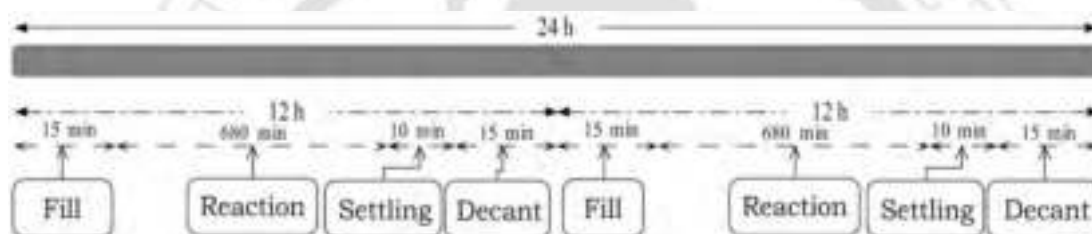
A-II.2.3.2 Mother reactor (MR)

After 30 d of growth phase reactor operation, aerobic sludge biomass has been transferred from GPR [Fig. A-II.1(a)] to mother reactor (MR) [Fig. A-II.1(b)]. A schematic diagram of MR has been shown in Fig. A-II.1(c). MR has been fabricated using an opaque PVC pipe of internal diameter 10.5 cm and height 100 cm (working volume 6 L) as given in Table A-II.3. MR has been operated with fill, aerate, settle, and decant modes with the periods of 15 min,

Table A-II.3 Basic design parameters of different reactors.

Sl No.	Parameters	Growth Phase Reactor	Mother Reactor
1	Total height (cm)	18	100
3	Internal diameter (D) (cm)	13.5	10.5
4	Working volume (L)	1	6
5	Cycle time (h)	24	12
6	No. of cycles/d	1	2

680 min, 10 min, and 15 min, respectively altogether 12 h for a complete cycle as shown in Fig. A-II.3. It has been fed with Lactogen 3 feed of COD approx. 500 mg/L per cycle. The MR content has been aerated and maintained in suspension using an air compressor and diffuser with an airflow rate of 2.5 L/min. The operation of MR has been carried out in ambient conditions. This operation has been continued to maintain suspended activated aerobic sludge biomass for carrying out experiments.

**Fig. A-II.3** Schematic representation of cycle time for Mother Reactor.

A-II.2.4 Sampling and monitoring frequency

After feeding, the reactors (GPR and MR) have been aerated for 2-3 min to provide uniform mixing, and then the aeration has been stopped to collect liquid samples for analyzing initial parameters. After that, aeration (at a rate of 2.5 L/min) in the reactors has been restarted and continued for the remaining aeration time period. The decanted liquid at the end of the cycle has been used for effluent parameters analyses. The frequency for analyses of parameters is given in Table A-II.4.

Table A-II.4 Analysis frequency of initial and effluent parameters.

Sl No.	Parameters	Frequency
1	Room temperature	Daily
2	pH	
3	Alkalinity	
4	Chemical oxygen demand (COD)	
5	Mixed liquor suspended solids (MLSS)	Every 7 th day
6	Mixed liquor volatile suspended solids (MLVSS)	
7	Interface settling velocity (ISV)	
8	Sludge volume index (SVI)	
9	Photographic evidence	Initially 7 th d and later 50 th d
10	Microscopic analysis	

A-II.2.5 Analytical methods

Various analytical techniques used in this investigation are presented in *Chapter 2*.

A-II.3 Results and Discussion

A-II.3.1 Reactor performance

The growth of biomass has been monitored in growth phase reactor (GPR) and mother reactor (MR). The variations in all the monitored parameters are presented in [Fig. A-II.4](#) and [Table A-II.5](#). The GPR and MR have been operated for 1-30 d and 31-1202 d, respectively. During growth phase of 1-7 d, 8-15 d and 16-30 d operations, initial COD have been 250 ± 11.54 mg/L, 540 ± 16.32 mg/L and 1022.85 ± 26.90 mg/L, respectively, while effluent COD of GPR for 1-7 d, 8-15 d and 16-30 d operations have been 93.75 ± 20.56 mg/L, 220 ± 36.51 mg/L and 94.28 ± 65.02 mg/L, respectively. The steady state has been observed in 35th d of operation for reactor MR. The initial, effluent and COD removal of MR have been 492.44 ± 22.92 mg/L, 56.83 ± 21.90 mg/L and $88.45 \pm 4.40\%$, respectively during 31-1202 d of operation.

Both reactors (GPR and MR) have been operated under uncontrolled (ambient) condition and the parameters such as temperature and pH have not been controlled throughout the operation (1-1202 d). The pH of the MR has not shown much variation and initial and effluent pH have ranged 7.16 ± 0.13 and 7.11 ± 0.14 , respectively. The initial and effluent alkalinity for GPR and MR have been observed in the ranged of 50.62 ± 10.87 to 67.50 ± 2.88 mg CaCO₃/L for 1-1202 d of operation. The room (ambient) temperature variation has been due to seasonal changes. The room temperature during filling and decanting of reactor have been observed as $24.91 \pm 4.99^\circ\text{C}$ and $21.47 \pm 6.79^\circ\text{C}$, respectively, for 1-1202 d of operation.

A-II.3.2 Biomass characteristics

The variation in biomass concentration and sludge volume index (SVI) of active aerobic sludge biomass is shown in [Fig. A-II.4](#). The solids estimation has been started from 15th d of GPR operation. The MLSS and MLVSS concentrations have been 844 and 701 mg/L, respectively, along with an SVI of 385 mL/g on 15th d of operation. Later on, biomass has adapted to the substrate and ambient conditions and started to grow. The MLSS has reached to a level of 2875 mg/L with an SVI of 231.30 mL/g on 29th d from the start of GPR operation. The SVI have been higher during GPR operation because biomass appears to be flocculent in nature with an abundance of filamentous microorganisms. The average MLSS and MLVSS of MR have been 5181.62 ± 1651.16 mg/L and 4832.41 ± 1561.46 mg/L, respectively, during 31-1202 d of operation. The characteristics of aerobic sludge biomass, i.e., interface settling velocity (ISV) and SVI, have been 1.87 ± 0.48 m/h and 121 ± 47 mL/g, respectively, during 31-1202 d of reactor operation as shown in [Fig. A-II.4](#). The thick flocs structure has resulted in increased ISV and lower SVI for biomass of MR. A sudden decline in solid concentration on 176, 587, 737, 767, and 857 d has been due to biomass withdrawal from MR for experimental studies.

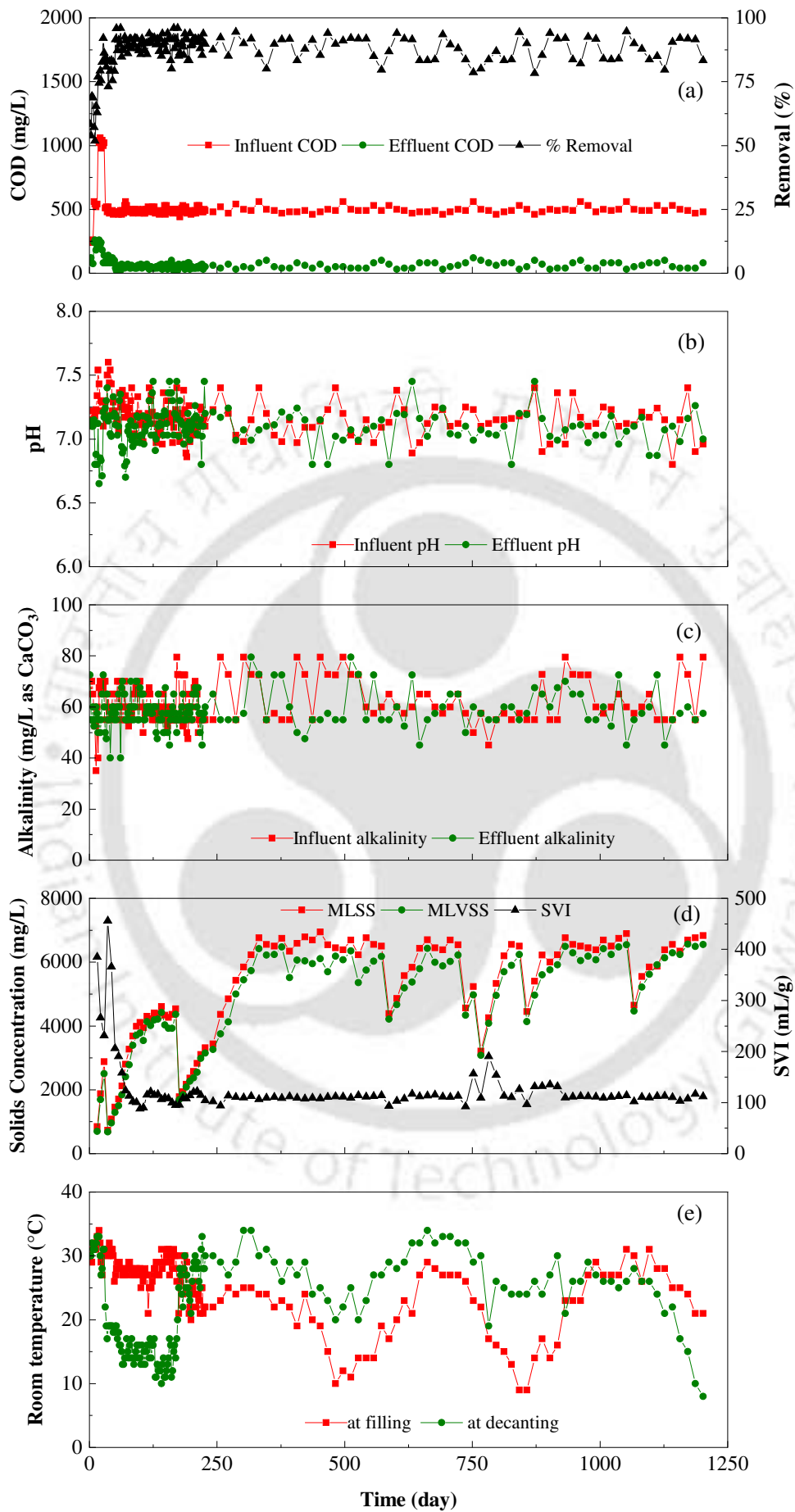


Fig. A-II.4 Variation in performance related parameters for GPR (1-30 d) and MR (31-1202 d).
(a) COD removal, (b) pH, (c) alkalinity, (d) biomass, and (e) room temperature.

