Biological sulfate reduction for batch and continuous removal of heavy metals from wastewater

A Thesis

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by

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Statement

I, hereby declare that the content embodied in this thesis entitled "Biological sulfate reduction for batch and continuous removal of heavy metals from wastewater" is the result of investigations carried out by me at the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of **Professor Kannan Pakshirajan sir (Supervisor) and Professor Gopal Das sir (Supervisor)**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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Certificate

It is certified that the work described in this thesis entitled “Biological sulfate reduction for batch and continuous removal of heavy metals from wastewater” by Mothe Gopi Kiran for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision at the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India, and this work has not been submitted elsewhere for a degree.

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Abstract

Heavy metals are extensively used for several applications, and as a consequence of which these metals are discharged into the environment from different industries, such as metallurgy, leather industries, mining, nuclear and electronic industries, electroplating plants, etc. Owing to their non biodegradable and non persistent nature, metals can merely be transformed to less toxic and insoluble forms. Besides heavy metals, sulfate is found abundant in wastewater streams which pose many environmental issues. Therefore, elimination of metals and sulfate from wastewater before their discharge into the environment is mandatory.

Existing technologies for metal removal from wastewater, which include chemical precipitation, adsorption, biosorption and oxidation-reduction, are either costly, less selective, less efficient, particularly at low concentrations, or generate secondary sludge disposal problems. Compared to these methods, sulfate reduction aided by sulfate reducing bacteria (SRB) enables the removal and recovery of metals as sulfides in treating metal containing wastewater. In this method, sulfate reduction occurs by oxidizing organic substrates or any electron donor, which result in the production of hydrogen sulfide (H_2S) and other sulfur species, thereby enhancing the metal precipitation.

This study was focused on the application of sulfidogenic bioreactor systems for heavy metal removal from metallic wastewater. Initial studies were carried out to screen suitable anaerobic biomass for the removal of different heavy metals and to investigate the mechanism of metal removal from both single and multi-component systems by SRB. Continuous metal removal studies were then carried out using two lab-scale sulfidogenic bioreactor systems with immobilized SRB to examine the metal removal efficiency and its applicability for treating heavy metal contaminated wastewater.

Biomass from different sources for the removal of Cd(II), Cu(II), Ni(II), Fe(III), Pb(II) and Zn(II) by biological sulfide precipitation was collected from (a) a wastewater treatment plant (WWTP) located at Indian Institute of Technology Guwahati, Guwahati, Assam, India, (b) a lab-scale upflow anaerobic packed bed reactor (UFAR) and (c) a lab-scale anaerobic packed bed reactor (APR) treating sulfate and arsenic rich water. Among the three biomass types investigated in this study, anaerobic biomass collected from the UFAR showed very high removal of different metals at both low and high initial concentrations and maximum sulfate reduction (> 90%) within 96 h with a maximum wastewater chemical oxygen demand (COD) removal of up to 92.8%. The heavy metal removal efficacy obtained was in the order Cu > Fe > Ni > Pb > Cd > Zn.

Field emission scanning electron microscopy (FESEM) and energy dispersive X-ray spectroscopy (EDX) analyses of the metal-loaded biomass showed that the precipitates formed during the process consist of mainly the metal sulfides. Transmission electron microscopy (TEM) along with energy dispersive spectroscopy (EDS) analysis confirmed that the metal precipitates were confined to either exterior or interior to the bacterial cell surface. The Fourier transmission infrared spectroscopy (FTIR) spectra of the biomass obtained during its growth clearly established the presence of sulfate ions. The heavy metal removal mechanism was attributed to the sulfate reducing capability of the biomass, which resulted in precipitation of the metals as their corresponding sulfide salts. Biomass from the same source was subsequently used for further heavy metal removal experiments.

In order to study heavy metal removal from multi-component system by SRB, different combinations of high and low concentration levels of six heavy metals as mentioned earlier were chosen employing the Plackett-Burman screening design. The results obtained showed a maximum removal of Cu(II) (98.9%), followed by Ni(II) (97%), Cd(II) (94.8%), Zn(II) (94.6%), Pb(II) (94.4%) and Fe(III) (93.9%). The heavy metal resistance by SRB varied with respect to the different metal species; the presence of Cu(II) and Ni(II) along with Pb(II) at their respective high concentration in the mixture inhibited Pb(II) removal; similarly, Ni(II) and Fe(III) at their respective high concentration inhibited the Fe(III) removal. All these effects of heavy metals on each other removal from mixture were attributed to the solubility product constant value of the corresponding metal sulfide salts.

The SRB biomass obtained from the mixture study was further characterized using FTIR, TEM-EDS and FESEM-EDX. FTIR spectroscopy analysis of the biomass confirmed the presence of functional groups in the SRB that were similar to that of an earlier reported SRB, *Desulfovibrio*. species. TEM-EDS and FESEM-EDX analyses of the bacteria following metal removal further confirmed that the metal precipitates formed were associated with the cell surface, and the heavy metal removal mechanism was attributed to the capability of the SRB to reduce sulfate to sulfide. A very high removal of the different metals was achieved at both low and high initial concentrations. However, at a high metal concentration combination, sulfate reduction and COD removal were inhibited, thus resulting in a slightly reduced removal of these metals by SRB, which also matched with the results obtained in the batch study.

For a successful application of this method, the choice of a suitable reactor system is essential. Therefore, continuous metal removal from synthetic wastewater by immobilized SRB was evaluated using two lab-scale sulfidogenic bioreactor systems: anaerobic rotating biological contactor (An-RBC) reactor and a downflow column reactor (DFCR) packed with immobilized SRB beads. Synthetic metallic wastewater containing heavy metals with different initial concentration was treated using the two bioreactor systems. The effect of different parameters, such as hydraulic retention time (HRT) and inlet metal concentration was carried out in this reactor study.

Heavy metal removal using sodium alginate immobilized SRB was evaluated under batch and continuous mode. Under batch conditions, more than 95% metal removal was achieved due to formation of insoluble metal sulfides exterior to the bead surface even at a high initial concentration, which is better than the results obtained using free/suspended biomass. Characterization of the metal loaded immobilized SRB beads using FESEM-EDX spectroscope established that the metals were precipitated exterior to the bead surface due to the formation of metal sulfides.

Continuous metal removal was achieved using DFCR packed with the immobilized SRB beads. Best heavy metal removal results were obtained at 48 h HRT than at 24 h HRT. Metal loading rates up to 4.29 mg/L·h in case of Cu(II) and up to 2.20 mg/L·h, in case of Fe(III), Pb(II), Ni(II), Zn(II) and Cd(II) were found to be favorable for their removal from single component system using the continuous column reactor. Statistically valid factorial design of experiments was carried out to understand the dynamics of metal removal from mixture using the DFCR. Copper removal was maximum (99%), followed by Zn(II) (95.8%) and other heavy metals at their respective low inlet concentrations. However, the removal values were reduced at a high inlet concentration of the heavy metals, which matched well with low COD and sulfate removal efficiencies. FESEM-EDX analysis of the metal precipitates formed in the reactor revealed the potential of SRB in precipitating metals as metal sulfides *via* sulfate reduction.

The performance of a continuously operated laboratory-scale An-RBC reactor immobilized with SRB was evaluated for heavy metal removal from synthetic wastewater under sulfate reducing condition at 24 h and 48 h HRT values. A very high metal removal efficiency was obtained at a low metal loading rate using the An-RBC reactor, which matched well with the results obtained using the DFCR; the metal removal efficiency order using this reactor was Cu > Cd > Pb > Fe > Zn > Ni. Sulfate and COD reduction values confirmed that a high metal loading is inhibitory to SRB activity, but the metal removal results were better than those results obtained using the DFCR. Metal loading rates greater than 3.64 mg/L·h in case of Cu(II) and 1.87 mg/L·h in the case of Fe(III), Pb(II), Ni(II), Zn(II) and Cd(II) proved toxic and inhibitory to SRB activity and therefore, were found detrimental to the reactor performance. V3-V4 metagenomics sequencing and analysis revealed that SRB immobilized in the An-RBC reactor is predominant with *Desulfovibrio vulgaris.* sp.

Combined effect of different heavy metals on the removal of metals, sulfate and COD by SRB using the An-RBC reactor was evaluated under continuous operating condition by employing the statistically valid fractional factorial design of experiments. Continuous metal removal from a mixture of the heavy metals showed that Cu(II) removal was maximum (> 98%), followed by Zn (II) (96%) and other heavy metals at their respective low inlet concentrations, and metal removal order in the mixture study was Cu > Zn > Cd > Pb > Fe > Ni, which matched well with the results obtained using the DFCR. The An-RBC reactor also yielded very high metal removal efficiencies even at high initial level concentration combination of the metals. Metal loading rates less than 3.7 mg/L·h in case of Cu(II) and less than 1.69 mg/L·h for Ni(II), Pb(II), Zn(II), Fe(III) and Cd(II) were favorable for achieving a stable and very good performance of the An-RBC reactor for metal removal from the mixture. These results strongly indicate that the passive biofilm based bioreactor (An-RBC reactor) could be preferred over DFCR for large-scale treatment of sulfate and metal rich wastewater.

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Abbreviations

(ΔG°): Gibbs free energy change
AAS: Atomic absorption spectrometer
ABR: anaerobic baffled reactor
ACP: anaerobic contact process
AFR: Anaerobic filter reactors
AFR: anaerobic fixed film reactors
AHR: anaerobic hybrid reactor
AMD: Acid mine drainage
AMP: adenosine monophosphate
ANOVA: Analysis of Variance
APHA: American public health association
APR: anaerobic packed bed reactor
APS: adenosine 5'-phosphosulfate
ATP: adenosine triphosphate
BESA: bromo ethane sulfonic acid
COD: chemical oxygen demand
CSTR: Continuously stirred tank reactor
DAF : dissolved air flotation
DFBR: downflow fluidized bed reactor
DFCR: downflow column reactor
DSFF: down flow stationary fixed film
EC: Electro-coagulation
ED: Electro-dialysis
PMMA: polymethyl methacrylate
PPi: Pyrophosphate
PRB: Permeable reactive barrier
PUF: polyurethane foam
RO: Reverse osmosis
RR: removal rate

PEI: polyethyleneimine

EDTA: ethylene diamine tetraacetic acid
EF: Electro-flotation
EPS: extracellular polymeric substances
FBR: fluidized bed reactor
FFD: fractional factorial design
g: gravitational acceleration
GLR: Gas lift reactor
HRT: hydraulic retention time
IFBR: inverse fluidized bed reactor
ILR: inlet metal loading rate
IMBR: immersed membrane bioreactors
MBR: membrane bioreactors
MEUF: Micellar enhanced ultra filtration
MIC: Minimum inhibitory concentration
NF: Nano-filtration
ORP: oxidation reduction potential
PAA: polyacrylic acid
PAC: polyaluminium chloride
PAM : polyacrylamide
PAP: phosphoadenosine 5'-phosphte
PAPS: phosphoadenosine 5'-phosphosulfate
PEUF: polymer enhanced ultra filtration
PFS : polyferric sulfate
SMBR: side stream membrane bioreactors
SRB: sulfate reducing bacteria
SRT: sludge retention time
UF: ultra filtration
UFAR: upflow anaerobic packed bed reactor
WWTP: wastewater treatment plant

UFAR: upflow anaerobic packed bed reacto

FESEM-EDX: field emission scanning electron microscopy energy dispersive X-ray spectroscopy

An-RBC: anaerobic rotating biological contactor reactor

FTIR: Fourier transforms infrared spectrometer

OVAT: one-variable-at-a-time

NTU: Nephelometric Turbidity Unit

TEM-EDS: transmission electron microscopy with energy dispersive spectroscopy

MLVSS: mixed liquor volatile suspended solids

UASB: upflow anaerobic sludge blanket reactor

PRESS: predicted residual error sum of squares

CHAPTER 1

INTRODUCTION

With the rapid industrialization and large-scale mining activities, heavy metal containing wastewater is directly or indirectly discharged into the environment leading to ecological imbalance. Though heavy metals are naturally present in the environment, large-scale release of these pollutants from human activities has far exceeded the carrying capacity of the environment. Effluent from metallurgical industries and acid mine drainage (AMD) from mining activities are, in general, acidic and rich in sulfate and dissolved metals (Svetlana et al., 2011). Discharge of such heavy metal containing wastewater results in a serious problem towards the environment and living things (Kikot et al., 2010). Hence, removal of metals from wastewater prior to its discharge into the environment is becoming inevitable.

1.1 Heavy Metals

Heavy metals play a vital and broad role in several fields in terms of their applications. Heavy metals are widely used as pigments, corrosion resistant materials, plastic stabilizers, alloys, semiconductors, conductors, antifouling agents, etc. Besides, these are used in computer systems, batteries, solar panels, electroplating industries, paint industries, electrical equipments, industrial machinery, housing, shipping, nuclear applications, etc. (Kikot et al., 2010). Metals, particularly Cd, Cu, Ni, Fe, Hg, Mn, Pb and Zn, are discharged in the effluent from different industries. These heavy metals are highly toxic even at low concentration and non bio-degradable, but can be transformed to less toxic and insoluble forms (Beyenal and Lewandowki, 2004; Min et al., 2008; Kieu et al., 2011). Hence, removal of metals from wastewater prior to its discharge into the environment is highly essential.

Environment protection rules formulated by Central Pollution Control Board (CPCB), India, (1989), recommended the general standards for discharge of environmental pollutants in the wastewater streams; the drainage standards for As, Cu, Cd, TCr, Fe, Hg, Mn, Ni, Pb, Se, V and Zn are 0.2 mg/L, 3 mg/L, 2 mg/L, 2 mg/L, 3 mg/L, 0.01 mg/L, 2 mg/L, 3 mg/L, 0.1 mg/L, 0.05 mg/L, 0.2 mg/L and 5mg/L, respectively.

1.2 Sulfate

Along with the heavy metals, sulfate is another compound abundantly found in wastewater, such as AMD and effluent from several other industrial processes. Generally, sulfate containing wastewater is generated from various industries, such as mining, explosives, gas scrubbing, flue gas scrubbing, paper and pulp industries, galvanic processing, pharmaceutical industries, battery, paint, textile, dye industries, etc. (Lens et al., 1998; Masigol et al., 2012). Several problems exist due to the discharge of sulfate rich wastewater, for example, sulfate reduction to hydrogen sulfide (H_2S), often leading to corrosion of pipe materials (Sulaiman et al., 2008). Hence, there is a continuous search for economical, effective and eco-friendly processes for the removal of heavy metals and sulfate from wastewater, which would ensure their presence in the environment below permissible limits.

1.3 Treatment Methods For Heavy Metal And Sulfate Containing Wastewater

Most of the industries treat metal containing wastewater, e.g. AMD, by precipitation with hydroxide or limestone because of process simplicity, low-cost and easy process control, which, however, results in sludge disposal problems. Other developed processes, such as reverse osmosis, ion exchange and electro-dialysis, etc., are available to treat AMD and other metal containing wastewater which are quite expensive, rarely used and less efficient for metal removal, particularly at low concentrations (Eccles, 2010; Fu and Wang, 2011). In view of the strict environmental legislations governing wastewater discharge standards and due to the technical and economical concerns related to the use of existing methods, biological processes seem to be more attractive than the afore-mentioned techniques for treating wastewater containing heavy metals and sulfate. Besides, biological methods are proving more economical over the physico-chemical methods for heavy metal removal (Pires et al., 2011).

1.4 Biological Sulfate Reduction For Heavy Metal Removal

In the recent years, biological methods are proving to be efficient for the treatment of mining wastewater containing sulfate and heavy metals. In particular, metal precipitation aided by sulfate reducing bacteria (SRB) has been found to be an attractive and promising alternative over physico-chemical and other methods in practice (Kieu et al., 2011). In this method, under anaerobic conditions and in the presence of an organic substrate, sulfate is reduced by SRB that mediates the metal removal *via* the resulting metal sulfide precipitation (Sahinkaya et al., 2011).

The ability of SRB to generate sulfide together with the high potential of sulfide to react with metal cations is considered effective for treating both metal and sulfate rich wastewater (Bai et al., 2008; Velasco et al., 2008; Jimenez-Rodriguez et al., 2009; Telcu et al., 2009). Biological sulfide precipitation of heavy metals offers several advantages over chemical precipitation which include low solubility product values of sulfide, even at a low pH, low residual sludge volumes, cost-effective and efficient removal, even at low metal concentrations (Tsukamoto et al., 2004; Gallegos-Garcia et al., 2009). However, heavy metal removal capacity by this method depends on several factors, such as the heavy metal, bacterial species, reactor configuration, etc. It is well reported in the literature that concentration of heavy metals in wastewater may range from a few mg/L to as much as 100 mg/L (Hao et al., 1994; Utgikar et al., 2002).