Table A-II.5 Summary of performance related parameters for growth phase reactors operated during 1-1202 d of operation.

Performance related parameters	Reactor	GPR			MR
	Day	1-7	7-15	16-30	31-1202
	Lactogen 3 (g/L)	0.24	0.5	1.0	0.5
Initial soluble COD (mg/L)		250±11.54 (4)	540±16.32 (4)	1022.85±26.90 (7)	492.44±22.92 (164)
Effluent soluble COD (mg/L)		93.75±20.56 (4)	220±36.51(4)	94.28±65.02 (7)	56.83±21.90 (164)
COD removal (%)		62.54±7.67 (4)	59.34±6.07 (4)	81.02±6.37 (7)	88.45±4.40 (164)
Initial pH		7.14±0.05 (4)	7.22±0.08 (4)	7.30±0.14 (7)	7.16±0.14 (164)
Effluent pH		7.11±0.01 (4)	6.90±0.16 (4)	6.93±0.22 (7)	7.10±0.17 (164)
Initial alkalinity (mg CaCO ₃ /L)		67.50±2.88 (4)	50.62±10.87 (4)	63.57±10.59 (7)	61.40±7.60 (164)
Effluent alkalinity (mg CaCO ₃ /L)		60.62±8.26 (4)	56.25±3.22 (4)	58.21±8.25 (7)	58.70±6.93 (164)
Room temperature (°C)		29.7±0.9 (4)	31.7±0.9 (4)	30.8±2.4 (7)	24.9±4.9 (164)
MLSS (mg/L)		-	-	1362.00±732.56 (3)	5181.62±1651.16 (93)
MLVSS (mg/L)		-	-	1196.26±701.08 (3)	4832.41±1561.46 (93)
SVI (mL/g)		-	-	325±84 (3)	121±47 (93)
Interface settling velocity (m/h)		-	-	0.99±0.30 (3)	1.87±0.48 (93)

[The data is presented in xx ±yy (zz) format where xx is the average value, yy is the standard deviation, zz is the number of data points considered for obtaining the average and standard deviation]

A-II.3.3 Biomass morphology

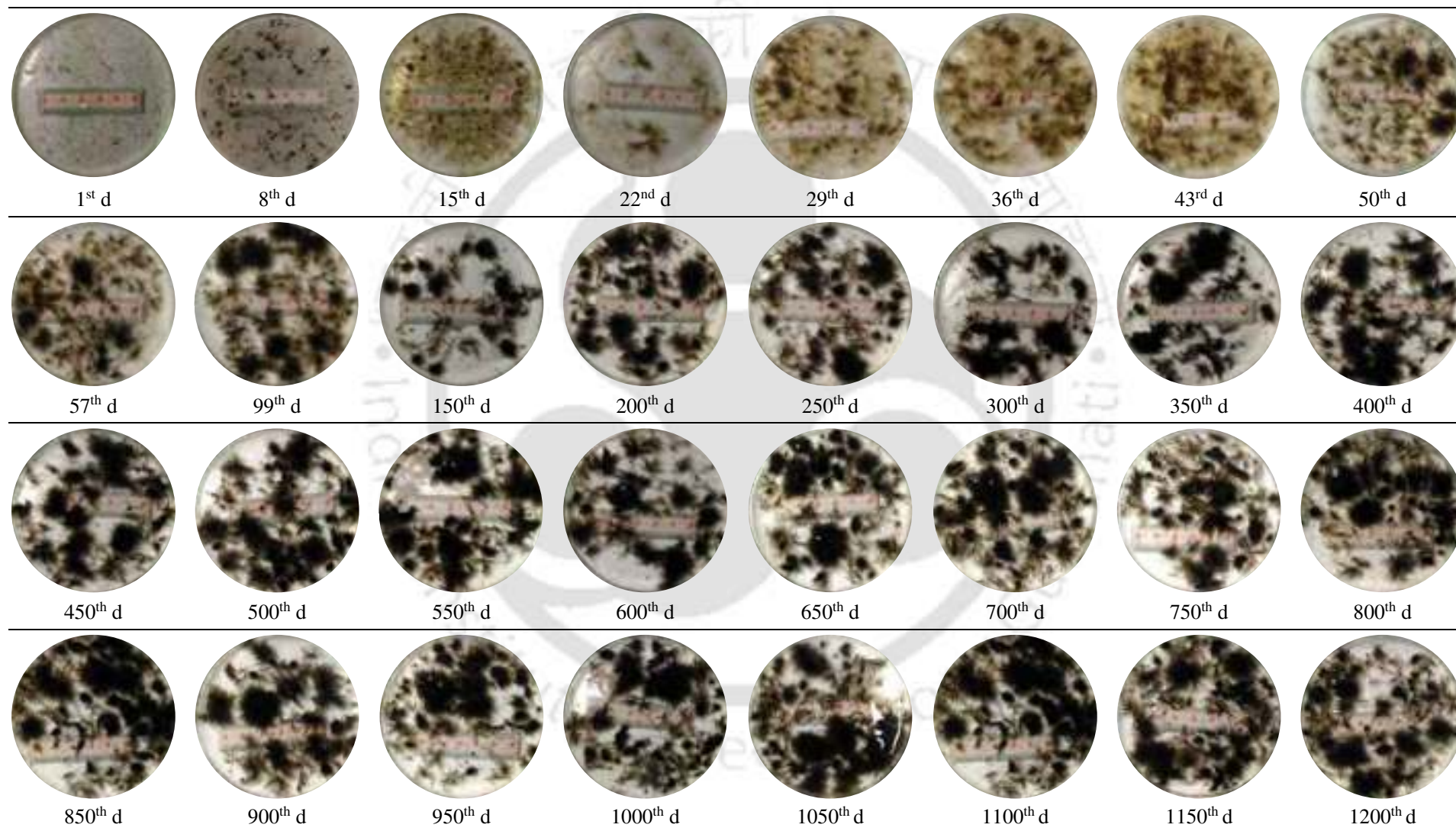
The seed sludge has contained very little biomass. Later on, biomass has started to grow on the Lactogen 3 feed. The microscopic and photographic observations of biomass samples withdrawn from GPR and MR have been carried out on every 7th d from the start of the growth phase; later on, the analysis frequency has been reduced to every 50th d. The microscopic observation reveals the presence of filamentous microorganisms in the reactor. The flocs size has increased from 1st d to 1202nd d as shown in Table A-II.6. The increased flocs size

Table A-II.6 Microscopic images of stained biomass sampled during 1-1202 d of operation.



and dense filaments have been observed with respect to time. The photographic images of aerobic sludge biomass have shown that the number of flocs and size increased day-wise (Table A-II.7). The biomass size almost remains the same during 57th to 600th d operation. Seed sludge has contained brown color flocculent biomass on the 1st d. Later on, the biomass color has changed from brown to dark green as observed on 43rd d. Microscopic images have confirmed the growth of filamentous microorganisms from 8th d onward. The compact structure of filamentous microorganisms has been observed from 50th d onward.

Table A-II.7 Photographic images of biomass sampled during 1-1202 d of operation.



A-II.4 Conclusions

The active aerobic sludge biomass has been successfully cultivated using GPR and maintained in MR. The MLSS and MLVSS content of the seed sludge have been very low. Later on, microorganisms have adapted to Lactogen 3 feed and started to grow. The biomass has been able to utilize more than 85% of COD under uncontrolled conditions. The photographic images have exhibited the dense filament structure of fully grown flocs. The microorganisms have grown continuously, and the size of flocs has increased with time. The microscopic analysis also confirms the existence of filamentous microorganisms.

References

Nestle, 2014. Lactogen 3. Nestle, New Delhi, India.



Appendix-III

Selection of Antibiotics

A-III.1 Background

Three sets of investigations have been carried out to choose the antibiotics and respective concentrations for the experiment. The experimental methodologies as well as results have been discussed below.

A-III.2 Materials and Methods

A-III.2.1 Chemicals and reagents

All chemicals and reagents used in this study are analytical grade, supplied by HiMedia Pvt. Ltd., India, and Merck India Ltd. The antibiotics named Azithral contains azithromycin (Alembic Pharmaceuticals Ltd, India), Hipen-Clav contains amoxicillin (Zydus Cadila, India), Cefuroxime contains cefuroxime (Sun Pharmaceutical Industries Ltd, India), and Alcipro contains ciprofloxacin (Alkem Laboratories Ltd, India) antibiotics have been purchased from local pharmacy shop in Guwahati, Assam, India.

A-III.2.2 Aerobic sludge biomass

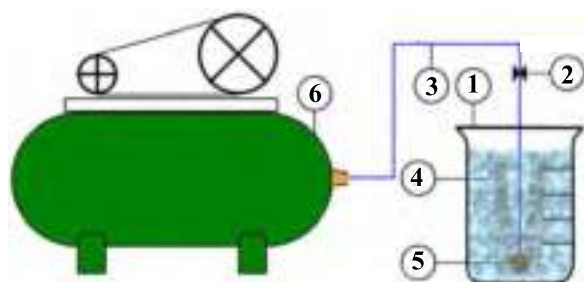
The aerobic sludge biomass withdrawn from the mother reactor (MR) has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in detail in *Appendix-II*.

A-III.2.3 Experimental conditions

The following tests have been carried out to evaluate the antibiotics toxicity for aerobic sludge biomass and their effect on reactor performance.

A-III.2.3.1 Test 1

The first test has been carried out to determine the impact of azithromycin (AZI) tablet on reactor performance and floc morphology. The experimental set-up consists of a batch reactor (height 14.5 cm×diameter 12.5 cm) with a working volume of 1 L (set-up shown in [Fig. A-III.1](#)). The reactor has been initially filled with ~160 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~1000 mg/L] withdrawn from mother reactor (MR). The synthetic feed has been prepared by using Lactogen 3 (0.50 g) and lukewarm water (~100 mL), properly mixed using magnetic stirrer and then added to the reactor. The composition of Lactogen 3 powder is presented in [Table A-II.2](#). Azithral has been used as



1. R_{azi20} 2. Air valve 3. Flexible PVC pipe
4. Aerobic sludge biomass 5. Diffused aeration 6. Air compressor

Fig. A-III.1 Schematic of reactor (R_{azi20}) setup used for Test 1.

a source of AZI for experiment. AZI [10 tablets of Azithral (each tablet contain 500 mg of azithromycin)] has been grinded using a mortar and pestle and stored in a airtight container. 20 mg of AZI has been mixed with ~50 mL of distilled water using magnetic stirrer. The Lactogen 3 feed solution (100 mL) and AZI solution (50 mL) have been added to the reactor, which has been designated as R_{azi20} . After addition of feed and AZI solution, the final volume of the reactor has been made to 1 L with tap water. For keeping the biomass in suspension and imparting mixing, air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffuser. The reactor R_{azi20} has been operated for 11 d at ambient conditions without pH control for two cycles per day with a fill time of 10 min, a react time of 675 min, a settling time of 10 min, a decant time of 15 min, and a wash time of 10 min (as shown in Fig. A-III.2). The steady-state conditions have been observed after 5 d of operation with respect to COD removal. Thereafter, mixed liquor of reactor R_{azi20} biomass has been divided (into two parts of 50% each) and poured in two separate 1 L batch reactor (height 14.5 cm×diameter 12.5 cm). The reactors have been designated as R_{azi500} and $R_{azi1000}$ (Fig. A-III.3) and operated similar to R_{azi20} ; however AZI concentration in reactors R_{azi500} and $R_{azi1000}$ have been increased to 500 mg/L and 1000 mg/L, respectively, in each cycle, keeping

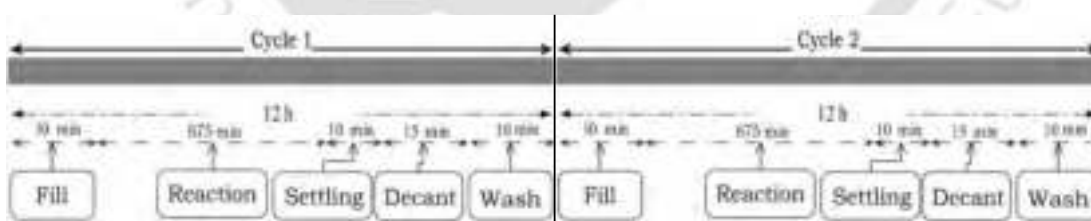
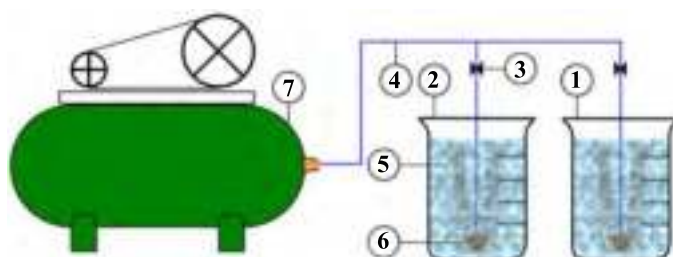


Fig. A-III.2 Schematic representation of cycle time for reactor operation.



1. R_{azi500} 2. $R_{azi1000}$ 3. Air valve 4. Flexible PVC pipe
5. Aerobic sludge biomass 6. Diffused aeration 7. Air compressor

Fig. A-III.3 Schematic of reactor (R_{azi500} and $R_{azi1000}$) setup used for Test 1.

the Lactogen dose same (0.50 g in each reactor). Both reactors R_{azi500} and $R_{azi1000}$ have been further operated for next 21 d.

A-III.2.3.2 Test 2

The second test has been performed to determine antibiotics among amoxicillin (AMX), cefuroxime (CFX), and ciprofloxacin (CIP), which have the most toxic effect on reactor performance as well as sludge morphology. The experimental set-up consists of four identical batch reactors (height 14.5 cm×diameter 12.5 cm) with a working volume of 1 L (set-up shown in Fig. A-III.4). The reactors have been designated as $R_{control}$ (fed with no antibiotics in the feed), R_{amx50} (fed with 50 mg/L of AMX), R_{cfx50} (fed with 50 mg/L of CFX), and R_{cip50} (fed with 50 mg/L of CIP) and operated at ambient conditions without pH control for two cycles per day with a fill time of 10 min, a react time of 675 min, a settling time of 10 min, a decant time of 15 min and a wash time of 10 min (as shown in Fig. A-III.2). Each reactor has been initially filled with ~160 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~1000 mg/L] withdrawn from mother reactor (MR).

The synthetic feed has been prepared by using Lactogen 3 (0.50 g) and lukewarm water (~100 mL), properly mixed using magnetic stirrer and then added to the reactor. The AMX solution has been prepared by injecting 10 mL of milli-Q water in Hipen-Clav vial (1.2 g) and thereafter vial has been hand shaken for proper mixing. Furthermore, 0.50 mL from AMX stock solution has been added to ~50 mL of tap water. Lactogen 3 solution (~100 mL) and AMX solution (~50 mL) have been added to reactor R_{amx50} and final volume has been made up to 1 L using tap water. CFX solution has been prepared by injecting 10 mL of milli-Q water in Cefuroxime Sodium (750 mg) vial and hand shaken for mixing. Furthermore, 0.66 mL from CFX stock solution has been added to ~50 mL of tap water. The Lactogen 3 solution (~100 mL) and CFX solution (~50 mL) have been added to reactor R_{cfx50} and final volume has been made up to 1 L using tap water. Alcipro contained 2000 mg/L of CIP (detail provided by Alkem Laboratories Ltd, India). Hence, 25 mL of Alcipro and Lactogen 3 solution (~100 mL) have been added to reactor R_{cip50} and final volume has been made up to 1 L using tap water. For keeping the biomass in suspension and imparting mixing, air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffuser. All four reactors have been operated for 30 d.

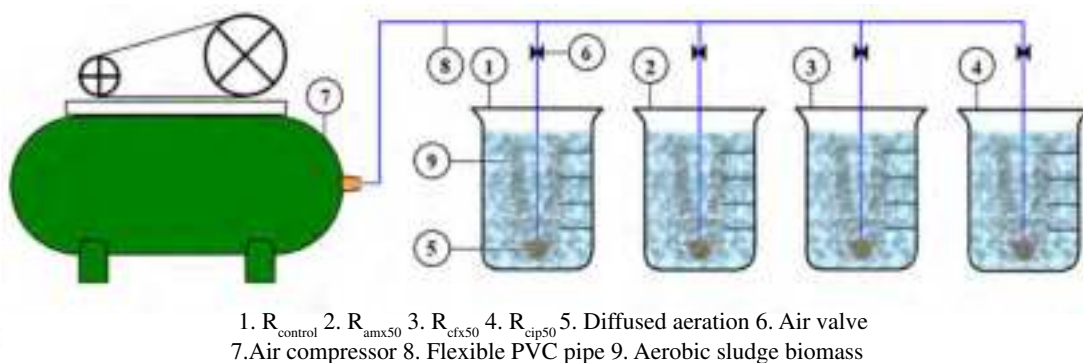


Fig. A-III.4 Schematic of reactor setup for used for Test 2.

A-III.2.3.3 Test 3

Based on the findings of Test 2, Test 3 has been conducted to evaluate the AMX concentration, which is more toxic impacting reactor performance and biomass morphology. The experimental set-up consists of four identical batch reactors (height 14.5 cm×diameter 12.5 cm) with a working volume of 1 L (set-up shown in Fig. A-III.5). The reactors have been designated as $R_{\text{amx}0}$ (fed with no antibiotics in the feed), $R_{\text{amx}250}$ (fed with 250 mg/L of AMX), $R_{\text{amx}500}$ (fed with 500 mg/L of AMX), and $R_{\text{amx}1000}$ (fed with 1000 mg/L of AMX) and operated at ambient conditions without pH control for two cycles per day with a fill time of 10 min, a react time of 675 min, a settling time of 10 min, a decant time of 15 min and a wash time of 10 min (as shown in Fig. A-III.2). Each reactor has been initially filled with ~160 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~1000 mg/L] withdrawn from mother reactor (MR). The synthetic feed has been prepared by using Lactogen 3 and lukewarm water (~100 mL), properly mixed using magnetic stirrer and then added to the reactor. The AMX solution has been prepared by injecting 10 mL of milli-Q water in Hipen-Clav vials (1.2 g) and thereafter vials have been hand shaken for proper mixing. The Lactogen 3 (2.50 g, 1.87 g, 1.25 g, and 0.1 g) and AMX solution (0, 2.5 mL, 5.0 mL, and 10.0 mL) have been added to reactor $R_{\text{amx}250}$, $R_{\text{amx}500}$, and $R_{\text{amx}1000}$, respectively, and final volume has been made up to 1 L using tap water. For keeping the biomass in suspension and imparting mixing, air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffuser. All four reactor have been operated until stabilization in COD removal have been observed.

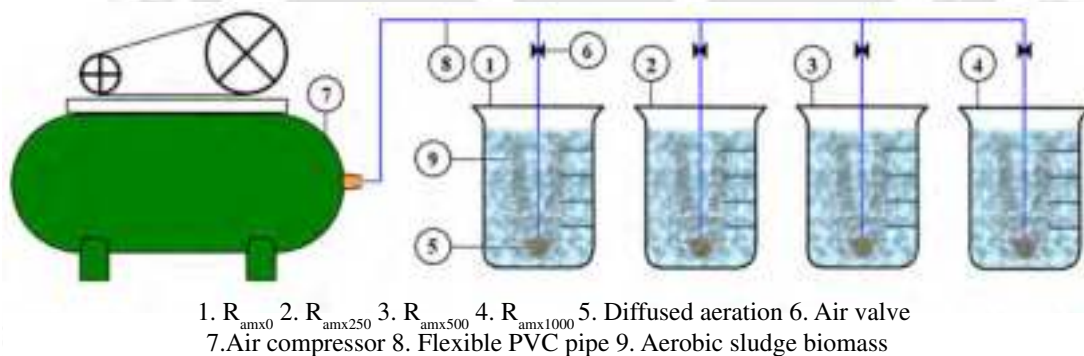


Fig. A-III.5 Schematic of reactor setup used for Test 3.