The precipitates formed are also more stable compared with the metal hydroxides formed during chemical processes (Cao et al., 2013). In addition to the removal of heavy metal associated toxicity from wastewater, it facilitates the metal recovery as metallic sulfides (Bijmans et al., 2011). Although the potential use of microbial sulfate reduction for treating metallic wastewater has been reported as early as 1969, development of SRB based passive or active treatment system for large-scale application is limited.

Moreover, there is still a need to explore more novel sources of SRB for the removal of heavy metals and sulfate and its characterization to further establish its application potential. This is particularly important because of its certain drawbacks, such as the inherent toxicity of metals on SRB growth and activity (Min et al., 2008). For instance, high metal concentrations have been shown to be inhibitory towards growth and activity of freely suspended SRB (Sani et al., 2001; Utgikar et al., 2001; Utgikar et al., 2002). The use of free/suspended SRB systems further give rise to problems, such as reduction in cell retention and cell washout in continuously operated reactors (Baskaran and Nemati, 2006), thereby, necessitating extended hydraulic retention time (HRT) in the system (Neculita et al., 2007).

In order to overcome the drawbacks associated with the use of free/suspended cells based SRB system, active cell immobilization using suitable naturally available polymeric matrix or biofilm development on a support material can be applied. Although a wide range of materials has been tested for immobilizing, the naturally available polymer, sodium alginate has received significant interest owing to its economical viability and inert nature to microbes (Chen and Lin, 1994; Lozinsky and Plieva, 1998).

However, active SRB immobilization using sodium alginate and its use in batch/continuous removal of heavy metals is very limited. Among the several bioreactor systems available to treat wastewater, rotating biological contactor (RBC) reactor is well known for treating various types of effluents (Alemzadeh et al., 2002; Kapdan and Kargi, 2002; Axelsson et al., 2006) as it offers a high interfacial area on its rotating discs for biomass growth and attachment, which enables sufficient contact between the microorganisms and contaminants present in the wastewater (Pakshirajan and Kheria, 2012).

Furthermore, RBC reactor is highly suitable for treating industrial wastewater and sewage (Banerjee, 1997) owing to the combined action of both free/suspended and fixed film microorganisms present in this reactor system. However, RBC reactors involve high initial investment cost; but simple process control, low operation and maintenance costs make these reactors technically feasible for large-scale applications (Borchardt, 1971; Wu and Smith, 1982; Wheatley, 1984).

The utility of RBC reactors has been examined at the laboratory scale for chemical oxygen demand (COD) removal from textile dying and synthetic wastewater (Pakshirajan et al., 2009). However, it has not been tested for the removal of heavy metals by biogenic sulfide precipitation. Therefore, this study is aimed at screening anaerobic biomass from different sources for sulfate reduction and heavy metal removal from aqueous solutions and further study the application potential of the anaerobic biomass for metal removal using different bioreactor systems.

CHAPTER 2

LITERATURE REVIEW

Water pollution has been a major point of concern since many decades owing to rapid urbanization and industrial activities associated with it. Heavy metals are categorized as one of the most toxic water pollutants and hence, their removal has been a topic of major concern. In view of exponentially increasing population and increasing consumption of resources, the need for controlling heavy metal emissions into the environment is even more obvious. Discharge of heavy metal containing wastewater at a low pH from several industries and acid mine drainage (AMD) may lead to environmental pollution and imbalance in the ecosystem (Kieu et al., 2011). Heavy metals can be classified as toxic and non-biodegradable inorganic pollutants as they tend to accumulate in the food chain and get absorbed by the living organisms, including humans, which result in serious health issues (Zhuang et al., 2009); moreover, they influence the aesthetic quality of potable water (Gray, 2008).

2.1 Heavy Metals

Heavy metals in water refer to the heavy, dense, metallic elements that occur in trace levels, but are very toxic with a high bioaccumulation potential; these elements are commonly referred to as trace metals. Globally, metal consumption rate is increasing exponentially, whereas metal resources are getting depleted as a consequence of the fast growing metal industries. This is mainly because heavy metals play a vital and broad role in several fields with respect to their applications. Besides, metals are also used in nuclear applications and even in warfare as chemical weapons.

In biological systems, heavy metals are necessary in trace amounts as they are the key constituents of enzyme complexes, blood pigments, biological electron transport and oxygen transportation systems, cofactors, bone formation and reproduction (Cohen and David, 2007). They also find application in medicinal fields for dietary vitamins, mineral supplements, and some lead to healing properties and increase immune system (DiSilvestro and Robert, 2004). Some of the heavy metals play a vital role as oxygen evolution in plants and are also used as preservatives and fungicides.

2.1.1 Sources

Heavy metals occur in the Earth’s geological formations and can, therefore, enter into streams, lakes, rivers and groundwater; very often these heavy metals are discharged into the environment due to various developmental activities. Wastewater from industries, such as mining, smelting, metal plating, battery manufacturing, etc., is a main cause of metals in the water (Kwon et al., 2010; Reddy et al., 2010; Abdel Salam et al., 2011). Table 2.1 presents various sources and activities through which heavy metals enter the environment.

Table 2. 1 Sources of heavy metals in the environment

S. No	Sources	Activities	Reference
1	Atmospheric	Volcanic activities, forest fires, dust particles etc.	Clark, 2001
2	Rivers	Erosion of rocks containing metals, surface runoff from naturally formed and anthropogenic metal sources, dredging, trawling	Clark, 2001
3	Groundwater seepage	Dissolved substances are carried *via* ground water movement – contamination in soil	Clark, 2001
4	Deliberate discharge	Contaminated waste dumping, industrial discharges, sewage	Clark, 2001
5	Industries and other anthropogenic activities	Coal fired power stations, mining, leather tanning, lead acid batteries, paints, e-waste, smelting operations, ceramics, bangle industry, chlor-alkali plants, fluorescent lamps, hospital waste, electrical appliances, sulfuric acid plant, incinerations and fuel combustion.	Gautam, 2011

2.1.2 Environment and health effects of heavy metals

Heavy metals tend to bio-accumulate, which means an increase in its concentration in a biological organism over time, compared to its concentration in the environment. These accumulate in living things and are stored faster than they are broken down (metabolized) or excreted. All these trace metals are highly toxic to humans, which include As, Cd, Cr, Hg, Ni, Pb, Sn, etc. Their presence in surface and ground water at a concentration above the permissible limit of 5 mg/L is undesirable. For some heavy metals, such as As, Cd, TCr, Hg, Ni, Pb, the permissible limits are 0.2 mg/L, 2 mg/L, 2 mg/L, 0.01 mg/L, 3 mg/L, 0.1 mg/L, respectively. Table 2.2 presents various environmental impacts posed by AMD from many mining operations, which are categorized as physical, chemical, biological, ecological and socioeconomic effects (Gary, 1997; Jarvis and Younger, 2000).

Table 2. 2 Various environmental impacts posed by AMD (Gary, 1997; Jarvis and Younger, 2000)

Physical	Chemical	Biological	Ecological	Socioeconomic
Reduction of oxygen diffusion	Change of natural mineral cycle operation	Acute and chronic toxicity	Modifications in the food chain	Corrosion and erosion problems
Metal adsorption on sediments	Production of different forms of sulfide	Problems related to reproduction	Modification in the ecosystems	Loss in the aesthetic value of the ecosystem
Increase in the turbidity	Increase in the acidity	Respiratory issues	Reduction in the primary production	Water supply issues
Decrease of light penetration	Metal lixiviation	Toxicity to sensitive species	Bioaccumulation in the food chain	Health problems

Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, change in blood composition, damage to lungs, kidneys, liver and other vital organs. Long-term exposure may result in slowly progressing physical, muscular and neurological degenerative processes which may result in dangerous diseases. Allergies are common and repeated long-term contact with some metals or their compounds may even cause cancer. Natural water bodies, such as rivers, lakes, estuaries, coastal areas, etc., and even groundwater resources are mostly affected by AMD streams. Low pH of the AMD streams directly cause toxic effects to the aquatic environment by increasing the solubility of toxic metals and affecting the natural mineral cycles (Johnson, 2000; Lens et al., 2002). Also, AMD affects ecological balance, biodiversity and the quality of the water used for industrial, recreational and agricultural applications (Jarvis and Younger, 2000).

2.2 Heavy Metal Removal Methods

Since the last few decades, it is becoming clear that conventional methods to treat polluted sites are too expensive. Conventional methods to treat heavy metal contaminated wastewater include chemical precipitation, ion exchange, adsorption, membrane technologies, electrochemical treatment, coagulation-flocculation, evaporation recovery, etc. (Atkinson et al., 1998; Crini, 2006). Figure 2.1 highlights various techniques used for heavy metal removal from wastewater.

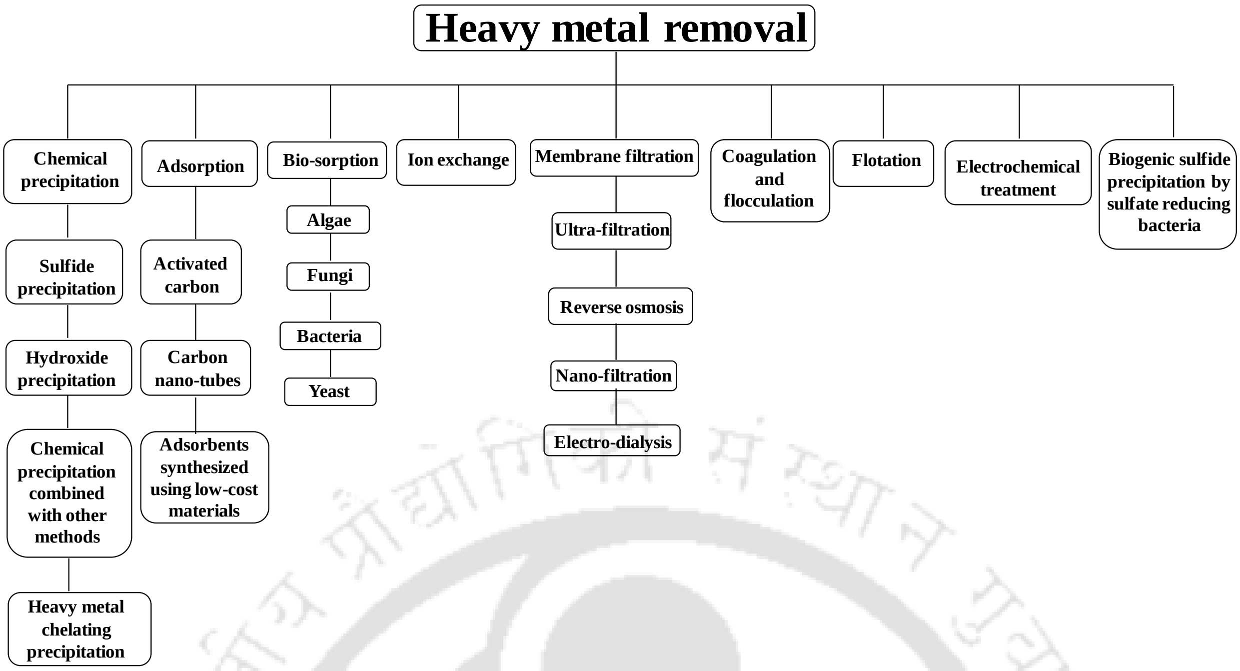


Figure 2. 1 Schematic showing an overview of different techniques used for heavy metal removal from wastewater.

2.2.1 Chemical precipitation

Chemical precipitation is a very effective and extensively used method in industries as it is relatively simple and inexpensive to operate (Ku and Jung, 2001). In this method, chemicals react with heavy metal ions to form insoluble precipitates. The precipitates formed can be separated from the water by sedimentation or filtration and the treated effluent is then decanted and reused or discharged into the environment. Hydroxide and sulfide precipitation are widely used industrially.

2.2.1.1 Hydroxide precipitation

Owing to the process simplicity, low-cost, ease of pH control and low solubility of metal hydroxides in the pH range 8-11, hydroxide precipitation is one of the widely used chemical precipitation technique in most of the industries (Huisman et al., 2006). The metal hydroxides can be removed by flocculation and sedimentation. Among the various agents used for metal precipitation from wastewater, lime is very commonly used at industrial scale owing to its low-cost and ease of handling (Baltpurvins et al., 1997).

Chen et al. (2009) reported that fly ash can be used as a seed material that enhances the efficiency of lime precipitation. It is reported that the fly ash-lime carbonation treatment increased the particle size of the precipitate and considerably enhanced the heavy metal removal efficiency. Coagulants, such as alum, iron salts and organic polymers enhance the heavy metal removal efficiency from wastewater, and chemical coagulation combined with lime precipitation has been used for treating synthetic wastewater containing Zn, Cd, Mn and Mg (Charerntanyarak, 1999).

Despite its wide use, hydroxide precipitation entails certain limitations which include (i) generation of large volumes of relatively low density sludge that can lead to dewatering and disposal problems (Kongsricharoern and Polprasert, 1995), (ii) inefficient removal due to a mixture of metals since the ideal pH for precipitating one metal may bring another metal back into solution and (iii) inhibition of metal hydroxide precipitation due to presence of any complexing agents in wastewater.

2.2.1.2 Sulfide precipitation

Sulfide precipitation is another efficient method for the removal of toxic heavy metals owing to the advantages associated with very low solubility product values of the metal sulfide precipitates over hydroxide precipitates and better thickening and dewatering characteristics than the corresponding metal hydroxide sludge. Hence, high degree of metal removal can be achieved by sulfide precipitation over a broad pH range compared with hydroxide precipitation. However, application of chemical sulfide precipitation for metal removal is limited due to the difficulty in controlling the dosage of sulfide (due to the sensitivity of the process to the dose, especially in chemical sulfide precipitation) and problems associated with the toxic and corrosive nature of excess sulfide (Veeken et al., 2003)

2.2.1.3 Chemical precipitation combined with other methods

A combination of chemical precipitation with the other existing methods has been proven to be successful over the individual methods for treating metal containing wastewater. Gonzalez-Munoz et al. (2006) employed sulfide precipitation followed by nano-filtration for both removal and recovery of heavy metals and reported that sulfide precipitation was efficient in reducing the metal content, whereas nano-filtration process yielded direct use of the treated water.

Ghosh et al. (2009) in an another study used chemical precipitation and electro-Fenton process together to treat rayon industry wastewater for reducing its chemical oxygen demand (COD) and Zn. Approximately, 88% COD was reduced and 99-99.3% zinc removal was attained in the pH range 9-10 using lime precipitation. Papadopoulos et al. (2004) in their study reported that use of ion exchange method showed only 74.8% of Ni removal, whereas a combination of ion-exchange and precipitation processes showed more than 95% of Ni removal.

2.2.1.4 Heavy metal chelating precipitation

Considering the increasing stringent environmental regulations towards the discharge of metal containing wastewater, conventional chemical precipitation methods are inefficient to treat heavy metal containing wastewater particularly that contain complexing agents. As a result, research is focused on using chelating precipitants to remove heavy metals from wastewater systems. Chang et al. (2002) used potassium ethyl xanthate for treating copper containing wastewater and reported that ethyl xanthate was suitable for the treatment of copper containing wastewater over a wide copper concentration range (50-1000 mg/L).

Matlock et al. (2002) in an another study investigated the effectiveness of three widely used commercial heavy metal precipitants, namely trimercaptotriazine, potassium/sodium-thiocarbonate and sodium-dimethyldithiocarbamate and reported a better metal removal efficiency. Xu and Zhang (2006) in their study synthesized a new organic heavy metal chelator, dipropyl dithiophosphate that yielded more than 99.9% heavy metal removal (Pb, Cd, Cu and Hg). Any newly developed or commercially available heavy metal precipitants may either lack the necessary heavy metal binding sites with respect to specific applications or often pose environmental risks. Hence, there is a definite need for new and more effective precipitants for heavy metal removal.

2.2.2 Ion-exchange

High treatment capacity, high removal efficiency and fast kinetics are the main advantages of ion-exchange technique for treating metallic wastewater (Kang et al., 2004). In general, synthetic resins are used in ion-exchange processes as they are very effective in removing heavy metals from wastewater (Alyuz and Veli, 2009). The most common cation exchangers are strongly acidic resins with sulfonic acid groups and weakly acidic resins with carboxylic acid groups. Hydrogen ions in carboxylic or sulfonic group of the resins serve to get exchanged with metal cation in wastewater for their removal. Compared with the expensive and synthetic ion-exchange resins, low-cost and naturally available materials, e.g. zeolites and silicate minerals have been widely used for heavy metal removal from aqueous solution.

It is reported that zeolites exhibit good cation-exchange capacities for heavy metal removal under different experimental conditions (Motsi et al., 2009; Ostroski et al., 2009; Taffarel and Rubio, 2009). Clinoptilolite is one of the most frequently studied natural zeolites that has received considerable attention due to its selectivity for heavy metals; it is reported that the use of amorphous Fe-oxide species can significantly improve the exchange capacity of clinoptilolite (Doula and Dimirkou, 2008; Doula, 2009). Doula (2009) employed clinoptilolite-Fe system for the removal of heavy metals (Cu, Mn and Zn) from drinking water and reported almost complete removal of these heavy metals.

Though the use of zeolites and montmorillonites as ion-exchange resins to remove heavy metals has been well reported in the literature, their application is limited only to lab-scale studies. Despite the popularity of the ion exchange method for the removal of heavy metals from wastewater, drawbacks, such as regeneration of ion-exchange resins following its exhaustion, secondary sludge disposal problem and high capital cost limit its application toward treating large volumes of wastewater containing low concentrations of heavy metals (Fu and Wang, 2011).

2.2.3 Adsorption

Adsorption is a well known method that is recognized as an effective and economical method for treating wastewater containing heavy metals owing to its simple process design and operation, high quality of the treated effluent and regeneration capacity of the adsorbents used. The following are some of the widely used adsorbents for heavy metal removal from wastewater.

2.2.3.1 Activated carbon

Large micropore and mesopore volumes and the resulting high surface area of activated carbon (AC) make this adsorbent extremely useful for the removal of heavy metals from wastewater. Additives of alginate (Park et al., 2007), tannic acid (Ucer et al., 2006), magnesium (Yanagisawa et al., 2010), surfactants (Ahn et al., 2009) and AC composite are very effective for treating metallic wastewater. Converting carbonaceous materials into AC for heavy metal remediation has been reported in the literature. For instance, Kongsuwan et al. (2009) developed and used AC from eucalyptus bark for metal removal from binary component system. Guo et al. (2010) prepared AC from poultry litter and reported a very high adsorption affinity and capacity for heavy metals than commercial AC derived from bituminous coal and coconut shell.

2.2.3.2 Carbon nano-tubes

The discovery of carbon nano-tubes (CNTs) by Iijima (1991) has led to various studies due to its excellent properties and application towards removing heavy metals from wastewater (Kandah and Meunier, 2007; Wang et al., 2007, Kabbashi et al., 2009; Kuo and Lin, 2009; Pillay et al., 2009; Li et al., 2010). It is reported that oxidation of CNTs by HNO_3, NaClO and $KMnO_4$ can significantly increase its sorption capacity.

Wang et al. (2007) in their study reported that acid treated CNTs showed better adsorption capacity for Pb over untreated CNTs. Pillay et al. (2009) investigated the adsorption capacity of functionalized multi-walled carbon nano-tubes (MWCNTs), non-functionalized MWCNTs and AC, and reported that both functionalized and non-functionalized MWCNTs showed a better adsorption capacity for Cr than that of AC. Despite the excellent properties of CNs for heavy metal removal from wastewater, extensive usage of CNTs for wastewater treatment may eventually result in the discharge of metal adsorbed CNTs to the environment which may further pose risk to the living things.

2.2.3.3 Low-cost adsorbents

Considering the high preparation cost of AC, the search for low-cost and easily available adsorbents for treating metal containing wastewater has been intensified. Till date, use of low-cost adsorbents, such as agricultural wastes, industrial by-products and natural products has been well reported for the treatment of wastewater containing heavy metals. Various industrial by-products, such as clino-pyrrhotite (Lu et al., 2006), lignite (Mohan and Chander, 2006), aragonite shells (Kohler et al., 2007), natural zeolites (Apiratikul and Pavasant, 2008a), kaolinite (Gu and Evans, 2008) and peat (Liu et al., 2008a), clay (Al-Jlil and Alsewailem, 2009), lignin (Betancur et al., 2009; Reyes et al., 2009), diatomite (Sheng et al., 2009), etc., have been successfully applied for treating metallic wastewater. However, industrial or large-scale use of these adsorbents is not well reported.

2.2.3.4 Biosorption

Biosorption has been proven to be a very promising method for the removal of heavy metals from aqueous solutions particularly at low concentration due to the use of highly effective and inexpensive bio-sorbents. Various forms of economical and non-living plant materials, such as black gram husk (Saeed et al., 2005), eggshell (Jai et al., 2007), potato peels (Aman et al., 2008), coffee husks (Oliveira et al., 2008), citrus peels (Schiewer and Patil, 2008), seed shells (Amudaa et al., 2009), sawdust (Kaczala et al., 2009), sugar-beet pectin gels (Mata et al., 2009), etc., have been broadly investigated for heavy metal removal. Due to its wide availability, low-cost and high metal sorption capacity, algae, a renewable natural biomass has drawn attention as a biosorbent for removing heavy metals (Apiratikul and Pavasant, 2008b).

Bio-removal of metals from wastewater has been investigated widely using several microbial species that include *Bacillus cereus* (Pan et al., 2007), *Pseudomonas aeruginosa* (Gabr et al., 2008; Tuzen et al., 2008), *Escherichia coli* (Quintelas et al., 2009; Souiri et al., 2009), etc. Fungi and yeasts are among the microbial species that are very easy to grow, yielding high amount of biomass and can be manipulated genetically and morphologically. Various fungal species, such as *Rhizopus arrhizus* (Aksu and Balibek, 2007; Bahadir et al., 2007), *Lentinus edodes* (Bayramoglu and Arica, 2008), *Saccharomyces cerevisiae* (Chen and Wang, 2008; Cojocaru et al., 2009), *Aspergillus niger* (Amini et al., 2009; Tsekova et al., 2010), etc., are reported to be very efficient at heavy metal removal from wastewater.

Maximum metal removal capacities for Pb, Cu and Cd (70.7, 43.7 and 70.8 mg/g, respectively) were reported by Pakshirajan and Swaminathan (2006) in their study using a continuous packed column reactor immobilized with *Phanerochaete chrysosporium*. However, the separation of bio-sorbents from metals would be difficult following the metal loading step (Fu and Wang, 2011).

Adsorption is a well recognized technique for handling wastewater containing low heavy metal concentration, but the application of AC is limited for treating wastewater on large-scale owing to its high cost. Even though several varieties of low-cost adsorbents have been developed and tested for treating heavy metal containing wastewater, metal removal efficiency depends on the type of adsorbent and also the wastewater characteristics. Biosorption which is based on the use of biosorbents has been proven to be a very promising method for the removal of heavy metal from wastewater since the last two decades (Fu and Wang, 2011).

2.2.4 Membrane filtration

Membrane filtration techniques, such as ultrafiltration, reverse osmosis, nano-filtration and electro-dialysis are found to be promising for heavy metal removal owing to their high efficiency, easy operation and very less space requirements.

2.2.4.1 Ultrafiltration

Dissolved and colloidal materials are removed from aqueous solution by ultrafiltration (UF) membrane technique which usually works at a low transmembrane pressure. Micellar enhanced ultra filtration (MEUF) and polymer enhanced ultrafiltration (PEUF) are proposed for handling metallic wastewater since the pore size of the more commonly used UF membranes are larger than dissolved metal ions, which exist in the form of hydrated ions or as low molecular weight complexes.

It is found that PEUF has also been proposed as a viable method for the separation of a wide variety of metals from aqueous solution. Finding a suitable polymer to achieve complexation with metal ions is the main limitation of the PEUF. However, complexing agents, such as humic acid (Kim et al., 2005), diethylaminoethyl cellulose (Trivunac and Stevanovic, 2006), polyethyleneimine (PEI) (Aroua et al., 2007; Molinari et al., 2008), polyacrylic acid (PAA) (Labanda et al., 2009), etc., have been proven to achieve selective separation and recovery of heavy metals with low energy requirements. The advantages of PEUF include high removal efficiency, high binding selectivity and highly concentrated metals for reuse, but its industrial or large-scale application is still not clear.

2.2.4.2 Reverse osmosis

Reverse osmosis (RO) technique normally works on the principle of osmotic pressure and in this process; a semi-permeable membrane allows the fluid that is being purified to pass through it, while retaining the contaminants. It accounts for more than 20% of the world’s desalination capacity (Shahalam et al., 2002). Dialynas and Diamadopoulos (2009) in their study used a pilot-scale membrane bioreactor system in combination with RO and reported very high heavy metal removal efficiencies. Use of RO systems for the removal of heavy metals has been extensively investigated but only at the laboratory scale. Other limitation of the RO systems is the high power consumption due to high pressure requirement for pumping the feed and restoration of the membranes.

2.2.4.3 Nano-filtration

Nano-filtration (NF) process is regarded as an intermediate process between UF and RO and it is well known for its advantages, such as ease of operation, reliability and relatively low energy consumption as well as high efficiency of pollutant removal (Erikson, 1988). Nano-filtration technology has been used for the removal of a wide variety of heavy metal ions from wastewater, such as Ni (Murthy and Chaudhari, 2008), Cr (Muthukrishnan and Guha, 2008), Cu (Cséfalvay et al., 2009; Ahmad and Ooi, 2010) and As (Nguyen et al., 2009; Figoli et al., 2010). A combination of NF and RO process has been reported to be applied for treating metallic wastewater (Liu et al., 2008b; Cséfalvay et al., 2009).

2.2.4.4 Electro-dialysis

Electro-dialysis (ED) is another membrane based method in which an electric field acts as the driving force for the separation of ions from one solution to another across a membrane. In most ED processes, two types of membranes, namely cation-exchange and anion-exchange membranes are used. In addition to its application in the production of drinking and process water from brackish and sea water, this method is also applied for treating industrial effluents and heavy metal containing wastewater, recovery of useful materials from effluents and salt production (Sadrzadeha et al., 2009).

Nataraj et al. (2007) employed an ED pilot plant comprising a set of ion-exchange membranes for the removal of hexavalent chromium ions and reported an excellent performance of the plant. High cost, process complexity, membrane fouling and low permeate flux are some limitations associated with membrane filtration processes which limits its use on a large-scale (Fu and Wang, 2011).

2.2.5 Coagulation and flocculation

Coagulation is defined as destabilization of colloids by neutralizing the forces that keep them apart. Following coagulation and flocculation, sedimentation/filtration is employed to remove heavy metals from wastewater. Many coagulants, such as aluminum, ferrous sulfate and ferric chloride are widely used in conventional wastewater treatment processes resulting in the effective removal of wastewater particulates and impurities by charge neutralization of particles. Samrani et al. (2008) investigated the removal of heavy metals from combined sewer overflow using ferric chloride solution and polyaluminium chloride (PAC) as coagulants. Flocculation aids in binding the particles into large agglomerates or clumps through the action of polymers to form bridges between the flocs. Once suspended particles are flocculated into larger particles, separation from solution is effected by filtration and straining or floatation. Well known flocculants, such as PAC, polyferric sulfate (PFS) and polyacrylamide (PAM) are widely used in the treatment of wastewater; however, the direct use of flocculants for heavy metal removal is difficult to apply at large-scale. Coagulation-flocculation process is in general, inefficient for removing the heavy metals from wastewater (Chang and Wang, 2007) and, therefore, additional treatment is required.

Plattes et al. (2007) employed combined precipitation, coagulation and flocculation processes using ferric chloride to treat industrial wastewater containing tungsten. Bojic et al. (2009) explored spontaneous reduction-coagulation process using micro-alloyed aluminium composite in a laboratory semiflow system to treat synthetic metallic wastewater. Although coagulation-flocculation process entails good sludge settling and dewatering characteristics, huge amount of chemical consumption and increased sludge volume generation are some of its drawbacks for large-scale applications (Fu and Wang, 2011).

2.2.6 Flotation

Flotation is a well known process which has found extensive use in wastewater treatment. Ion flotation, dissolved air flotation (DAF) and precipitation flotation are the main flotation processes utilized for the removal of metals from wastewater. Flotation method has been employed to separate heavy metals from a liquid phase using bubble attachment originating in mineral processing. In a typical DAF process, micro bubbles of air are used to attach to the suspended particles in the water, allowing it to form agglomerates which further aid in the formation of less dense flocs that can be easily removed as sludge (Lundh et al., 2000). Flotation method offers many advantages over several other conventional methods, such as high metal selectivity, high removal efficiency, high overflow rates, low detention periods, low operating cost and production of more concentrated sludge (Rubio et al., 2002), but its use is limited due to high initial capital cost as well as high maintenance and operation costs.

2.2.7 Electrochemical treatment

Electrochemical methods basically involve the plating out of metal ions on a cathode surface and can recover metals in the elemental form. Electro-coagulation (EC) is one such electrochemical method that involves the generation of coagulants in-situ by dissolving either aluminum or iron ions from aluminum or iron electrodes electrically (Chen, 2004). The metal ion generation takes place at the anode and hydrogen gas is released from the cathode. The hydrogen gas helps in separating the flocculated particles from solution by making them easily float (Chen, 2004).

Electro-flotation (EF) is another electrochemical method in which solid/liquid separation occurs and pollutants float to the liquid surface due to the generation of hydrogen and oxygen gases as tiny bubbles by water electrolysis. Electro-flotation has a wide range of applications in heavy metal removal from industrial wastewater. Electro-deposition is another method which is regarded as a clean technology for the recovery of metals from wastewater with no secondary sludge generation (Issabayeva et al., 2006).

Electrochemical treatment techniques are widely applied for the treatment of heavy metal containing wastewater due to its several advantages, such as rapid separation of metals, easy process control, requirement of few chemicals, good reduction yields and low sludge generation. But its use is restricted due to high initial capital investment and the need to supply electricity, which is expensive. Thus, it is clear from the afore-mentioned techniques that it is very essential to consider various factors, including the initial metal concentration, wastewater composition, capital investment and operational cost, plant flexibility and reliability, and environmental impact for handling heavy metal containing wastewater (Kurniawan et al., 2006).

2.3 Sulfate

Sulfur is one of the most abundant elements available on Earth. Generally, sulfate (SO_4^{2-}) is the final oxidation product, which gets accumulated in minerals and in the ocean. Different sources are known to emit sulfur directly into the atmosphere. These sources include natural, such as volcanic eruptions and evaporation of water and anthropogenic sources; for example, burning of fuels releases large quantities of sulfur dioxide into the environment, contributing extensively to air pollution and even leads to acid rain ultimately affecting the aesthetic value of the environment (Lloyd, 2006).

2.3.1 Effects of sulfate

High levels of sulfate in mine tailings from metal bearing ores and some coal ores are readily oxidized by water and oxygen, resulting in acid drainage disturbing the ecological balance (Masigol et al., 2012). Surface and ground water sources get polluted due to the release of excessive quantities of sulfate, thereby posing threat to various life forms. Hence, it is necessary to treat sulfate containing wastewater prior to its discharge into the environment by maintaining its level within the permissible limits (Moon et al., 2013). The functioning of the natural sulfur cycle would be effected due to the release of excessive sulfate and sulfide formed due to sulfate reduction in the aquatic environment.

Presence of sulfate ions increases the conductivity and corrosion potential of receiving water bodies and may contribute to the mineralization of water (Silva et al., 2010). In addition, excess sulfate in the water promote corrosion and scaling in pipes, fouling and deposition in boilers and acidification of soils and blockage of soil pores, hindering irrigation or water drainage (Bowell, 2000). Sulfate in its reduced form, e.g. hydrogen sulfide (H_2S), may result in toxicity, odor and corrosion problems (Sawyer et al., 2009). Hydrogen sulfide is highly toxic to humans, causing death within 30 minutes at gaseous concentrations of only 800-1000 mg/L, and instant death at higher concentrations (Speece, 1983).

2.4 Sulfate Removal Methods

Several physicochemical and biological methods are available for the treatment of sulfate containing wastewater, and these methods are widely applied on large-scale. Physicochemical methods are broadly classified into two types, which include membrane and filtration techniques and chemical precipitation. However, biological treatment is preferred over these physicochemical methods due to certain limitations, such as separation and disposal of the sludge generated and relatively high cost and energy consumption (Silva et al., 2002).