A-III.2.4 Analytical methods

The analytical techniques such as COD and solids estimation, microscopic and photographic observation of aerobic sludge biomass, and aerobic sludge biomass activity test have been discussed in Chapter 2.

A-III.3 Results and Discussion

All three tests have been carried out for a total of 69 d to choose antibiotic and its suitable concentration for experiment. The reactors have been operated during Test 1, Test 2, and

Test 3 for a total of 32 d, 30 d, and 7 d, respectively. The results obtained during reactor operation have been discussed below.

A-III.3.1 Test 1 observations

During Test 1, reactor R_{azi20} has been fed with 20 mg/L of AZI tablet for 11 d, while reactors R_{azi500} and $R_{azi1000}$ have been fed with 500 and 1000 mg/L of AZI tablet, respectively, for 21 d as shown in Fig. A-III.6. The COD removal for reactor R_{azi20} has shown variation during initial period. On 1st d of operation, 45.8% of COD removal has been observed for reactor R_{azi20} , which has further increased to 92.0% of COD removal on 5th d. The variation in COD removal for initial 5 d operation could be due to acclimatization of biomass. The aerobic

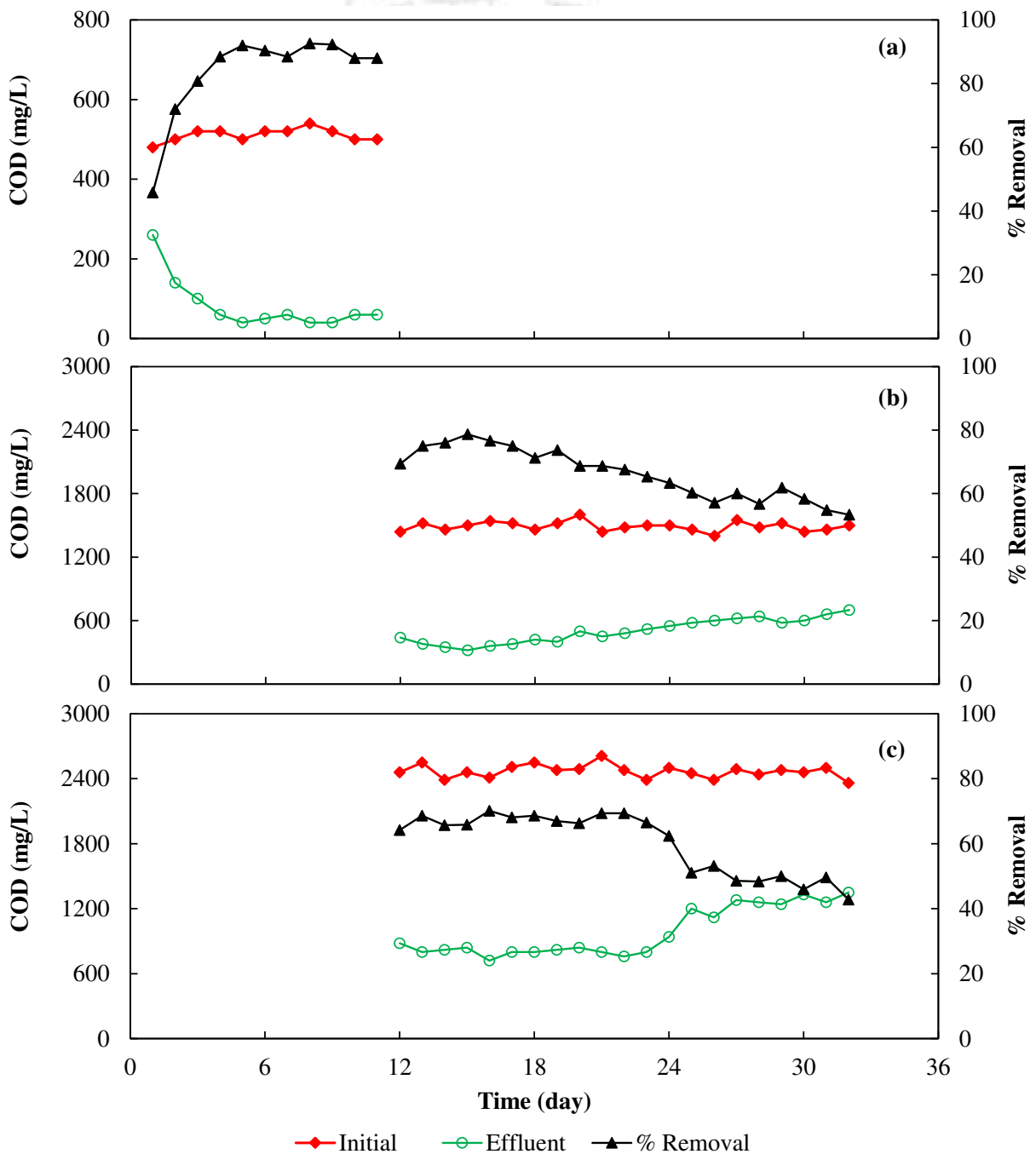


Fig. A-III.6 Variation in COD removal for reactors (a) R_{azi20} , (b) R_{azi500} , and (c) $R_{azi1000}$ during 32 d of operation in Test 1.

sludge biomass have taken 5 d to adapt to the new condition and later on uniform COD removal has been observed for next 6 d for reactor R_{azi20} [Fig. A-III.6(a)]. The average initial, effluent, and % COD removal for reactor R_{azi20} has been observed as 510.90 mg/L, 82.72 mg/L, and 83.5%, respectively, in 11 d of operation. After 11 d of operation, mixed liquor of reactor R_{azi20} has been equally divided and poured into reactors R_{azi500} and $R_{azi1000}$. The COD removal for both reactors R_{azi500} and $R_{azi1000}$ have been observed to be stabilized till 24 d of operation. The average COD removal for reactors R_{azi500} and $R_{azi1000}$ have been observed as 71.5% and 67.0%, respectively, for 12-24 d of operation. COD removal for both reactors have started to deteriorate gradually after 24 d [Fig. A-III.6(b)-(c)]. The average COD removal for reactors R_{azi500} and $R_{azi1000}$ have been observed as 57.8% and 48.7%, respectively, for 25-32 d of operation. The deterioration in COD removal could be due to AZI toxicity to the microorganisms and also AZI is a complex compound that could not be easily metabolized by microorganism. AZI particle settling, a big concern, has been observed during Test 1. AZI in low concentration (at 20 mg/L) is readily dissolved in water, however it is difficult to dissolve AZI at higher concentrations (500 and 1000 mg/L) at neutral pH, which leaves white particle settled at the bottom of the reactors.

The solids characteristics have been estimated on every 10th d, as presented in Table A-III.1. AZI at 20 mg/L has no impact on biomass growth of reactor R_{azi20} and it has increased from 1138 to 2461 mg/L with in 10 d. The sludge volume index (SVI) has also deteriorated in 11 d of operation and it has increase from 97 mL/g to 220 mL/g. There have been no impact observed of 500 mg/L and 1000 mg/L of AZI on biomass growth (Table A-III.1). The SVI for reactor R_{azi500} and $R_{azi1000}$ biomasses have been observed as 215 and 210 mL/g, respectively, on 20th d of operation.

Table A-III.1 Variation in solids characteristics during 1-32 d of operation in Test 1.

AZI concentration	Day	MLSS (mg/L)	MLVSS (mg/L)	Settled Vol. (mL)	SVI (mL/g)
20 mg/L	1	1138.08±38.80	1008.06±28.94	110	97
	10	2460.60±80.70	2255.56±36.84	540	220
500 mg/L	11	1260.46±44.72	1180.76±74.80	290	230
	20	2610.58±62.84	2380.22±64.84	560	215
1000 mg/L	11	1330.64±36.88	1220.38±34.86	300	226
	20	2520.34±44.78	2210.76±42.82	530	210

[The data is presented in xx ±yy (zz) format where xx is the average value, yy is the standard deviation]

MLSS: Mixed liquor suspended solids; MLVSS: Mixed liquor volatile suspended solids.

The microscopic images have been taken at 5× and 100× magnifications of stained aerobic sludge biomass and presented in Table A-III.2. Images illustrate that neither 20 mg/L nor 500 mg/L and 1000 mg/L of AZI has affected the morphology of aerobic sludge biomass. The biomass images taken on 30th d suggest that there has been abundance of filaments and AZI has not affected it even at higher concentration. The continuous growth of filaments have been observed in both reactors R_{azi500} and $R_{azi1000}$ till 30th d of operation.

Table A-III.2 Microscopic images of stained aerobic sludge biomass taken during 1-32 d of operation in Test 1.

Mag.	1 st d	30 th d	
	R _{azi20}	R _{azi500}	R _{azi1000}
5×			
100×			

A-III.3.2 Test 2 observations

To overcome the problem of AZI dissolution observed during Test 1, Test 2 has been carried out with intravenous antibiotic forms with 3 different antibiotics. The reactors R_{cfx50}, R_{amx50}, and R_{cip50} have been fed with 50 mg/L of CFX, AMX, and CIP, respectively, while R_{control} has been fed with only carbon source. The reactors have been operated for 30 d and results has been shown in Fig. A-III.7. The reactor R_{control} have shown uniform COD removal throughout the operation [Fig. A-III.7(a)]. The average COD removal for reactor R_{control} has been observed as 90.4% for 30 d of operation. The addition of antibiotics have hindered the COD removal efficiency of reactors from 1 to 12 d of operation and a stabilized COD removal has been observed for all the reactors. The average COD removal for reactors R_{cfx50}, R_{amx50}, and R_{cip50} have been observed as 73.6%, 75.3%, and 83.2%, respectively, for 1-12 d of operation. The initial variation in COD removal could be due to acclimatization of the biomass to the toxicity of antibiotics and as the biomass has adapted to the condition, it has started to degrade the organic matter. The average COD removal during 12-30 d for reactors R_{cfx50}, R_{amx50}, and R_{cip50} have been observed as 74.6%, 53.5%, and 65.5%. These results suggest that AMX has a significant impact on COD removal as compared to CFX and CIP.