Chemical precipitation

Gypsum (lime or limestone), barium sulfate and ettringite are commonly used for the removal of sulfate by precipitation. Sulfate removal using lime is very limited due to the production of highly soluble $CaSO_4$ (Rubio et al., 2009). Even though sulfate removal by barium precipitation is highly efficient, high cost of barium compounds and toxicity of any residual barium ions limits its use on large-scale. Requirement of alkaline pH for sulfate removal is the main drawback associated with the use of ettringite or barium sulfate precipitation method. Generation of high volumes of sludge is another common problem associated with the use of all these chemical precipitating agents.

Membrane separation

Membrane technologies, such as ED and RO are known to be applied for the treatment of sulfate containing wastewater (Silva et al., 2012). However, use of both these processes on large-scale is not reported mainly due to the requirement of a pre-treatment step to prevent fouling and microbial growth, and the cost required for treating a large amount of water.

Biological sulfate removal

Biological sulfate removal is regarded as one of the most cost effective alternative over the expensive physicochemical removal methods for treating high sulfate rich wastewater (Maree et al., 1991). Bacterial sulfate reduction is considered a vital bioprocess for eliminating both sulfate and metals from AMD by means of sulfate reducing bacteria (SRB) (Tuttle et al., 1969; Wakao et al., 1979; Herlihy and Mills, 1985; Hedin et al., 1989). Due to the natural occurrence of the SRB, sulfate reduction techniques can be utilized in-situ for the treatment of AMD provided with the conditions that enhance the bacterial activity. The mechanism of sulfate removal is based on the capability of SRB to oxidize organic substrates using sulfate as the terminal electron acceptor in anaerobic respiration (Barton and Tomei, 1995). Thus, sulfide generated aids in precipitation of heavy metals as metal sulfides (Cabrera et al., 2006).

2.5 Heavy Metal Removal by Biological Sulfate Reduction

In view of the existing conventional methods, microorganisms are considered as important mediators in determining the form and distribution of metals in the environment (Sterritt and Lester, 1980). They play a major role in the modification, activation and detoxification of heavy metals. Although metals cannot be degraded, they may, as a result of biological action, undergo changes in valency (less toxic form) or converted into organo-metallic compounds (Gadd, 1992).

Several biological processes have been well demonstrated to eliminate metals from wastewater that include biosorption, intracellular uptake and accumulation, complexation, oxidation and reduction, methylation combined with volatilization, and extracellular precipitation (Gadd, 1992; White, 1995; Mallick, 2002). Also, a number of biological processes are proven to be potential to generate alkalinity or consume acidity and, therefore, have potential use in neutralizing metallic wastewater (Johnson, 2000).

The metal binding capacities of various microorganisms and several biomass sources have shown a very good potential to remove hazardous metals from wastewater (Darnall et al., 1986; Lembi and Waaland, 1988; Volesky, 1990). Biological sulfate removal can be used simultaneously to treat industrial effluents and achieve neutralization, and eliminate sulfate and metals from wastewater. Among the novel approaches developed for the removal of metals from wastewater, technologies based on biological methods for metal removal and/or recovery have emerged as cost effective substitutes (Popuri et al., 2007). Biological sulfate reduction is increasingly replacing chemical unit processes in mining biotechnology. Sulfate reducing bacteria can be used for treating ground and surface water contaminated with metal containing wastewater and for recovering metals from wastewater and process streams.

The use of various low-cost substrates together with newer bioprocess designs have evaluated the potential application of SRB based bioreactors in handling metallic wastewater and for selective metal recovery (Kaksonen and Puhakka, 2007). Biological systems exploit hydrogen sulfide production by SRB in order to precipitate metals as sulfides (Johnson and Hallberg, 2005), whereas biogenic bicarbonate alkalinity neutralizes acidic water.

2.6 Sulfate Reducing Bacteria

Sulfate reducing bacteria are obligate anaerobes and members of a heterogeneous group of eubacteria and archaebacteria which are characterized by their use of sulfate as a terminal electron acceptor during anaerobic respiration (Hansen, 1994; Akagi 1995; Colleran et al., 1995; Cypionka, 1995; Hamilton, 1998). SRB form a group of sulfate reducing prokaryotes and the main genus is *Desulfovibrio*. *Desulfovibrio desulfuricans* is often used to immobilize dissolved heavy metals as metallic sulfides.

Although many bacteria can produce sulfide, only a few are capable of producing sulfide at a rate that is sufficient for large-scale applications. These rapid sulfide generating bacteria are able to conserve energy by the reduction of sulfate (Widdel and Hansen, 1992), and they are generally termed as SRB. Sulfate is activated by means of adenosine triphosphate (ATP). The enzyme ATP sulfurylase catalyzes the sulfate to phosphate of ATP, leading to the formation of adenosine 5'-phosphosulfate (APS) as shown in Fig. 2.2 (Madigan et al., 2003). Even though ATP gets hydrolyzed, end products formed are to be removed for the completion of the reaction since the reaction is energy requiring. Pyrophosphate (PPi) can be hydrolyzed to phosphate by pyrophosphate (Cypionka, 1995).

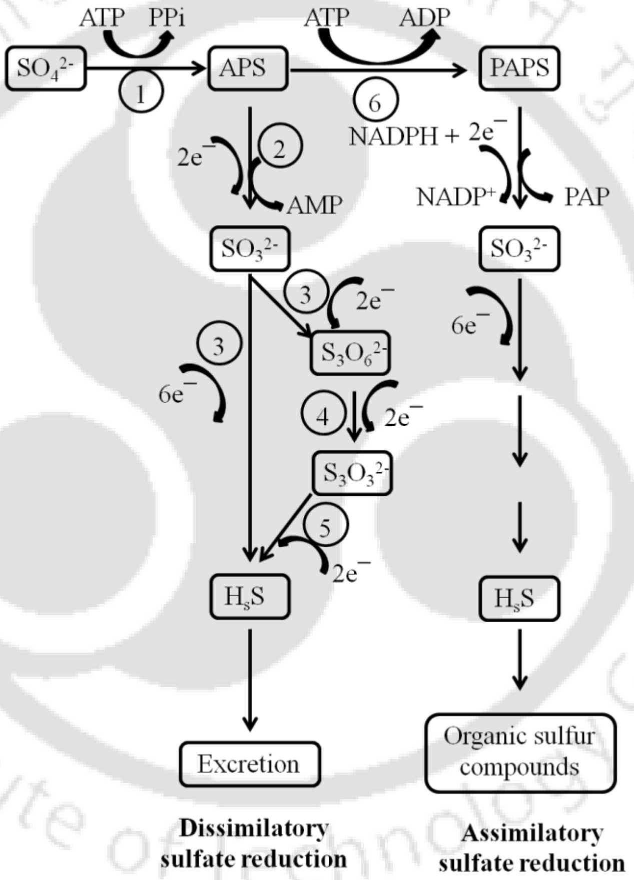


Figure 2. 2 Dissimilative and assimilative patways of sulfate reduction. The enzymes catalyzing the reactions include (1) ATP sulfurlyase, (2) APS reductase, (3) sulfite reductase, (4) trithionate reductase, (5) thiosulfate reductase, and (6) APS kinase (Akagi, 1995; Madigan et al., 2003).

The sulfate moiety of APS is reduced directly to sulfite (SO_3^{2-}) by the enzyme APS reductase with the release of adenosine monophosphate (AMP) in dissimilative sulfate reduction. Whereas in assimilative reduction, another phosphate group is added to APS to form phosphoadenosine 5'-phosphosulfate (PAPS), where sulfate moiety gets reduced to sulfite with the release of phosphoadenosine 5'-phosphte (PAP) (Madigan et al., 2003). Two hypotheses are proposed for the reduction of sulfite to different forms of sulfide; one hypothesis is that a direct six electron reduction of sulfite to sulfide ocurs with the aid of the enzyme, sulfite reductase without the formation of any ioslable intermediate compounds (Akagi, 1995).

In the second hypothesis, two intermediates, trithionate and thiosulfate are formed in which trithionate pathway involves a recycling mechanism of sulfite that is released during the reduction of trithionate to thiosulfate by the trithionate reductase, and the reduction of thiosulfate to sulfide by the thiosulfate reductase (Akagi, 1995). Hydrogen sulfide is excreted into the environment in the case of dissimilative sulfate reduction, whereas in case of the assimilative reduction, H_2S formed is immediately converted into organic sulfur compounds, such as amino acids (Madigan et al., 2003)

The SRB are broadly categorized into two types depending on their oxidative capability: the genera in the first group (*Desulfovibrio, Desulfomonas, Desulfotomaculum* and *Desulfobulbus*) consume lactate, pyruvate, ethanol and certain fatty acids as carbon source but are not capable of oxidizing acetate to carbon dioxide (CO_2). The genera in the second group (*Desulfobacter*, *Desulfococcus*, *Desulfosarcina* and *Desulfonema*) are specialized in the oxiding short chain fatty acids, particularly acetate. SRB are capable of surviving in a wide range of pH conditions, more particularly their growth is optimum between pH 5 and 9 (Postgate, 1984). Sulfate reducing bacteria populations have been found to occur at temperatures ranging from the psychrophilic to the hyper thermophilic range (Kolmert, 1999).

2.6.1 Classification of SRB

The SRB represents a group of chemoorganotrophic and strictly anaerobic bacteria, which include representatives of the genera *Desulfovibrio*, *Desulfomicrobium*, *Desulfobacter*, *Desulfosarcina*, *Desulfotomaculum*, *Thermodesulfobacterium*, etc. (Odom and Singleton, 1993). Generally, SRB are classified into different groups depending on their applications as detailed further:

Extremophilic SRB: Among the diversity of sulfate reducing prokaryotes, the acidophilic, thermophilic and psychrotolerant bacteria are extremophiles that could improve the performance of existing treatment systems.

Acidophilic SRB: Oxygen normally enters the deep geological environments during mining activities and aids in chemical and biological oxidation processes. Hydrogen ions and sulfate are produced which lower the pH significantly in the range of 2-3 (Kolmert, 1999; Madigan et al., 2000). At present, biological AMD treatment uses mostly the neutrophilic SRB that are highly sensitive to acidic conditions (Jong and Parry, 2006). In these type of systems, treatment is carried out in two stages; first the SRB grow in separate tanks where hydrogen sulfide is produced and the sulfide is transferred to a second reactor containing the metal contaminated water which results in precipitation of metal sulfides. Acidophilic or acid-tolerant bacteria are capable of growing in acidic environment, and the use of these type of extremophiles will simplify the system and keep the process economics low (Kolmert et al., 2001; Kimura et al., 2006).

Thermophilic SRB: Some industries typically discharge wastewater at high temperatures of 50 to 70 ºC and even above 90 ºC. The thermophilic SRB are capable of sustaining particularly at high temperatures and these can be directly applied for treating wastewater at high temperatures. It eliminates cooling of the process water and allows direct use of the treated water without the need of any additional reheating. These systems normally produce less sludge and are capable of treating high organic loading rates with high removal efficiency (Vallero, 2003; Pender et al., 2004).

Cold-adapted SRB: Treatment of industrial wastewater in countries with cold environments is found to be different from other countries and it is recognized that psychrophilic SRB generally have an optimum growth temperature of 18 ºC, whereas the optimum temperature for sulfate reduction is 28-30 ºC. However, bacteria reducing sulfate below 4 °C have been identified. A low reaction rate of the sulfate reduction process at low temperatures could be compensated by an increased number of bacteria (Knoblauch et al., 1999; Sahm et al., 1999).

2.6.2 Sulfate removal mechanism and its significance to heavy metal removal from wastewater

Generally, SRB are capable of converting sulfate or sulfite (SO_3^{2-}) to different forms of sulfide ($S^{2-}/HS^-/H_2S$) by using electron donating substrates in the form of COD already present in wastewater or by using low-cost externally added substrates. In this process, sulfate acts as an electron acceptor to support anaerobic respiration as shown in the equation

2.1. The substrates are either partially oxidized (e.g. to acetate) or fully oxidized to CO_2 based on the type of bacteria.

$$SO_4^{2-} + COD \rightarrow S^{2-}/HS^{-}/H_2S + CO_2 \qquad 2.1$$

Factors, such as availability of growth nutrients, age and physiological state of bacterial cells, environmental conditions (pH, ionic strength and temperature), presence of competitive ions and concentration of the biomass can influence the sulfate removal mechanism. Once sulfate-reducing conditions are established, sulfide precipitation becomes the predominant mechanism of metal removal from AMD or metallic wastewater (Machemer and Wildeman, 1992; Bechard et al., 1994; Kaksonen and Puhakka, 2007).

Biogenic sulfide generated by SRB can form highly insoluble metal precipitates. Thus, the sulfides can precipitate soluble heavy metals in wastewater streams or polluted groundwater to insoluble sulfide precipitates, thereby decreasing the bioavailability of toxic metals (Mizuno et al., 1994), as represented in the equation 2.2. Since the metals ions are highly concentrated in the precipitate as insoluble form, the resulting metal sulfide precipitates can be removed and recycled back to industry for reuse. Extensive care should be taken in handling the hydrogen sulfide produced and separate provision should be provided for the recovery of metals from the insoluble precipitates formed.

$$S^{2-} + M^{n+} \rightarrow MS\downarrow \qquad 2.2$$

where, M is metal, n is the valency and MS is metal sulfide precipitation.

In view of existing methods for the removal of heavy metals and sulfate from wastewater, biological processes exhibit some key advantages over the conventional treatment methods, such as (i) metal specific, (ii) efficient in terms of very low residual metal concentration compared to other common physico-chemical processes, (iii) less intensive in terms of energy and materials consumption, and (iv) very low production of secondary sludge.

The key advantages of the use of SRB in biological heavy metal removal by sulfate reduction can be summarized as follows:

1. Overall low treatment costs together with a very high treatment efficiency
2. Reduction or elimination of costs associated with metal sludge disposal

3. The elimination of wastewater treatment sludge avoids the geotechnical costs of pond construction and the costs associated with providing valuable land to sludge ponds (Bratty et al., 2006).

Although the potential use of microbial sulfate reduction for treating heavy metal loaded wastewater has been reported as early as 1969, development of SRB based on passive and active treatment system is quite recent.

2.7 SRB Based Treatment Systems

Bioreactors are classified based on the retaining capacity of the microorganisms that accomplish desired chemical conversions (Lens et al., 2002). Figure 2.3 lists the active and passive treatment systems along with different reactor types applied for treating heavy metal containing wastewater.

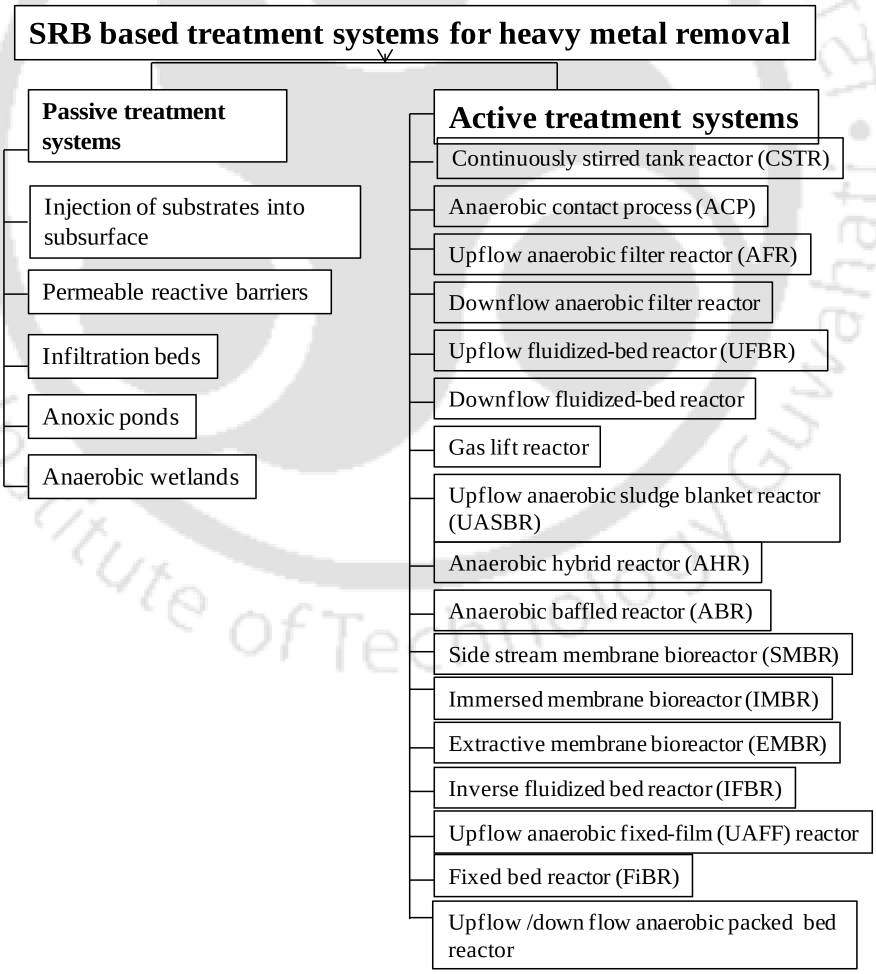


Figure 2. 3 An overview of different SRB based treatment systems for treating heavy metal containing wastewater (Johnson and Halberg, 2005; Kaksonen and Puhakka, 2007).