The solids characteristics have been estimated on every 7th d and presented in Table A-III.3. Both reactors R_{control} and R_{cip50} have shown continuous biomass growth with continuous increase in SVI from 1st d to 24th d. It indicates that CIP has no significant inhibitory effect on biomass growth. The reactor R_{cfx50} has shown slower biomass growth as compared to reactor R_{control} and R_{cip50} biomasses. Whereas, AMX in reactor R_{amx50} has significantly affected the biomass growth and reduced it by almost 50% as compared to reactor R_{control} and R_{cip50} biomasses. Similarly, SVI for reactor R_{amx50} biomass has yielded lower values as compared to reactor R_{control}, R_{cfx50}, and R_{cip50} biomasses throughout the operation (Table A-III.3).

The microscopic and photographic observations have been presented in Table A-III.4 and Table A-III.5, respectively. The biomass of all the reactors have shown limited filaments and loose flocs structure on 1st d (Table A-III.4). On the other hand, abundance of filamentous

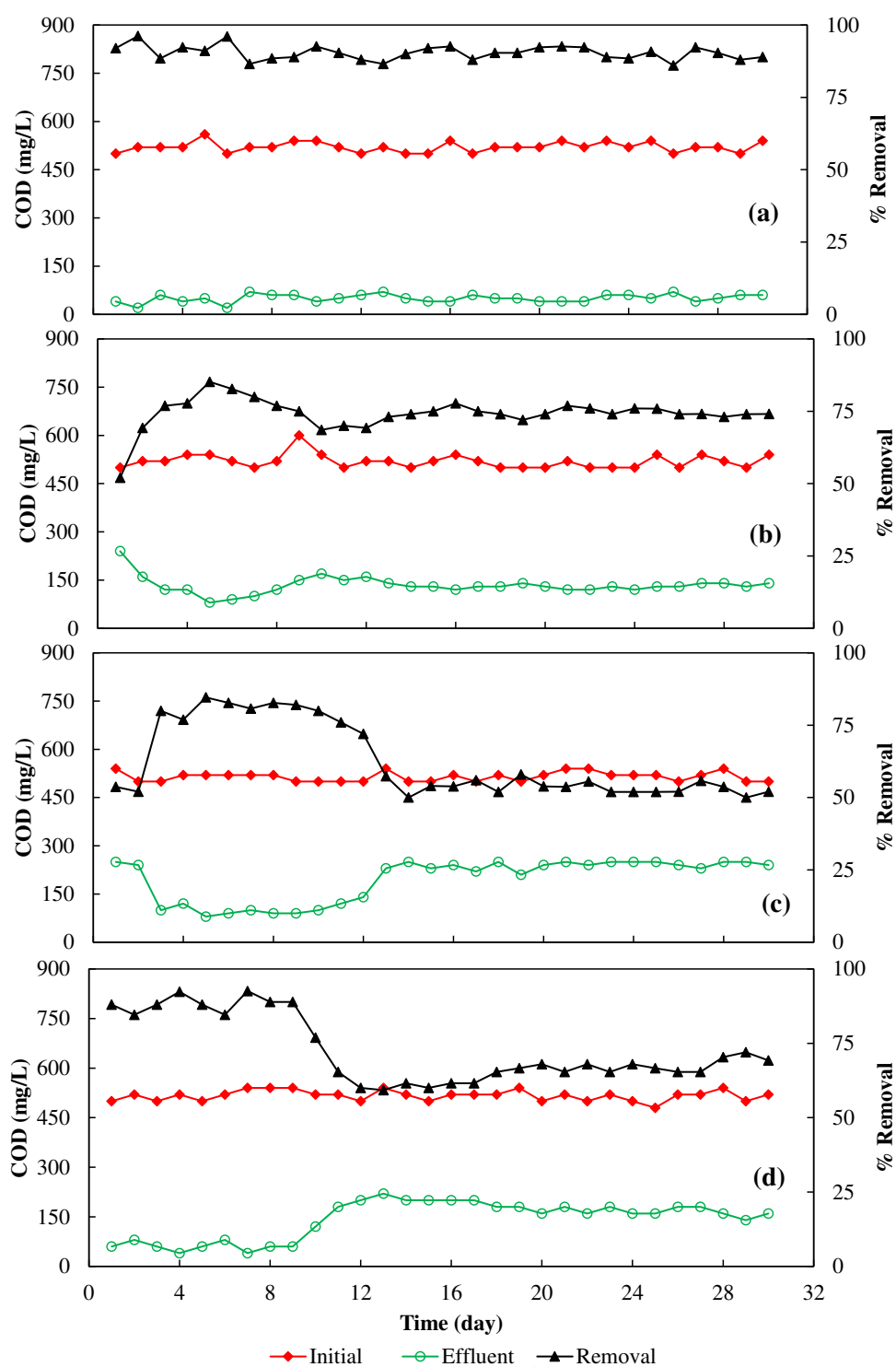
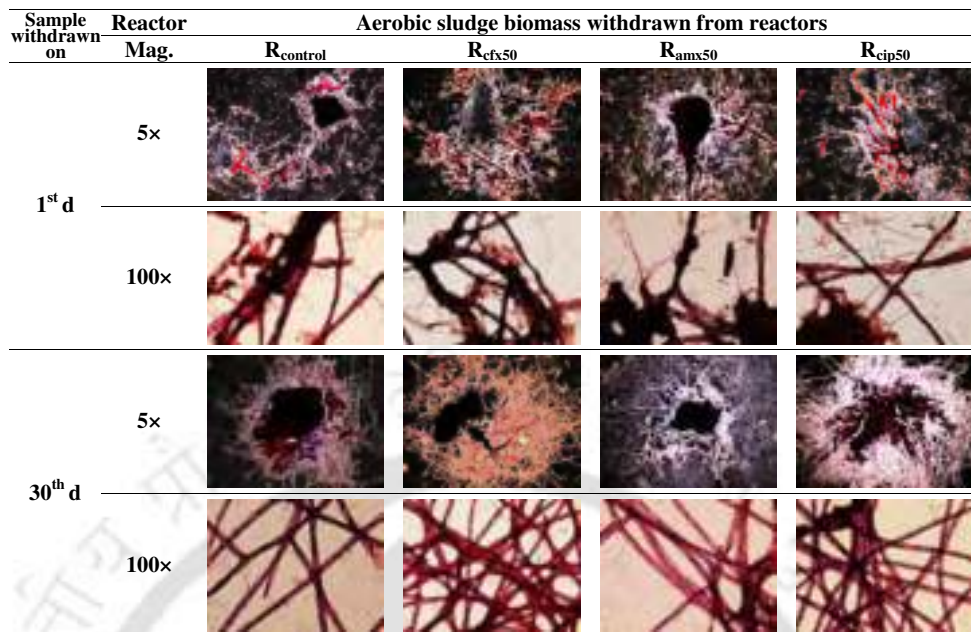


Fig. A-III.7 Variation in COD removal for reactors (a) R_{control} , (b) R_{cfx50} , (c) R_{amx50} , and (d) R_{cip50} during 30 d of operation in Test 2.

Table A-III.3 Variation in solids characteristics during 1-30 d of operation in Test 2.

Sample withdrawn on (d)	MLSS (mg/L)				MLVSS (mg/L)				SVI (mL/g)			
	R_{control}	R_{cfx50}	R_{amx50}	R_{cip50}	R_{control}	R_{cfx50}	R_{amx50}	R_{cip50}	R_{control}	R_{cfx50}	R_{amx50}	R_{cip50}
1	1160	1048	1105	1010	1090	972	1012	900	129	134	127	119
8	1820	1248	1180	1350	1740	1136	1006	1196	137	160	136	200
16	2214	1415	1290	1890	2058	1244	992	1640	226	254	155	201
24	2548	1685	1340	2480	2406	1520	980	2210	259	392	194	266

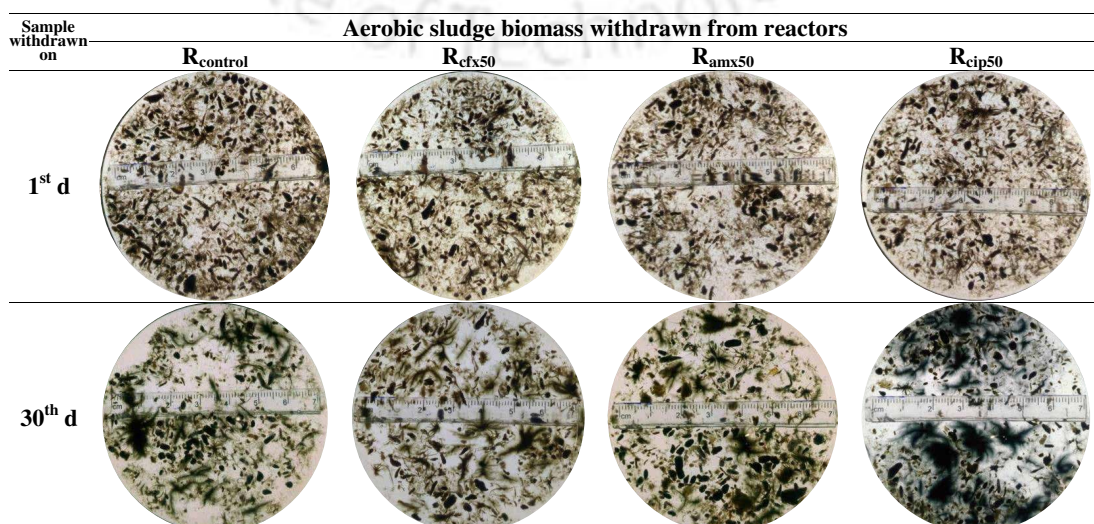
Table A-III.4 Microscopic images of stained aerobic sludge biomass observed during 1-30 d of operation in Test 2.



biomass has significantly increased in reactors R_{cfx50} and R_{cip50} as compared to reactor R_{control} and R_{amx50} on 30th d of operation. The microscopic images suggest that AMX is more toxic for filaments and has inhibited the filamentous biomass growth in reactor R_{amx50} as compared to other antibiotics (CFX and CIP).

The photographic images (Table A-III.5) have indicated uniform floc morphology on the 1st d of operation. The floc size has been observed in the range of 1.0–4.0 mm for all four reactors on 1st d. The addition of CFX and AMX to reactor R_{cfx50} and R_{amx50}, respectively, have slightly changed the morphology, whereas the morphology of reactor R_{cip50} biomass has drastically changed as compared to reactor R_{control} biomass. The addition of CIP has stimulated filamentous growth and floc size has increased in the range of 1.0–8.0 mm;

Table A-III.5 Photographic images of aerobic sludge biomass observed during 1-30 d of operation in Test 2.



however, 1.0–5.0 mm of floc size has been observed in reactor R_{control} , R_{cfx50} , and R_{amx50} biomasses. According to the findings, AMX tends to be more harmful than the CFX and CIP antibiotics.

A-III.3.3 Test 3 observations

Test 3 has been carried out for a week based on the COD removal of reactors. The reactors R_{amx0} , R_{amx250} , R_{amx500} , and R_{amx1000} have been fed with 0, 250, 500, and 1000 mg/L of AMX, respectively, along with the feed for 7 d. The variation in COD removal of reactors has been

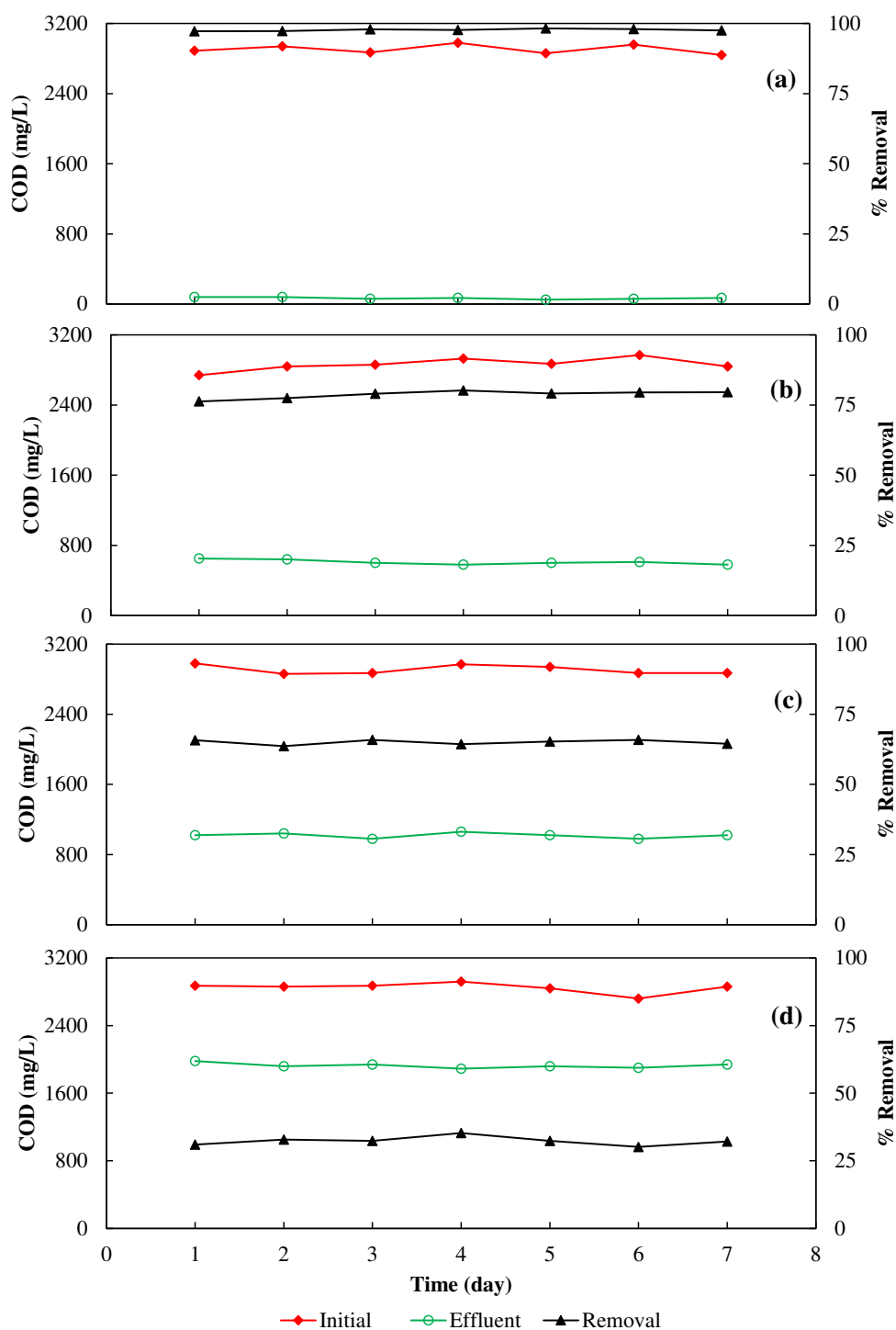


Fig. A-III.8 Variation in COD removal for reactors (a) R_{amx0} , (b) R_{amx250} , (c) R_{amx500} , and (d) R_{amx1000} during 7 d of operation in Test 3.

shown in Fig. A-III.8. The control reactor ($R_{\text{amx}0}$) has shown uniform COD removal for 7 d [Fig. A-III.8(a)] and the average COD removal has been observed as 97.7% in a 7 d of operation. Other three reactors $R_{\text{amx}250}$, $R_{\text{amx}500}$, and $R_{\text{amx}1000}$ have shown decrease in COD removal with increase in AMX concentration in the feed from the 1st d [Fig. A-III.8(a)-(c)]. The average COD removal for reactors $R_{\text{amx}250}$, $R_{\text{amx}500}$, and $R_{\text{amx}1000}$ have been observed as 78.7%, 65.0%, and 32.3% in 7 d of operation. The results suggest that increase in AMX concentration has deteriorated the COD removal efficiency of reactors.

Aerobic sludge biomass activity (ASBA) test has been performed on 7th d of operation to assess the organic degrading capability of aerobic sludge biomass. The ASBA results for all the reactors have been shown in Fig. A-III.9 and Table A-III.6. The ASBA results suggest that presence of AMX has severely affected the aerobic sludge biomass as compared to control

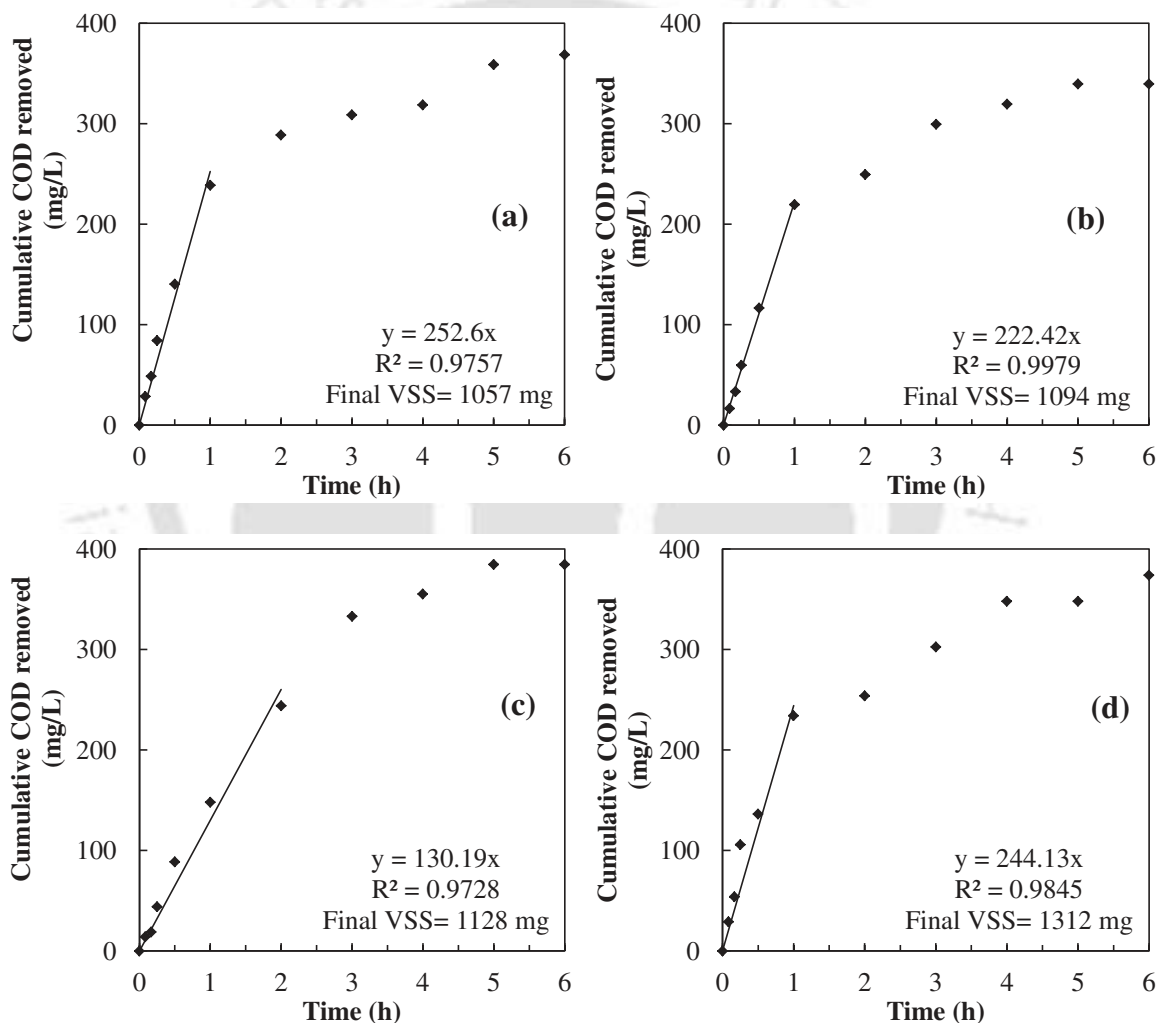


Fig. A-III.9 Aerobic sludge biomass activity test for reactors (a) $R_{\text{amx}0}$, (b) $R_{\text{amx}250}$, (c) $R_{\text{amx}500}$, and (d) $R_{\text{amx}1000}$ biomasses withdrawn on 7th d of operation in Test 3.