2.7.1 Passive biological treatment systems

2.7.1.1 Anoxic ponds

Anoxic ponds are the water bodies appended with organic substrates. Mining wastewater has been treated with SRB employing an open pit as a large-scale basin (Riekkola-Vanhanen and Mustikkamaki, 1997). Liquid manure was used as a source of SRB and press juice from silage was added as an electron donor. Riekkola-Vanhanen and Mustikkamaki (1997) reported that there was an increase in the water pH with the prevalence of sulfate reducing activity, which as well resulted in the decrease of sulfate, Zn, Fe and Mn concentrations and redox potential. The drawbacks of anoxic ponds are: (a) requirement of a large land area for treatment, (b) requirement of proper sludge removal and treatment and (c) unsuitable for cold weather conditions (Varon and Mara, 2004). Figure 2.4 shows various SRB based passive treatment systems used for heavy metal removal.

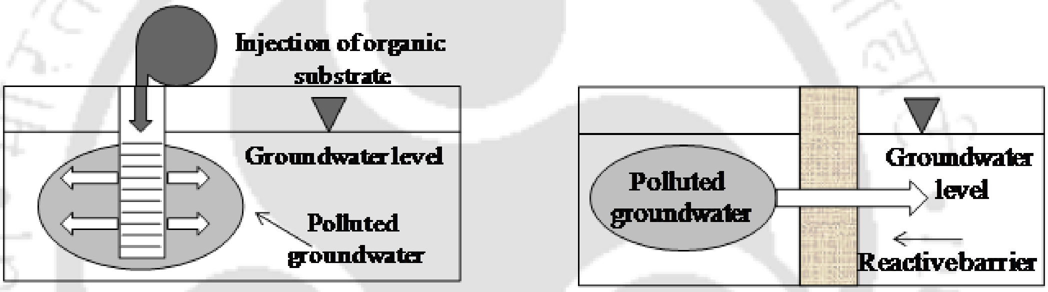
2.7.1.2 Wetland systems

Utilization of constructed wetland systems for feasible wastewater treatment is perhaps a significant progress towards an environmental friendly and sustainable technology (Bailey, 1976). For several years, wetlands have served as economical designs to enhance the treated AMD characteristics (Huntsman et al., 1978; Wieder and Lang, 1982). Also, these designs have already conceded lab-scale (Gazea et al., 1996) and employed for the elimination of metals, radionuclides and sulfate from mining wastewater on large-scale (Noller et al., 1994). Wetland systems are classified as anaerobic and aerobic; anaerobic system uses SRB for treating wastewater. The mechanisms through which heavy metal bearing wastewater are treated in wetland systems include a wide range of techniques, such as adsorption, filtration, sedimentation, uptake by plant biomass and precipitation of metals by geochemical processes (Stottmeister et al., 2003). Wetland treatment systems encounter certain problems, such as that being ineffective in arid and semiarid climatic conditions and metal dissolution upon exposure of metal sulfides to oxygen during the drought periods.

In a study using constructed wetland, Sheoran and Sheoran (2006) reported 75-99% removal of Cd, 76% of Ag, 67% of Zn and 26% of Pb. Romero et al. (2011) reported almost 100% removal of heavy metals using a constructed wetland. Using marsh constructed wetlands, Hafeznezami et al. (2012) obtained heavy metal removal efficiencies of 17.6% of

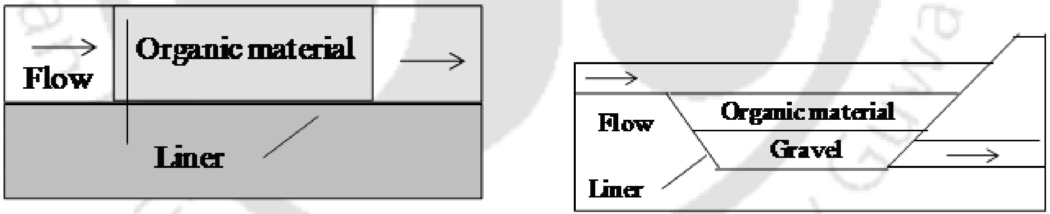
Zn, 23.9% of Cd and 10.6% of Cu. Metal removal was associated with their accumulation in the lateral roots, rhizomes with roots and shoots with the maximum content accumulated in the leaves, whereas shoots accumulated the least amount of metals (Zachritz et al., 2006).

Among the different passive treatment systems, constructed wetlands and compost bioreactors have so far been employed for full-scale wastewater treatment (Johnson and Hallberg, 2005). In a study using a laboratory scale vertical subsurface flow constructed wetland system, 97.2-98.3% of Cr(VI) removal was obtained at a 2 day hydraulic retention time (HRT) with an influent pH of 7. The highest accumulated Cr concentration (464.33 mg/kg) was found in plants grown in the system at pH 7.0, whereas the minimum accumulated Cr concentration (249.21 mg Cr/kg) was in plants grown in the system at pH 4.0 (Sinha et al., 2017).



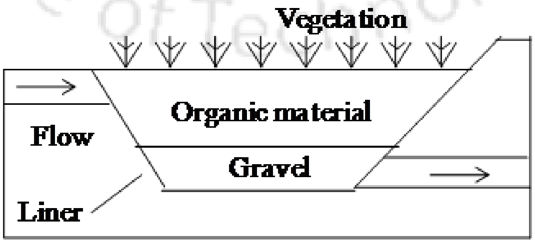
Injection of substrates into the subsurface system

Permeable reactive barrier system



Infiltration bed system

Anoxic pond system



Anaerobic wetland system

Figure 2. 4 Various SRB based passive treatment systems used for heavy metal treatment (Robb and Robinson, 1995; Gazea et al., 1996; Waybrant et al., 1998; Canty, 1999; Vestola, 2004; Kaksonen and Puhakka, 2007).

2.7.1.3 Substrate injection into subsurface

This method has been tested to treat groundwater polluted by AMD through the enhancement of SRB activity for sulfate reduction by injecting or placing substrates into the subsurface by boreholes (Groudev et al., 1998; Canty, 1999) or using permeable reactive barrier systems put up against the source of ground water flow (Herbert et al., 1998; Waybrant et al., 1998; Benner et al., 1999; Amos et al., 2003). Canty (1999), in their study with mining wastewater flowing through the organic substrate placed in mine shafts, reported high removal efficiencies of aluminium (Al), Cd and Zn along with a raise in the pH. However, as a consequence of high stream flow rates in the spring period and surface water, reduction in pH and re-dissolution of the metal precipitates occurred (Canty, 1999). In these types of systems, every attempt should be made to cut down the potential for leaks, spill outs and any other form of unwanted discharge of substrates into the ground surface (Canty, 1999).

2.7.1.4 Infiltration beds

Infiltration bed structures are employed for the treatment of surface water contaminated with AMD in the similar way as reactive barrier systems are used for treating ground water (Vestola, 2004). Infiltration bed systems can be constructed as channels of mining area zones, as an effect of which the contaminated water run over the bed material. The bed comprises of organic substrates that sustain SRB growth and activity. Organic compounds can also be supplemented with nutrients and SRB to increase the effectiveness of the infiltration bed. Using an infiltration bed system, Riekkola-Vanhanen (1999) reported that metal removal efficiency of water flowing through the bed followed the order: Cu (94-99%) > Zn (76-97%) > Fe (85-96%) > Mn (76-96 %) and sulfate removal was 72-94%. Vestola (2004) examined AMD contaminated surface water with Cu, Zn, Fe and Mn using infiltration beds and reported 94-99% of Cu, 76-97% of Zn, 85-96% of Fe and 76-96% of Mn removal, respectively. Subsurface infiltration beds are not recommended when there is a significant risk of basement seepage or flooding. The infiltration bed should be constructed in such a way that it should avoid the re-dissolution of previously precipitated metal (Vestola, 2004).

2.7.1.5 Permeable reactive barrier systems

Permeable reactive barrier (PRB) systems comprise of regions of reactive substance positioned transversely to the flow of polluted groundwater (Richardson et al., 2002). Once the ground water contaminated with AMD enters this region, SRB uses electron donor present in the barrier for sulfate reduction and produce sulfide. As a result, the metals precipitate as sulfides. The choice of the reactive mixture influences the permeability and reactive nature of the barrier (Waybrant et al., 1998; Amos et al., 2003). The main concern with the reactive barriers is that they often depend on the natural groundwater flow (hydro-geological conditions) for the transport of pollutants to the treatment region, which leads to prolonged treatment periods. As a result of substrate depletion and blockage of the barrier owing to metal precipitation, the longevity of the system may get affected, which is another drawback of reactive barrier systems (Richardson et al., 2002).

2.7.1.6 Other passive treatment systems

The very early passive bioreactors used animal manure and mushroom compost as substrates as they provide significant alkalinity (Dvorak et al., 1992; URS Report 2003). Later, a combination of sawdust, limestone and alfalfa was used to provide electron donor, alkalinity, high hydraulic conductivity; these substances also served as a good energy source for the bacterial community (URS Report 2003).

Wood Cadillac biofilter and Cadillac molybdenite passive bioreactor are two field scale application of passive treatment systems which were successfully installed and operated on mine sites of Northern Quebec, Canada. Cadillac biofilter (50 m × 57 m area and 1m depth) was constructed during 1999-2000 with yellow birch barks serving as the substrate and operated at 25 h HRT (Isabel et al., 2000; Germain and Cyr, 2003). The reactor removed 90-95% As and sulfate to less than 250 mg/L at a pH in the range 5.5-6.5 (Germain and Cyr, 2003). Cadillac molybdenite passive bioreactor was operated with 5-day HRT using wood chips, limestone, hay and manure as substrates (Kuyucak et al., 2006). The reactor showed maximum removal of all the metals (Cu, Al, Ni, Fe and Zn) and less than 360 mg/L of sulfate. However, Mn removal was less using this reactor system.

Two anaerobic reactors (1930 m^3 each) were built in USA using a combination of composted cow manure, saw dust, inert limestone and alfalfa as substrates for treating mildly contaminated AMD from a lead-zinc mine area (Gusek et al., 1999). The reactors were able to precipitate about 87% Pb(II) and 75% Zn(II), respectively. A pilot scale cylindrical reactor was set up in 1997 for treating copper containing acid mine wastewater using a mixture of sawdust, cattle manure, limestone, hay and alfalfa (Reinsinger et al., 2000). The reactor showed more than 99% Cu(II) removal for two years even at very low temperatures.

2.7.2 Active bioreactor systems

The sulfidogenic bioreactors signify a very different approach for treating wastewater contaminated with heavy metals (Boonstra et al., 1999; Johnson, 2000). Treatment of heavy metal contaminated wastewater using anaerobic sulfate reduction process depends on a wide range of factors, such as sulfate concentration, heavy metal species and their concentration, pH, temperature and carbon source/substrate/electron donor.

The potential advantages of active biological treatment systems are selective recovery of heavy metals from wastewater, predictable and readily controllable performance and significantly lowered sulfate concentrations. Hence, the application of sulfidogenic bioreactor systems for the elimination of heavy metals is widely applied on large-scale compared with the passive treatment systems. On the downside, the construction, operation and maintenance costs of these systems are substantial.

Different designs of sulfidogenic reactors employed for biological sulfate reduction and metal removal have been extensively described in the literature as outlined in Fig. 2.5. Table 2.3 summarizes the various sulfidogenic reactors developed for treating sulfate and metal containing wastewaters along with their target wastewater, inoculum used, carbon source, HRT and sulfate and metal removal efficiencies. All these sulfidogenic reactors have been employed for metal removal and recovery from a lab to field scale. The merits and demerits of these different sulfidogenic bioreactors are presented in Table 2.4. Some of the salient features of these bioreactors are discussed in the following section.

2.7.2.1 Upflow anaerobic sludge blanket (UASB) reactor

In terms of biomass retention, upflow anaerobic sludge blanket (UASB) reactors are the best as the biomass is present in the form of granular sludge with excellent settling characteristics (Lettinga et al., 1980; Speece, 1983). These UASB reactors do not have flow channeling, clogging and sludge problems; these type of reactors also do not use any biomass support.

Various UASB reactor types with different operating conditions and target wastewater treated are presented in Table 2.3. Due to these advantages, high treatment rates can be achieved using UASB reactors. Besides, they also entail the following advantages: (i) low land requirements, (ii) minimum odor problems, (iii) mechanical simplicity, (iv) high sludge quality, (v) simple operation and process control (vi) low maintenance and, low investment and operational costs.

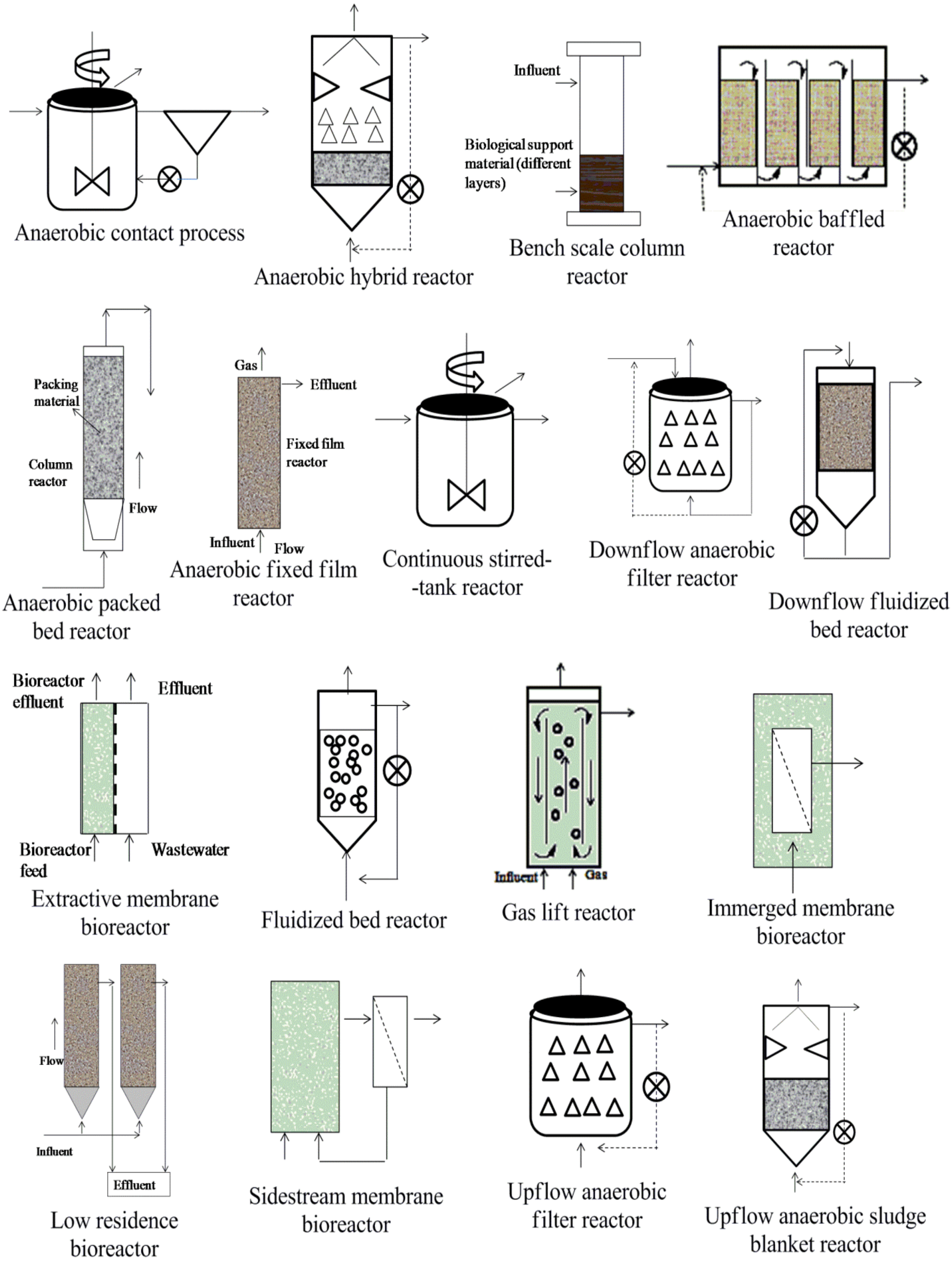
The following are, however, the main drawbacks of UASB reactors: economically not viable in colder regions, sensitive towards toxic substances, high skill labor requirement, delayed startup period to achieve steady state operation, particularly when sufficient activated sludge is unavailable (Naturgerechte Technologien and Bau-und Wirtschaftsberatung, 2001) (Table 2.4) (Speece, 1983; Barnes et al., 1991a).