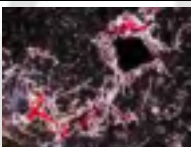
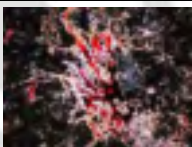
Table A-III.6 Aerobic sludge biomass activity test results carried out on 7th d of operation.

Biomass withdrawn on	7 th d			
Reactor	$R_{\text{amx}0}$	$R_{\text{amx}250}$	$R_{\text{amx}500}$	$R_{\text{amx}1000}$
ASBA (mg COD removed/mg VSS d)	5.74	4.88	4.10	2.95

reactor. The ASBA values for reactor R_{amx0} , R_{amx250} , R_{amx500} , and $R_{amx1000}$ biomasses have been observed as 5.74, 4.88, 4.10, and 2.95 mg COD removed/mg VSS d, respectively. The AMX has imparted a negative impact on aerobic sludge biomass and resulted into decreased ASBA values. In addition, impact of AMX on ASBA has increased with increase in AMX concentration.

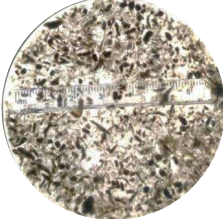
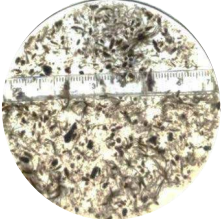
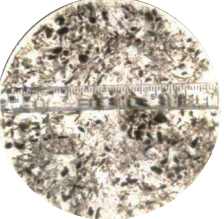
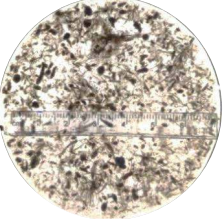
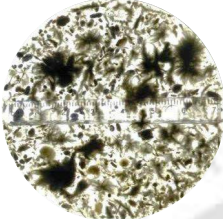
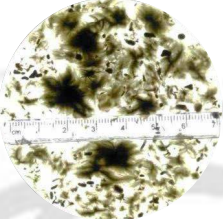
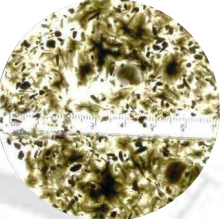
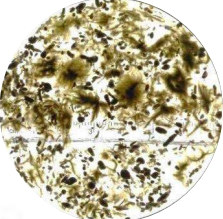
The microscopic observations have been presented in Table A-III.7, that shows all the four reactors have limited filamentous biomass on 1st d of operation. The abundance of filaments have increased with in a week for all the four reactors. The microscopic images taken at 5× magnification (on 7th d) suggest that lower concentration of AMX (250 mg/L) has no significant impact on floc morphology; however, it has stimulated the filamentous growth in reactor R_{amx250} biomass. The reactors R_{amx0} and R_{amx250} has shown similar biomass morphology on 7th d. The floc size has been affected and has not increased for reactor $R_{amx1000}$ biomass as compared to reactor R_{amx0} biomass due to high doses of AMX.

Table A-III.7 Microscopic images of stained aerobic sludge biomass observed during 1-7 d of operation in Test 3.

Sample withdrawn on	Mag.	Reactors			
		R_{amx0}	R_{amx250}	R_{amx500}	$R_{amx1000}$
1 st d	5×				
	100×				
7 th d	5×				
	100×				

The photographic observations have been presented in Table A-III.8, which suggest that flocs size for reactor R_{amx0} , R_{amx250} , R_{amx500} , and $R_{amx1000}$ biomasses have been observed almost same on the 1st d of operation and ranged between 1.0-4.0 mm in size. On the other hand, flocs size has been significantly increased with 7 d of operation for all the reactors (Table A-III.8). In comparison to the reactor R_{amx0} biomass, reactor R_{amx250} biomass has a similar morphology and floc size (1.0-20.0 mm on 7th d). The flocs size has shrunk for reactor R_{amx500} and $R_{amx1000}$ biomasses as AMX concentration in the feed hiked. The flocs size for reactor R_{amx0} , R_{amx250} , R_{amx500} , and $R_{amx1000}$ biomasses have been observed in the range of 1.0-14.0 mm on 7th d of operation.

Table A-III.8 Microscopic images of stained aerobic sludge biomass observed during 1-7 d of operation in Test 3.

Sample withdrawn on	Reactors			
	R _{amx0}	R _{amx250}	R _{amx500}	R _{amx1000}
1 st d				
7 th d				

A-III.4 Conclusions

Each of the three experiments offers useful information to help in selection of antibiotic for the experiment. Test 1 indicates that lower concentration of AZI has no impact on COD removal (83%), whereas higher doses 500 and 1000 mg/L of AZI has shown 71.5% and 67.0% of COD removals. The major issue encountered during the Test 1 has been the dissolution of AZI at neutral pH. Literature suggests that AZI can be completely dissolved at pH ~5.5. In every experiment, reactors have been operated without pH adjustment (average pH ranged 6.45-7.21) and therefore reducing the pH to 5.5 or less for dissolution of AZI is challenging. As a result, AZI is not suitable for the experiment. Test 2 suggests that AMX has severe toxic effect on aerobic sludge biomass as compared to CFX and CIP. The inclusion of CFX, AMX, and CIP in the feed has lowered COD removal to 73.6%, 75.3%, and 83.2%, respectively, at 50 mg/L of each antibiotic during 1-12 d of operation. AMX has also affected floc morphology and filaments concentration in aerobic sludge biomass. Biomass exposed to AMX has shown limited growth; however, CIP exposed biomass has shown accelerated biomass growth. Based on AMX toxicity to biomass and reactor performance, it has been selected as the suitable antibiotic for the experiment out of the three options i.e., CFX, AMX, and CIP. Test 3 aids in determining AMX concentration ranges inducing more harm to aerobic sludge biomass. In a 7 d of operation, higher doses of AMX has far more toxic effects on COD removal than lower doses. At higher concentrations, it has also affected the structure and morphology of the flocs. The ASBA findings also show that higher AMX concentrations have a negative impact on biomass, which has yielded decline in ASBA values as AMX concentrations have increased. Based on the results of Test 3, 500, 1000, and 2000 mg/L concentrations of AMX have been selected for the experiments.



Appendix-IV

Selection of Pesticide Doses

A-IV.1 Background

This investigation has been carried out to choose the pesticide [Chloripyrifos (CPS)] doses for the experiment. The experimental methodologies as well as results have been discussed below.

A-IV.2 Materials and Methods

A-IV.2.1 Chemicals and reagents

All chemicals and reagents used in this study are analytical or HPLC grade, manufactured by HiMedia Pvt. Ltd., India, and Merck India Ltd. The list of chemicals is presented in *Appendix-I* (Table A-I.1). Tricel (chlorpyrifos 20% EC) has been purchased from a local agriculture shop in Guwahati, manufactured by Excel Crop Care Limited, Mumbai, India.

A-IV.2.2 Aerobic sludge biomass

The aerobic sludge biomass withdrawn from the mother reactor (MR) has been used in this experimental investigation. The cultivation and maintenance of aerobic sludge biomass have been presented in *Appendix-II*.

A-IV.2.3 Experimental conditions

The experimental set-up consists of two identical batch reactors (height 14.5 cm×diameter 12.5 cm) with a working volume of 1 L (set-up shown in Fig. A-IV.1). The reactors have been designated as R_{Control} (no CPS in the feed) and R_{CPS} (50 mg/L of CPS in the feed) and operated at ambient conditions without pH control for two cycles per day with a fill time of 10 min, a

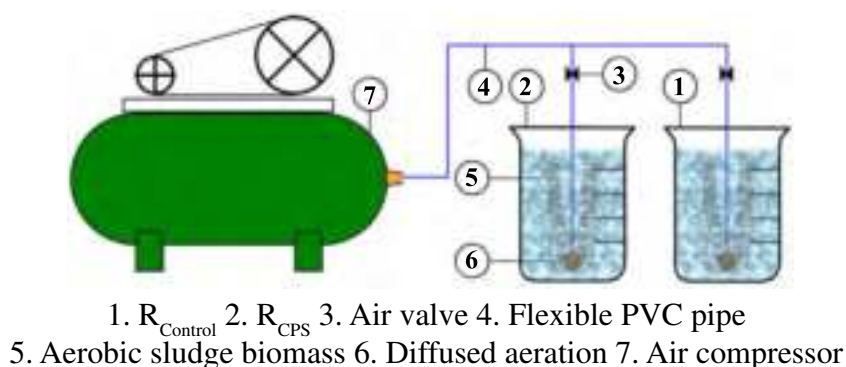


Fig. A-IV.1 Schematic of reactor setup used for experiment.

react time of 675 min, a settling time of 10 min, a decant time of 15 min and a wash time of 10 min (as shown in Fig. A-IV.2). Each reactor has been initially filled with ~160 mL of mixed liquor [amounting to mixed liquor suspended solids (MLSS) ~1000 mg/L] withdrawn from mother reactor (MR). The synthetic feed has been prepared by using Lactogen 3 (0.50 g) and lukewarm water (~100 mL), properly mixed using magnetic stirrer and then added to the control reactor. Tricel has been used as the source of CPS in the feed, which contains 21.5 g/L of CPS. The reactors R_{Control} and R_{CPS} have been fed with 0 and 2.32 mL (2 mL+320 μL giving 50 mg/L CPS) of CPS solution in each cycle. After the addition of CPS solution, the final volume of the reactors has been made to 1 L with tap water. For keeping the biomass in suspension, compressed air has been supplied using air compressor at a flow rate of 2.5 L/min through air diffusor. Both reactors have been operated for 30 d.