2.7.2.2 Fluidized bed reactor (FBR)

Fluidization of the inert biomass carrier with re-circulated liquid avoids channeling and clogging (Somlev and Tishkov, 1992) and the fluidized carrier material results in a greater surface area for the biofilm formation (Speece, 1983). Compared to AFR, fluidized bed reactor (FBR) offers better sulfate reduction rates and higher carrier surface area (Somlev and Tishkov, 1992). In FBR, the recycle flow dilutes high influent concentrations due to which FBRs are well suited for treating acidic wastewater containing metals and sulfate (Marin et al., 1999; Kaksonen et al., 2003a; Sahinkaya et al., 2007).

In downflow fluidized bed reactors (DFBR), well known as inverse fluidized bed reactor (IFBR), carrier material which is light in weight is fluidized downward with a downflow of liquid. Foamed plastic materials are commonly used as the bio-support materials in DFBRs (Garcia-Calderon et al., 1998). Owing to the recovery of metals as metal sulfides, DFBR is found to be promising improvement in view of FBRs (Celis-Garcia et al., 2007).

Using DFBR with ethanol as the carbon source, Garcia et al. (2009) examined the treatment of synthetic acidic wastewater containing Fe, Zn, Cd and reported more than 99% metal removal. So far, several types of FBRs have been used widely on large-scale for treating heavy metal contaminated wastewater, as detailed in Table 2.3. High energy required for fluidization of carrier material and chances of biomass loss due to shear forces are some of the demerits of FBR (Table 2.4) (Melin et al., 1997; Melin et al., 1998; Marin et al., 1999).



Anaerobic contact process
Anaerobic hybrid reactor
Bench scale column reactor
Anaerobic baffled reactor
Anaerobic packed bed reactor
Anaerobic fixed film reactor
Continuous stirred--tank reactor
Downflow anaerobic filter reactor
Downflow fluidized bed reactor
Extractive membrane bioreactor
Fluidized bed reactor
Gas lift reactor
Immerged membrane bioreactor
Low residence bioreactor
Sidestream membrane bioreactor
Upflow anaerobic filter reactor
Upflow anaerobic sludge blanket reactor

Figure 2. 5 Various SRB based active treatment systems used for heavy metal treatment (Speece, 1983; Christensen et al., 1996; Jung et al., 1997; Garcia-Calderon et al., 1998; Chuichulcherm et al., 2001; Weijma et al., 2002; Jong and Parry, 2003; Mack et al., 2004; Celis-García et al., 2007; Kaksonen and Puhakka, 2007; Cao et al., 2009; Mayes et al., 2011).

2.7.2.3 Anaerobic filter reactor (AFR)

Anaerobic filter reactors (AFRs) are operated under horizontal upflow or downflow modes (Hammack et al., 1992). Table 2.3 presents the literature on various types of AFRs employed for treating heavy metal containing wastewater under different operating conditions. In AFRs, biomass retains on the packing material as biofilm and also unattached in the space between the packing materials (Speece, 1983). These AFRs offer several advantages which include low shear forces, prolonged sludge retention time and the utilization of gravitational force under downflow mode (Speece, 1983; Anderson et al., 1990; Barnes et al., 1991a). The main drawbacks of AFRs include flow channeling and clogging of the reactor channels by the precipitates formed (Table 2.4).

2.7.2.4 Anaerobic hybrid reactor (AHR)

In general, a unification of UASB and AFR represents anaerobic hybrid reactor (AHR), in which the filling substance is on the upper segment and granular sludge bed is in the lower segment of the reactor (Colleran et al., 1998; Steed et al., 2000). Steed et al. (2000) evaluated the viability of the AHR, AFR and UASB for the elimination of metals from AMD. The AHR showed better removal efficiency of metal and sludge concentration (Steed et al., 2000). Though, high metal removal rates were achieved with the UASB reactor, it did not serve to improve the clarity of the treated effluent, and, therefore, the level of suspended solids high in the effluent (Steed et al., 2000). Compared with AFR, sludge removal is easier and problem due to clogging is minimum in AHR (Steed et al., 2000) (Table 2.4). Steed et al. (2000) reported more than 95% removal of Fe, Mn, Zn and Cu using AHR with recycle and acetate as the sole carbon source.

2.7.2.5 Continuously stirred tank reactor (CSTR)

In continuous flow reactors, the reactor configuration usually influences sludge retention time (SRT)/HRT (Speece, 1983). The process loading rates are mainly governed by the biomass retention rate in any reactor (Lettinga et al., 1980). Maximum possible biomass or sludge retaining capacity is advantageous for the stability of system and minimum sludge production. As such, small HRTs help reduce the reactor volume requirement, and hence minimize the capital cost (Speece, 1983) (Table 2.4). In most of the process, washout of active biomass is a major concern with CSTR (Speece, 1983).

2.7.2.6 Anaerobic baffled reactor (ABR)

Anaerobic baffled reactor (ABR) symbolize UASB reactor with a little modification in which retaining capacity of the biomass is improved by allowing the water to different sections (Barber and Stuckey, 2000). The two reactors (AHRs and ABRs) have been employed for the treatment of sulfate and heavy metal containing wastewater; however, their ability to eliminate metals, sulfate and acidity simultaneously has not been examined in detail (Colleran et al., 1998; Barber and Stuckey, 2000; Steed et al., 2000). Table 2.3 presents the various operating conditions used for treating heavy metal containing wastewater using ABR. Advantages of ABR include a) extended sludge retention time, b) no need of biomass carrier and c) good handling of hydraulic shock loading and organic loading rates (Speece, 1983; Barber and Stuckey, 2000) (Table 2.4).

2.7.2.7 Anaerobic contact process (ACP)

Biomass retention can be improved by providing in situ placed sedimentation systems or by using flocculating agents (White and Gadd, 1997). Anaerobic contact process (ACP) systems enable the separation of biomass from the effluent and recirculate it to the reactor which enhances the HRT/SRT (Speece, 1983). Numerous techniques have been applied for the biomass recovery, such as flocculation, centrifugation, sedimentation (Vegt and Buisman, 1996) and magnetic separation (Watson et al., 1996; Bahaj et al., 1998). ACP results in better biomass retention in contrast to CSTRs; however, it suffers from the breakdown of flocs and sludge (Speece, 1983) (Table 2.4).

2.7.2.8 Anaerobic packed bed reactor and column bioreactors

Several immobilized biomass reactors have been developed for treating wastewater on a large-scale based on the slow growth rate of SRB (Kaksonen et al., 2003a). Anaerobic fixed bed or packed bed bioreactors have been proved to be viable and efficient in the remediation of wastewater (Foucher et al., 2001; Kolmert and Johnson, 2001). However, their long term performance is affected due to clogging and channeling problems (Kolmert and Johnson, 2001). Table 2.3 presents the salient features of these reactors for treating different types of wastewater.

Table 2. 3 Different SRB based sulfidogenic reactor systems reported for treating metal contaminated wastewater

Reactor generic name	Target wastewater	Scale of operation	Nature of biomass	Carbon source	HRT	% Metal removal	% Sulfate removal	Reference
Anaerobic filter reactor (AFRs)								
Four sequential downflow AFRs	Wastewater containing Al and Fe	Not Available (NA)	SRB	Dextrose, urea and saccarose	20 h	> 9%	NA	Bechard et al., 1993
Upflow AFRs along with recycling unit	Wastewater containing Fe, Zn, Mn, Al, Cr, Ni and Pb	NA	SRB	Molasses	12 h	95.9% of Fe, 92.2% of Zn, 92.7% of Mn, 9.6% of Al, 68.2% of Cr, 50.0% of Cd, 63.0% of Ni, 63.0% of Pb	84%	Maree and Strydom, 1987
Downflow AFRs with limestone (Palmerton-pilot, USA)	Wastewater containing Zn, Ni, Cd and Mn	Pilot-scale 200 L	SRB	Mushroom compost	216 to 408 h	> 95%	20%	Dvorak et al., 1992
Upflow AFRs (pilot plant Colorado, USA)	Wastewater containing Zn, Mn and Cd	NA	SRB	Cow manure, paper products	50 to 100h	96-99% of Zn, 71-91% of Mn, 95-99% of Cd	70%	Farmer et al., 1995
Upflow AFR	Wastewater containing Fe, Mn, Zn, Cu, Cd and As	Laboratory scale (LS) 40-50 L	SRB	Cow manure, saw dust, cheese whey	115 to 190 h	84% of Fe, 40% of Mn, 99.7 % of Zn, 99 % of Cu, 99% of Cd, 89% of As	80 to 98%	Drury, 1999
Upflow anaerobic sludge blanket reactor (UASBR)								

UASB with a calm zone above sludge	Zn, Cd, Co and Cu containing wastewater	NA	SRB	Ethanol, nutrients	4 h	Zn, Cd, Co and Cu >99%	79	Barnes et al., 1991a
UASBR	Metal and sulfate containing mine wastewater	LS-500 mL	Granular sludge	Glucose	8 h	30-99%	23-72%	Tuppurainen et al., 2002
UASBR	Fe, Zn containing wastewater		SRB	Lactate and ethanol	16 h	>99% of Fe and Zn	75%	Kaksonen et al., 2003b
UASBR	Synthetic wastewater containing Cd and Zn	Continuous bench scale reactor-13 L	Anaerobic sludge	Lactate and stillage	18h	> 99%	93.75%	Goncalves et al., 2007
UASBR	Synthetic wastewater containing Pb	LS-5.7 L	SRB	Sodium lactate	NA	85–95%	50%	Hoa et al., 2007
UASBR	Synthetic wastewater containing selenium	LS	SRB	Sodium lactate	6 h	> 95%	27-95%	Lenz et al., 2008
UASBR	Synthetic metal bearing solution containing Pb	LS-5 L	Granular sludge from waste-water treatment plant	Ethanol	4 days	99%	94%	Velasco et al., 2008

Fluidized bed reactor (FBR)								
FBR	Wastewater containing Cd	NA	SRB	Lactate and yeast extract	8h	99%	NA	Ma and Hua, 1997
FBR	Wastewater containing Ni, U, Cu, Cd, Zn, As and Pb	LS-6.6 L	SRB	Molasses	6.8 h	93.0% of Ni, 66.7% of U, 93.3% of Cu, 60.0% of Cd, 76.7% of Zn, 95.0% of As, and 90.0% of Pb	92%	Somlev and Banov, 1998
FBR	Fe, Zn containing wastewater	LS-0.5 – 1.3 L	SRB	Lactate and ethanol	16 h	>99% and 99%	65% and 81%	Kaksonen et al., 2003a; Kaksonen et al., 2003b
Sulfidogenic fluidized bed reactor	Acidic wastewater containing Fe	LS-425-625 mL	SRB	Lactate or ethanol	24 h	> 99%	~50%	Sahinkaya et al., 2007
Downflow fluidized bed reactor (DFBR)	Synthetic acidic wastewater containing Fe, Zn, and Cd	LS-2.5 L	SRB	Ethanol	1-2 days	> 99%	41%	Garcia et al., 2009
UFBR	Zn and Cu containing wastewater	LS-20 L	SRB	Lactate		> 99%	60-86%	Erkan Sahinkaya and Murat Gungor, 2010
DFBR	Zn and Cu containing wastewater	LS-20 L	SRB	Lactate		> 99%	40-88%	Erkan Sahinkaya and Murat

								Gungor, 2010
Sulfidogenic fluidized bed reactor	AMD containing Al, Co, Ni, Pb, Zn and Mn	LS-300 mL	SRB	Ethanol	12 and 24 h	99.9% of Al, Fe, Ni, Cu, Pb, and Zn and, 94% of Mn	90%	Sahinkaya et al., 2011
Inverse fluidized bed reactor (IFBR)	Synthetic wastewater containing heavy metals- Cu, Pb, Cd and Zn	LS-5 L	Anaerobic sludge from a digester treating activated sludge	Sodium Lactate	24 h	98.4%, 96.5%, 96%, and 97.9% of Cu, Zn, Pb and Cd	68-88%	Villa-Gomez et al., 2011
Three-stage sulfidogenic fluidized-bed reactor system	Synthetic AMD containing Fe and Cu	LS-600 and 250 mL	SRB	Lactate or ethanol	24 h	> 99%	60-90%	Ucar et al., 2011
Sulfidogenic fluidized-bed reactor	Synthetic acid mine drainage	LS-600 mL	Sulfate reducing sludge from a laboratory scale sulfidogenic ABR	Ethanol or landfill leachate	15-28 h	80-99.9%	> 90%	Sahinkaya et al., 2013
IFBR	Synthetic acid mine drainage	LS-2.5 L	SRB	Ethanol	24 h	> 90%	> 50%	Janyasuthiwong et al., 2016
Anaerobic baffled reactor (ABR) and Anaerobic hybrid reactor (AHR)								
Anaerobic hybrid	AMD- Fe, Cr	NA	SRB	Ethanol	24h	> 90%	NA	Colleran

reactor (AHR)								et al., 1998; Steed et al., 2000
AHR with recycle	Wastewater containing Fe, Mn, Zn and Cu	LS-16.8 L	SRB	Acetate	310 h	> 95%	77%	Steed et al., 2000
Sulfidogenic anaerobic baffled reactor (ABR)	Zn containing acidic wastewater	LS-20 L	Effluent of anaerobic digester	Sodium lactate	2 days	> 99%	62–90%	Bayrakdar et al., 2009
ABR	AMD containing Cu and Zn	LS-20 L	Anaerobic digester effluent	Ethanol	48 h	84-98%	NA	Sahinkaya et al., 2009
Sulfidogenic ABR	Acidic wastewater containing Cu and Zn	LS-20 L	Inoculum from ABR	Ethanol	2 days	> 99%	70-92%	Erkan Sahinkaya and Zeynep Yucesoy, 2010
ABR	Synthetic spent refinery catalyst leach liquor with Mo, Ni, Co and V	LS-28 L	SRB	Lactate	5 days	Mo (36-72%), Ni (20%), Co (21%) and V (81%)	Varied between 20 to 50%	Cibati et al., 2013
Continuous stirred tank reactor (CSTR)								
CSTR	Synthetic wastewater	LS-500 mL	Anaerobic	Ethanol	SRT (10	>99%	40-70%	Sahinkaya, 2009

	containing Zn		digester effluent		days)			
Anaerobic semi-continuous stirred tank reactors	Synthetic wastewater containing mixtures of Cu, Zn, Ni, and Cr	LS-2 L	SRB	Sodium lactate	20 days	96 - 100% of Cu, 94 - 100% of Zn and Ni and 96 - 100% of Cr	43-67%	Kieu et al., 2011
Anaerobic fixed-film reactor								
Upflow anaerobic fixed-film (UAFF) reactor	Synthetic wastewater containing heavy metals-Cu, Pb, Cd, Ni, Cr and Zn	LS-9.8 L	SRB	Acetic acid	5- 20 h	90-100%	NA	El Bayoumy et al., 1999a
Anaerobic fixed bed reactor	Bioleaching solution containing Fe, Ni and Cu	LS-1-2 L	Anaerobic sludge containing SRB	Sodium lactate	NA	More than 90%	More than 50%	Cao et al., 2009
Fixed bed anaerobic reactor	Heavy metals (Fe, Cu, Zn and Al) of the AMD	LS-7 L	Acid mine drainage water and cheese whey wastewa-ter	Cheese whey wastewater	8 days	91.3%, 96.1%, 79.0% and 99.0%	NA	Rodríguez et al., 2009
Fixed-bed sulfate reducing bioreactor	Synthetic metal-bearing solutions	LS-250 mL	SRB	Ethanol	0.8h	99%	85%	Kousi et al., 2011

	containing Fe, Zn, Cu and Ni							
Fixed-bed bioreactor	Synthetic AMD	LS-Column pore volume: 1.5 L	SRB	Sodium lactate	18 months	> 99%	± 50%	Viggi et al., 2010
Anaerobic fixed bed reactor	Fe, Cu, Zn, Ni, Co and Cd containing wastewater	LS-1.2 L	SRB	Sodium lactate	NA	> 90%	> 90%	Bratkova et al., 2013
Fixed bed reactors	Cr(VI) contam-inated water	LS-6.65 L	SRB	Ethanol	NA	> 95%	> 65%	Pagnanelli et al., 2012
Anaerobic packed bed reactor								
Upflow anaerobic packed bed reactor	mildly acidic contam-inated waters (Cu, Zn, Ni, Fe, Al Mg and As)	LS-4.78 L	SRB	Sodium lactate	16.16 h	97.5% of Cu, Zn and Ni, > 82% of Fe and > 77.5% of As	> 80%	Jong and Parry, 2003
Anaerobic down-flow packed bed reactors	AMD dominated with Fe, Cu and Zn	LS-400 mL	SRB	Ethanol	8 days	61–91% of Fe and 97% of both Zn and Cu.	> 90%	Costa et al., 2009
Up-flow anaerobic packed bed system	Fe, Cu and Zn containing AMD	LS-87 mL	SRB	Wine wastes	NA	99%	85%	Martins et al., 2011
Up-flow anaerobic packed bed system	Synthetic AMD	LS	SRB	Sodium lactate	18-24 h	> 99%	61- 88%	Zhang et al., 2016
Column reactors								
Batch reactor	AMD containing Fe,	LS-1.25 L	SRB	Whey and cow manure	203 days	More than 90%	19 to 27%	Christensen et al.,

	Cu, Zn and Al							1996
Column reactor	Synthetic water containing U and Mo	LS-100-150 mL	SRB	Formate or lactate	24-36 h	> 99%	48-70%	Tucker et al., 1998
Glass column bioreactor	Synthetic wastewater containing Cr	LS-450-500 mL	SRB	Sodium lactate	1h	> 99%	NA	Brunet et al., 2002
Column Studies	Fe containing AMD	LS-1.2 L	SRB	Ethanol and Methanol	17-29 h	Fe 93%	50 to 84%	Tsukamoto et al., 2004
Sulfate reducing columns	Heavy metal contam-inated water	LS-40 mL	SRB	Woody material and alfalfa	NA	> 99%	> 99%	Pruden et al., 2007
Various other sulfidogenic reactors								
Infiltration beds	AMD contam-inated surface waters-Cu, Zn, Fe and Mn	NA	SRB	NA	NA	94–99% ,76–97%, 85–96% and 76–96%	NA	Vestola, 2004
Gas-lift bioreactor (GLR)	Zn containing synthetic wastewater	LS-4 L	Sulfate reducing sludge	CO_2	24.2	99%	> 90%	Bijmans et al., 2011
Low residence time bioreactors	Zinc-rich acid mine water also containing Pb and Cu	LS-2.9 L	Anaerobic digested sludge	Farm manure	11-14 h	>99% of the dissolved Pb and Cu, 99% of the dissolved Zn	NA	Mayes et al., 2011
Sulfate reducing bioreactor	Synthetic AMD containing Fe, Cu, Zn, Ni, Co and Mn	Bench scale-1 L	SRB	Manures, wood-chips, millet fodders and sugar-cane waste)	10 days	84.85% - 99% of Cu, 35.11-99% of Zn, 51.49%-99.32% of Fe, 87–99.14% of Ni, 63.55–99.02% of Co and 12.68–73.86% of Mn	43- 54%	Choudhary and Sheoran, 2012

Packed cage rotating biological contactor system	Synthetic industrial wastewater containing Ni and Pb	LS-43 L	Bio-sludge	NA	8h	85%, 89%	NA	Suntud Sirianuntapiboon and Sudarat Chumlaong, 2013
Sulfidogenic bioreactor	Cu containing wastewater	Batch reactors 1.5 L	SRB	Lactate	12 – 120 h	> 99%	69% - 99%	Bai et al., 2012
Up-flow anaerobic multiple-bed (UAMB) reactor	AMD containing Cu, Fe and Mn	LS-2.15 L	SRB	Lactate	12 – 96 h	99% of Cu, 86% of Fe and 52. 6% of Mn	61%	Bai et al., 2013

Table 2. 4 Merits and demerits of the different sulfidogenic bioreactors

Reactor	Merits	Demerits	Reference
FBR	Large surface area for the formation of biofilm; High biomass retention; Large mass transfer rates; small pressure drops and no flow channeling; No clogging	Fluidization of carrier needs energy; Biomass loss due to shear forces; Due to the inert nature of carrier, less volume will be accessible for biomass compared with UASB	Rittmann, 1982 Speece, 1983; Yoda et al., 1989; Anderson et al., 1990; Melin et al., 1997; Melin et al., 1998; Marin et al., 1999
ACP	Better biomass retaining capacity in contrast to CSTR	Breakdown of flocs and sludge due to shear force	Speece, 1983
AFR	Small shear forces; enhanced SRT; utilizes gravitation force in downflow mode	Channeling effect due to shear force; Large pressure drops	Speece, 1983; Anderson et al., 1990; Barnes et al., 1991a
UASB	No flow channeling and low chances of sludge compacting; Does not require biomass carrier; Nil clogging; treatment rates are higher	Chances of biomass wash out; added vulnerability to the transformations of influent quality	Speece, 1983; Barnes et al., 1991a; Jhung and Choi, 1995
CSTR	High performance; consistent and rapid equilibrium conditions	Inefficient retention of biomass	Speece, 1983; Barnes et al., 1991b; Barnes et al.,1991a
ABR	Extended SRT; Does not require biomass carrier; Withstands high hydraulic and organic loading rates	Not available (NA)	Speece, 1983; Barber and Stuckey, 2000
GLR	Imparts good mixing and mass transfer	High pressure drop in case of gaseous substrate	Dijkman et al., 1999; Lens et al., 2002
AHR	Less susceptible to clogging; Sludge removal easier; Better biomass retention than in UASB	NA	Steed et al., 2000
MBR	Better biomass retainment; Avoids direct contact between SRB and toxic water (Extractive MBR)	Need backwashing due to chances of membrane fouling (due to microbes or metal precipitates)	Chuichulcherm et al., 2001; Mack et al., 2004; Tabak and Govind, 2003; Vallero et al., 2005

2.7.2.9 Anaerobic fixed-film reactor

Fixed film reactors utilize support medium for biomass growth, such as rock, plastic, wood, or other natural or synthetic solid material on its surface and within its porous structure (Shafi et al., 2009). Another type of anaerobic fixed film reactors (AFR) is down flow stationary fixed film (DSFF) reactor which is a high rate anaerobic reactor with active biomass retention. Unlike other fixed film reactor systems, the unique features of the DSFF reactor are the structural design of the packing material used (biofilm support), the downflow mode of process and the absence of suspended biomass (Vigneswaran et al., 1986).

The downflow approach reduces the possibility of column clogging by means of allowing any settleable particles which may otherwise get accumulated in the system. Rising gas bubbles in the system facilitate complete mixing of the effluent, thus avoiding the use of any unnecessary agitation unit (Vigneswaran et al., 1986). The system shows better mixing properties and reduces the accumulation of inhibitors or volatile acids by means of counter current two phase flow (Hall and Melcer, 1984). Various AFR reactors reported in the literature for treating heavy metal containing wastewater are listed in Table 2.3.

2.7.2.10 Gas lift reactor (GLR)

Gas lift reactor (GLR) systems impart good mixing and enhance the mass transfer rates (Dijkman et al., 1999). It comprises of a riser column, a down comer column and gas is flushed from the bottom end of the riser. The variation in the density formed due to the pressure difference in the riser and the down comer due to gas holdup is considered as the governing factor for the flow of liquid in the reactor (Van Houten and Lettinga, 1995). A GLR can be employed with or without carrier substance. Possibility of pressure drops avail during the use of gaseous substrates which is the main drawback of GLRs (Table 2.4) (Van Houten et al., 1994; Dijkman et al., 1999; Lens et al., 2002).

Bijmans et al. (2011) used GLR for treating Zn containing synthetic wastewater using carbon dioxide as the carbon source and achieved 99% removal of Zn and 90% removal of sulfate from the wastewater. One of the largest sulfate reducing bioreactor for treating zinc containing wastewater is a 500 m^3 gas lift reactor (Budel Zink B. V, The Netherlands) with a reported maximum conversion rate of 350 kg SO_4/h (Weijma et al., 2002).

2.7.2.11 Membrane bioreactor (MBR)

The integration of a membrane to a bioreactor is a major step towards the development of new bioreactors (Mack et al., 2004). Bioreactors with a membrane unit for biomass separation are the widely used membrane bioreactors (MBR) (Kaksonen and Puhakka, 2007). These MBRs form a union of a suspended growth system and a membrane filtration unit. Membrane unit can be immersed in the bioreactor (IMBR) or it can be placed in a side stream (SMBR). In contrast to the SMBR, the IMBR does not contain any recycling unit and biomass separation occurs within the reactor (Kaksonen and Puhakka, 2007).

In general, compared with SMBR, IMBR possess significantly a large membrane area per unit volume. IMBRs entail the advantage of functioning at low trans-membrane pressure and at low fluid cross flow velocities and, hence, requires less energy input and involves low-cost of operation (Mack et al., 2004). Another type of membrane bioreactor is extractive membrane bioreactor (EMBR) which has been employed to avoid contact between SRB and wastewater (Chuichulcherm et al., 2001).

In the EMBR, the wastewater containing metal ions is allowed to flow above one face of a selective permeable membrane, whereas inoculum is grown on other face of the reactor (Mack et al., 2004). This membrane permeates H_2S from the microbial culture section in to the metallic wastewater chamber and precipitates the metals. Due to the impermeable nature of the membrane to charged molecules in the wastewater stream, it prevents SRB from coming into contact with any toxic metals or high salinity in the wastewater (Chuichulcherm et al., 2001). Zinc removal from wastewater was examined using EMBRs. Zinc sulfide precipitation on the effluent side of the membrane resulted in lowered H_2S mass transfer (Chuichulcherm et al., 2001).

Due to the impermeable nature of the membrane to the charged molecules, sulfate cannot pass through the membrane and, as a result, electron acceptor has to be supplied externally. In view of the growing interest in sulfidogenic bioreactor application for treating metal contaminated wastewater, SRB plays a key task in enhancing the reactor performance. Table 2.5 presents different sulfate reducing genus identified from various sulfidogenic bioreactors treating heavy metal containing wastewater. *Desulfovibrio*. species are found to be the most common and predominant SRB genera in these sulfidogenic bioreactors.

Table 2. 5 Different sulfate reducing genus identified from sulfidogenic bioreactors treating heavy metal containing wastewater

Sulfidogenic reactor type	**SRB genus identified**	**Reference**
Glass column bioreactor	*Desulfomicrobium norvegicum*	Brunet et al., 2002
Fluidized bed sulfate reducing bioreactor	*Desulfovibrio alcoholovorans, Desulfovibrio sulfodismutans Desulfovibrio amnigenus, Desulfovibrio acetoxidans*	Kaksonen et al., 2003a; Kaksonen et al., 2003b
Sulfidogenic fluidized bed reactor	*Desulfotomaculum and Thermanaeromonas, Desulfobulbus spp.*	Sahinkaya et al., 2006
Sulfate reducing columns	*Desulfovibrio vulgaris*	Pruden et al., 2007
Anaerobic down-flow packed bed reactors	*Desulfovibrio fructosovorans, Desulfovibrio aminophilus and Desulfovibrio desulfuricans*	Costa et al., 2009
Gas lift reactor	*Desulfovibrio carbinoliphilus, Clostridium bowmanii, Lactonifactor longoviformis, Thioclava pacifica, Parabacteroides goldsteinii Desulfovibrio burkinensis, Methanobrevibacter arboriphilus Petrimonas sulfuriphila, Desulfovibrio gigas*	Bijmans et al., 2011
Fixed-bed sulfate reducing bioreactor	*D. postgatei, D. fructosovorans, D. carbinolicus, D. burkinensis Desulfomicrobium, D. fructosovorans, D. carbinolicus Desulfovibrio baculatus and Desulfobulbus propionicus*	Kousi et al., 2011
Up-flow anaerobic packed bed system	*Desulfovibrio, Clostridium, Citrobacter and Cronobacter genera*	Martins et al., 2011

2.7.3 Passive vs. Active treatment systems

Table 2.6 compares the main features of active and passive systems for the treatment of wastewater containing metals by biological sulfate reduction. It is obvious that passive treatment systems come with relatively reduced labor, low-cost, low operation and low maintenance cost. However, passive treatment systems entail drawbacks, such as requirement of a larger area for treatment, difficulty in metal recovery, poor process control and unpredictable performance (Johnson and Halberg, 2002).

On the contrary, active treatment systems offer several advantages which include less area for treatment, ease of metal recovery, good process control and good performance predictability (Table 2.6). However, the latter requires more expenditure and skilled manpower for operation and maintenance (Gazea et al., 1996; Johnson, 2000; Kaksonen et al., 2004).

Table 2. 6 Main features of active and passive systems for biological treatment of heavy metal containing wastewater (Gazea et al., 1996; Johnson, 2000; Kaksonen and Puhakka, 2007)

Parameter	Active system	Passive system
Operation and maintenance expenditure	High	Low
Labor requirement	More	Less
Treatment area	Small	Large
Metal recovery	Possible	Difficult
Process control	Good	Poor
Degree of predictability	Good	Poor
Cost	High	Low

2.8 Factors Affecting The Selection of Sulfidogenic Bioreactors For Heavy Metal Removal

Figure 2.6 highlights the different factors that govern the selection and design of an appropriate bioreactor that influence the reactor performance. If the key goal of the sulfidogenic reactor is to precipitate the metals as metal sulfides, sustenance of SRB is the governing step for subsequent sulfide formation. Nature of the precipitates formed also plays a vital role in metal recovery. Again, both these factors depend on several parameters (Fig. 2.6) which are very vital for the design of different bioreactors. For example, pH governs the survival of microbial communities in the reactors and directly affects the quality of the precipitates formed along with the concentration levels of metals and sulfide generated (Andrea et al., 2014). Therefore, pH plays a vital role while designing a reactor. Sustenance of SRB community depends on the metal toxicity, HRT and sulfide toxicity. It is also essential to consider the possible toxicity that may be generated due to the formation of intermediate compounds during the incomplete oxidation of electron donors by SRB (Kaksonen and Puhakka, 2007).

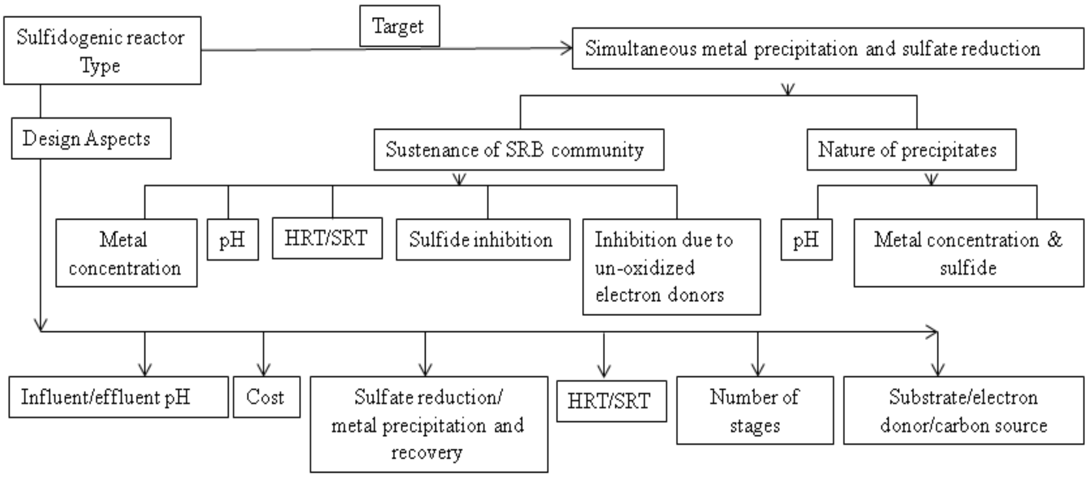


Figure 2. 6 Different factors that govern the selection of sulfidogenic reactors for sulfate and heavy metal removal from wastewater (Andrea et al., 2014).