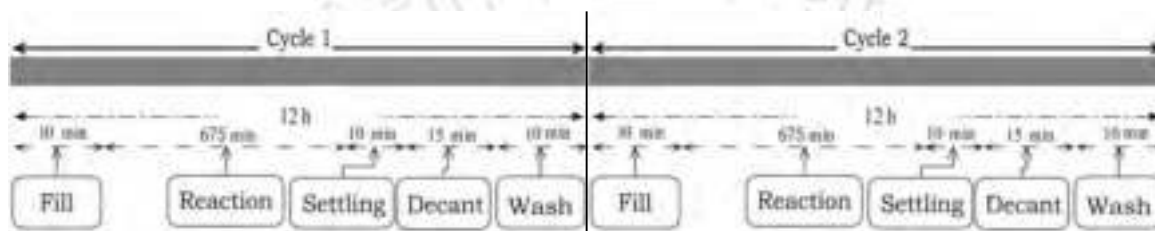


Fig. A-IV.2 Schematic representation of cycle time for reactor operation.

A-IV.2.4 Analytical methods

The analytical techniques such as COD and solids estimation, microscopic and photographic observation of aerobic sludge biomass have been discussed in *Chapter 2*.

A-IV.3 Results and Discussion

The reactors have been operated for 30 d and results obtained during reactor operation have been discussed below. The variation in COD for both the reactors have been shown in Fig. A-IV.3. The reactor R_{Control} have shown uniform COD removal throughout the operation [Fig. A-IV.3(a)]. The average COD removal for reactor R_{Control} has been observed as 90.4% for 30 d of operation. Whereas, the COD removal for CPS fed reactor (R_{CPS}) has been hindered due to addition of CPS in feed between 1-15 d of operation. The average COD removal for reactor R_{CPS} has been observed as 48.2% during 1-15 d of operation [Fig. A-IV.3(a)]. After 15th d, the COD removal has been stabilized and 39.4% of COD removal has been observed for 15-30 d of operation. The initial variation in COD removal could be due to change in feed composition and adaptation of the biomass to the new feed.

The solids characteristics have been estimated on every 7th d and data has been presented in Table A-IV.1. Both reactor biomasses have shown continuous biomass growth with continuous increase in SVI from 1st d to 24th d of operation. The reactor R_{CPS} biomass has shown slower biomass growth as compared to reactors R_{Control} biomass. It indicates that CPS at 50 mg/L has imparted slight inhibition on biomass growth. Similarly, SVI for reactor R_{CPS} biomass has yielded lower values as compared to reactor R_{Control} biomass throughout the operation (Table A-IV.1).

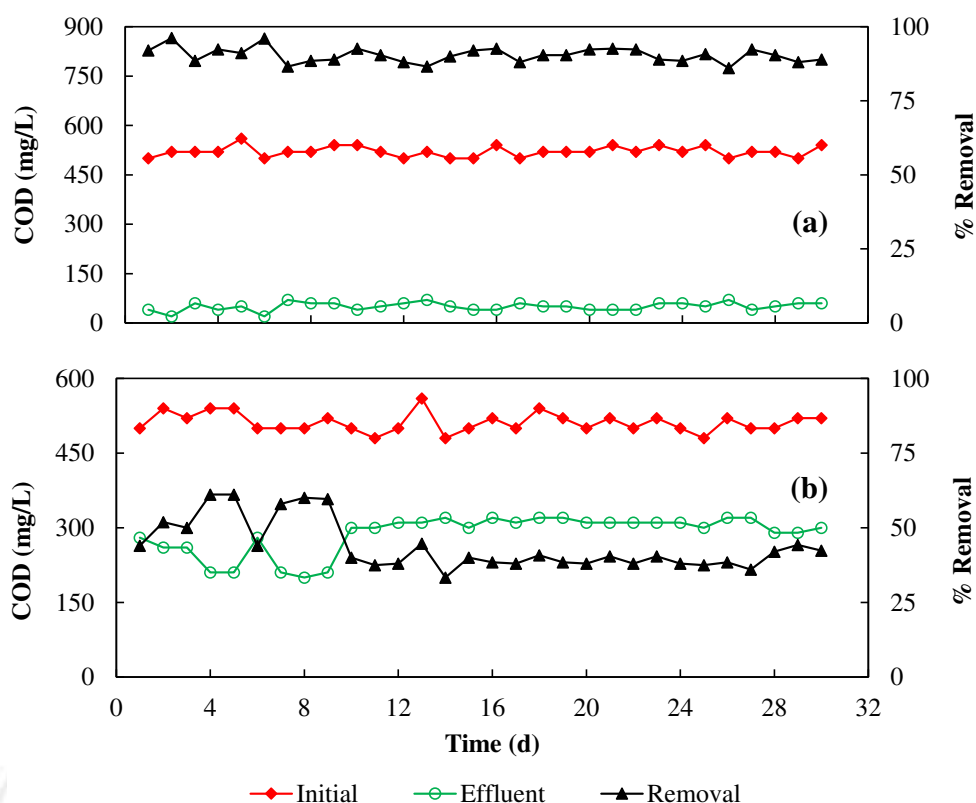


Fig. A-IV.3 Variation in COD removal for reactors (a) R_{Control} and (b) R_{CPS} during 30 d of operation.

The microscopic observation has been made at 5× and 100× magnifications with stained aerobic sludge biomass, which shows the overall floc morphology and filament density, respectively, as presented in Table A-IV.2. On the 1st d of operation, similar floc morphology and filament density have been observed for both the reactor biomasses. Whereas, on 30th d of operation, floc size and abundance of filaments have increased for reactor R_{Control} biomass and disintegration of flocs has been observed for reactor R_{CPS} biomass (Table A-IV.2). The abundance of weak filamentous biomass in reactor R_{CPS} could be due to the CPS toxicity, which has also resulted in to disintegrated flocs.

The photographic observation has been carried out on weekly basis, which has been presented in Table A-IV.3. The images suggest that flocs size for reactor R_{Control} and R_{CPS} biomasses have been observed almost same on the 1st d of operation and ranged between 1.0-4.0 mm in size. On the other hand, flocs size has increased for reactor R_{Control} biomass. In comparison to the reactor R_{Control} biomass, reactor R_{CPS} biomass has granular biomass on 30th d. The flocs size has reduced for reactor R_{CPS} biomass due to presence of CPS in feed.

Table A-IV.1 Variation in solids characteristics during 1-30 d of operation.

Sample withdrawn on (d)	MLSS (mg/L)		MLVSS (mg/L)		SVI (mL/g)	
	R _{Control}	R _{CPS}	R _{Control}	R _{CPS}	R _{Control}	R _{CPS}
1	1160	1136	1090	1062	129	134
8	1820	1348	1740	1128	137	130
16	2214	1615	2058	1334	226	130
24	2548	1885	2406	1624	259	140

The flocs size has been observed in the range of 1.0-14.0 mm for reactor $R_{Control}$ biomass and 1.0-7.0 mm for reactor R_{CPS} biomass.

Table A-IV.2 Microscopic images of stained aerobic sludge biomass observed during 1-30 d of operation.

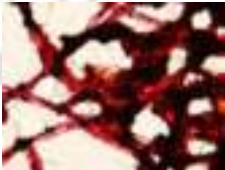
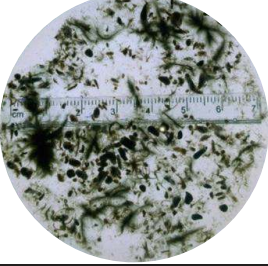
Withdrawn on	Mag.	Aerobic sludge biomass withdrawn from reactors	
		$R_{Control}$	R_{CPS}
1 st d	5×		
	100×		
30 th d	5×		
	100×		

Table A-IV.3 Photographic images of aerobic sludge biomass observed during 1-30 d of operation.

Withdrawn on	Aerobic sludge biomass withdrawn from reactors	
	$R_{Control}$	R_{CPS}
1 st d		
30 th d		

A-IV.4 Conclusions

According to this study, CPS at 50 mg/L in feed has a significant impact on COD removal, which can inhibit the performance by up to 40%. Furthermore, when compared to the control reactor, CPS has slowed biomass growth and reduced SVI. Along with the reduction in floc size, disintegration of flocs and decreased filament density have been observed due to CPS in feed. As a result, a minimum dosage of 50 mg/L of CPS has been chosen for the present study. In addition, a literature survey has led to the selection of two additional CPS doses of 100 and 200 mg/L. The studies are available with CPS concentrations up to 100 mg/L. As a result, CPS concentrations of 50, 100, and 200 mg/L have been selected for present the study.





Appendix-V

Table A-V.1 Minimum laboratory equipment needed for tests at plant sites of treatment plant located in India [Source: CPHEEO (2013)].

Equipment	Types of plant	
	5 MLD*	>5 MLD*
Analytical Balance	✓	✓
Autoclave	×	✓
Centrifuge	×	✓
Chlorine comparator	✓	✓
Colony counters	×	✓
Demineraliser	✓	✓
Dissolved oxygen sampler	✓	✓
Drying oven (hot air)	✓	✓
Fume cupboards	✓	✓
Gas liquid chromatograph	×	✓
Hot plates	✓	✓
Incubator 20°C (BOD)	✓	✓
Incubator 30°C (Bacteriological)	×	✓
Kjeldahl digester unit	✓	✓
Magnetic stirrers	✓	✓
Microscope, binocular with oil immersion and movable stage counting cell	×	✓
Membrane Filter Assembly	×	✓
Muffle Furnace	✓	✓
Orsat or equivalent gas analysis apparatus	×	✓
pH comparator (Colorimetric)	✓	✓
pH meter with reference & spare electrodes	×	✓
pH meter portable	✓	✓
Refrigerator	✓	✓
Sedwick Rafter funnel	×	✓
Sludge sampler	×	✓
Soxhlet extraction unit	×	✓
Spectrophotometer (atomic absorption)	×	✓
Spectrophotometer with or without U-V range or photo electric colorimeter	×	✓
Total organic carbon analyser	×	✓
Turbidimeter	✓	✓
Vacuum pump	✓	✓
Water bath (thermostat controlled)	✓	✓

*Million liters per day

References

CPHEEO. (2013). [Manual on Sewerage and Sewage Treatment Systems: Part A Engineering](#). Technical report, Ministry of Urban Development, Govt. of India, New Delhi, India.