From Fig. 2.6, selection of an appropriate sulfidogenic reactor depends on various design aspects, such as influent pH, HRT, sulfate reduction/metal precipitation, number of stages, carbon source used and cost involved. The sustenance of microbial biofilm activity and performance of a reactor is mainly dictated by the HRT/SRT ratio (Lettinga et al., 1980). Number of reactor stages plays a vital role in metal recovery and its maintenance (Fig. 2.6). Based on the number of stages, reactors can be broadly classified as single stage or multi stage process. In a single stage process, sulfate reduction is carried out by SRB to produce sulfide, thus, aiding in metal precipitation as metal sulfides within the reactor (Kaksonen et al., 2003a).

In multi or two stage reactors, sulfate reduction occurs in one reactor and the produced sulfide is diverted to another reactor to precipitate the metals as sulfides at controlled conditions (Andrea et al., 2014). In single stage reactors, homogenous sulfide concentration is produced by SRB which aid in better settling of the metallic sulfides, whereas in a two stage or multi stage process, when sulfide is dosed to another reactor as a gas phase, it results in less thickening and settling of the metal sulfides (Tabak et al., 2003). Also, single stage process offers advantages, such as reduction in the investment costs, easy process design and control, does not require any accessories for the transport of sulfide to another reactor and less possibility of sulfide inhibition of SRB activity due to metal precipitation (Andrea et al., 2014). Finally, cost of the reactor depends on whether the reactor operates for sulfate reduction alone or for both metal precipitation and metal recovery (Fig. 2.6).

2.9 Effect of Different Parameters on The Performance of Sulfidogenic Bioreactors

Table 2.7 presents the different parameters and their effect on the performance of a sulfidogenic reactor. These parameters are grouped under microbial composition, influent characterization, reactor design aspects and operational conditions.

Table 2. 7 Effect of different parameters on sulfidogenic metal precipitation and sulfate reduction by SRB

Parameters	Effect(s)	References
Microbial composition		
Biofilm properties of microorganisms	The capacity of microbial species to colonise is variable	Isa et al., 1986 ; Omil et al., 1997
Influent parameters		
pH	Low pH may result in toxicity to SRB; high pH may support SRB over methanogens	Visser et al., 1992; van Houten et al., 1995; Omil et al., 1996; Omil et al., 1997; Crine et al., 1999; Marchal et al., 2001; García et al., 2001
Inlet sulfate concentration	Low concentrations of sulfate may out compete SRB; high concentration may lead to inhibition	Overmeire et al., 1994; White and Gadd, 1996a; Crine et al., 1999
COD/SO_4^{2-}	Influences the sulfate reduction process	Hao et al., 1996
Metal concentrations	High metal concentrations may lead to toxicity; Ca^{2+} and Mg^{2+} in the media may improve the dominance of SRB over methanogens	de Smul and Verstraete, 1999; Hao, 2000; Marchal et al., 2001
Trace element	Needed in electron transport, redox-active metalloenzymes and composition of some protein and enzymes	Postgate, 1984; Bridge et al., 1999; Biswas et al., 2009
Salinity	Adversely affects sulfate reduction	Kerkar and Loka Bharathi, 2007; Sorensen et al., 2004
Reactor design aspects		
Surface area for microorganism immobilization	Influences the potential of active SRB populations, thus affecting sulfate reduction and metal precipitation	Bass et al., 1996; Lyew and Sheppard, 1997
Operational conditions		
Temperature	Affects the growth and activity of SRB; high temperature helps SRB outcompete methanogens	Visser et al., 1992; Visser et al., 1993; Crine et al., 1999;

		Dean, 1999
Flocculant addition	Influences the settling of solids	Scheeren et al., 1993
Substrate/carbon source concentration and loading rate	Increase in substrate concentrations enhances sulfate reduction	Harada et al., 1994; White and Gadd, 1996a; Kalyuzhnyi and Fedorovich, 1997; Omil et al., 1998; El Bayoumy et al., 1999b; Crine et al., 1999
Different carbon source	Lactate, ethanol, propionate, butyrate, hydrogen and glucose can favor the growth of SRB; Lactate helps to obtain high biomass yields of SRB; Ethanol enhances sulfide production	Polprasert and Haas, 1995; Omil et al., 1996; White and Gadd, 1996b; Greben et al., 2000
Upward liquid velocity (V_{up})	Biomass washout at high V_{up} (4-6 m/h) in granular sludge reactors	Omil et al., 1996
H_2S concentration	High concentration of H_2S inhibits growth of SRB	Omil et al., 1996; van Houten et al., 1997
Exposure to oxygen	A short term exposure to oxygen helps SRB outcompete methanogens	Omil et al., 1997
Hydraulic retention time (HRT)	High HRTs: sulfide toxicity and small HRTs: biomass washout, affects sulfate reduction rate	Kalyuzhnyi and Fedorovich, 1997; Fedorovich et al., 2000
N and P addition	Optimum nutrient levels (C:N:P ratio) enhances the growth of SRB; toxicity due to exposure of high NH_4^+ concentrations	El Bayoumy et al., 1999b; Lens et al., 1999
Stirring conditions	Alters the growth and decreases substrate uptake	Marchal et al., 2001
N_2 flushing	Enhances anaerobic conditions and decreases H_2S toxicity	Marchal et al., 2001
Oxidation reduction potential (ORP)	Affects the activity of SRB and competition between SRB and mathanogens	Gerhardt et al., 1994; Khanal and Huang, 2006; Huan et al., 2013.
Sludge retention time (SRT)	Influences competition between SRB and other bacteria; influences the reactor's performance and sludge production	Esposito et al., 2003;Weijma et al., 2002

2.9.1 Microbial composition

Microbial composition of a biomass differs due to the capacity of microbes to colonize and form biofilm. In this respect, mixed consortia entail several advantages over pure cultures, such as the existence of a variety of resistance mechanisms, availability in the natural environment, no contamination problem, presence of reducing conditions due to complete oxidation of the carbon sources (Bruins et al., 2000), ease of maintenance of culture conditions, etc. (Kieu et al., 2011). Therefore, it is essential to select the best source of biomass for efficient treatment of wastewater using a bioreactor.

2.9.2 Influent parameters

Influent parameters, e.g. low pH results in a toxic effect on SRB and high pH (> 7) favors growth of SRB over methanogens. Moreover, the pH determines the binding characteristics and overall charge of the metals and biomass (Table 2.7). High sulfate concentration may inhibit SRB growth and a low sulfate concentration may favor methanogens over SRB. A high concentration of metal cations and oxyanions may result in toxicity to the SRB (Table 2.7).

The toxicity of metals is due to many factors, such as type of metal and its concentration, biomass quantity, temperature, pH, concentrations of electron donor and sulfate (Hao, 2000). Toxicity of metals, particularly oxyanions, also depends on their oxidation state, for example Cr(VI), As(V) and Se(VI) are more toxic than Cr(III), As(III) and Se(IV), respectively (Collins and Stotzky, 1989). However, trace concentration of certain metals, e.g. Cu(II), Zn(II) and Fe(III) are needed in electron transport and for the function of redox-active metallo-enzymes. Salinity of the media adversely affects the sulfate reduction rate (Table 2.7).

Ratio of COD to sulfate often defines the interaction of SRB with carbon source available and the electron acceptor. Chemical oxygen demand represents the concentration of oxygen essential for the oxidation of available organic matter and indicates the preferred metabolic route for energy production (Barbosa et al., 2014). A minimum COD/sulfate ratio of 0.67 is considered as the ideal stoichiometric proportion for complete sulfate reduction and degradation of organic substrates (Rinzema and Lettinga, 1988); however, a COD/sulfate ratio in the range 0.7 to 1.5 is common, depending on the type and source of electron donor used (Hao et al., 1996).

The selection of an optimum COD/sulfate ratio is the key element in assuring the efficiency of any system designed for treating metals and sulfate from wastewater (Barbosa et al., 2014). Therefore, when ethanol is used as the carbon source, the optimum COD/sulfate ratio range between 0.55 and 0.84 (Greben and Maree, 2000; Greben and Maree, 2005); if organic waste products (such as activated sludge and municipal compost) are used as carbon sources, optimum COD/sulfate ratio varies between 1.6 and 5 (Ani, 1994; Henry and Prasad, 2000).

2.9.3 Reactor design and operational parameters

Operational parameters, such as pH and temperature influence the SRB physiological state and, consequently, their resistance to metal toxicity. High temperature helps SRB to outcompete methanogens. Flocculant addition and stirring may alter the SRB growth, decrease substrate uptake and influence the solid settling. Upward liquid velocity plays a vital role in case of FBR, GLR and granular sludge reactors. Biomass washout may prevail at high upward liquid velocities. A high sulfide concentration (either in gas phase or liquid phase) inhibits the growth of SRB. The presence of fine solid particles in the vicinity of the SRB cells at a high sulfide concentration can act as a physical barrier, thereby, partially blocking the transportation of sulfate and other substrates into the cell cytoplasm (Utgikar et al., 2002; Jong and Parry, 2003; Barbosa et al., 2014) (Table 2.7).

Nitrogen (N_2) flushing enhances the anaerobic condition and decreases H_2S toxicity, whereas short term exposures to oxygen support SRB to outcompete methanogens (Table 2.7). The SRB may be inhibited due to sulfide toxicity at high HRTs; whereas small HRTs may result in biomass washout and affect sulfate reduction rate. Oxidation reduction potential (ORP) and SRT affect the activity and competition between SRB and methanogens. Also, SRT influences reactor performance and sludge production. Surface area for microbial immobilization, which is one of the key aspects of the reactor design, influences the activity of SRB population, thus affecting the efficiency of sulfate reduction and metal precipitation.

Selection of an appropriate carbon source is another key factor affecting any reactor performance. Mostly, heavy metal containing wastewater is deficient in carbon source which is necessary for the growth of microorganisms. Thus, addition of external carbon source is very much essential. In passive treatment systems, locally and economically available organic compounds are used in order to promote long term activity for achieving a high treatment efficiency (Neculita et al., 2007). The selection of suitable carbon source is based on (a) accumulation capability of organisms present, (b) availability in sufficient quantities and (c) added load in case of incomplete oxidation of the carbon source (van Houten et al., 1994; Dries et al., 1998; Dijkman et al., 1999). Table 2.8 presents the Gibbs free energy change ($\Delta G°$) of different electron donors for the sulfate reduction reactions. From Table 2.8, a high value of $-\Delta G°$ of the lactate oxidation reaction with sulfate indicates that lactate is the most preferred carbon source and electron donor for SRB (Villa-Gomez et al., 2011).

Lactate also acts as a key carbon source in terms of biomass production; a diverse range of SRB are capable of oxidizing lactate either entirely or partly in sulfate rich environment (Okabe et al., 1995; Kaksonen et al., 2003b). High cost and high proportion of un-dissociated lactate in metal containing wastewater, such as AMD, which leads to inhibition of SRB, are some of the drawbacks of lactate as the carbon source (Bruser et al., 2000). From Table 2.8, next to lactate, ethanol and acetate are the preferred carbon sources, which are available in cheap and extensively studied for treating metallic wastewater (Widdel, 1988; Nagpal et al., 2000; Kaksonen et al., 2004).

Table 2. 8 Gibbs free energy change of different electron donors for sulfate reduction reactions (Stefanie et al., 1994)

Sulfate reduction reaction between electron donor and acceptor	**-ΔG° (kJ/mol)**
Lactate + ½ SO_4^{2-} → Acetate + HCO_3^- + ½ HS^- + H^+	80.0
Ethanol + ½ SO_4^{2-} → Acetate + HCO_3^- + ½ HS^- + ½ H^+ + H_2O	66.4
Acetate + SO_4^{2-} → $2HCO_3^-$ + HS^-	47.6
4 H_2 + SO_4^{2-} + H^+ → HS^- + $4H_2O$	38.1
Propionate + ¾ SO_4^{2-} → Acetate + HCO_3^- + ¾ HS^- + ¼ H^+	37.7
Butyrate + ½ SO_4^{2-} → 2Acetate + ½ HS^- + ½ H^+	27.8

2.10 Biofilm Composition in Attached Growth Sulfidogenic Bioreactors

Sulfidogenic reactors are considered as potential alternative systems for treating a wide range of industrial wastewater where metal precipitation, sulfate reduction and wastewater neutralization can be addressed simultaneously on large-scale. These systems also enable easy sludge management and facilitate metal recovery (Kousi et al., 2015). The performance of a sulfidogenic bioreactor is highly dependent on the microbial biofilm formation and its regeneration capacity. Therefore, biofilm formation in a bioreactor is key to the treatment capacity of the system on a large-scale for prolonged period.

In general, biofilms are very versatile and heterogeneous in nature. They encompass several microorganisms which grow and attach on various surfaces under different conditions (Flemming, 1995). Microorganisms in biofilm undergo chemical interactions with their surrounding environment, entailing both metal sorption and mineral precipitation (Konhauser et al., 1993). In general, a biofilm comprises of about 25% of bacteria and 75%

of extracellular polymeric substances (EPS). They envelop the supporting material partly and form a gel type film, mainly involving EPS, shaped by biofilm organisms.

Therefore, the sorption characteristics of biofilms notably determine the overall process, which makes it a vital feature in successfully running the process (Flemming, 1995; Sutherland, 2001; van Hullebusch et al., 2003). Extracellular polymeric substances of SRB typically comprise of amino sugars and proteins with cationic functional groups (e.g., -NH_3^+), uronic acids and proteins with anionic functional groups (such as $-COO^-$, $-HPO_4^-$) (Flemming, 1995; Sutherland, 2001).

SRB structure consists of an outer membrane, murein, cell wall, cytoplasmic membrane and cytoplasm. The outer membrane of SRB, which is a typical Gram negative bacteria, is mostly composed of lipo-polysaccharides, proteins and phospholipids (Flemming, 1995). Phosphoryl, carboxyl and amino groups constitute the major functional groups of SRB outer membrane (Beveridge et al., 1997; Langley and Beveridge, 1999; Smith and Ferris, 2001). Figure 2.7 describes the different stages involved in SRB biofilm formation on a bio-support material.

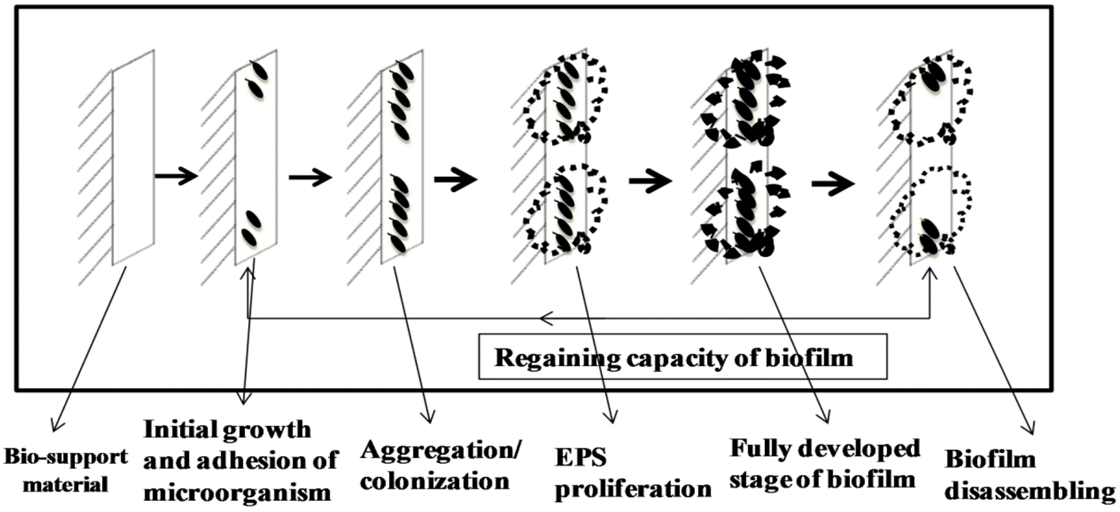


Figure 2. 7 Different stages involved in the development of biofilm formation on a bio-support material.

In the first stage of biofilm formation, SRB initially grow on the bio-support material through adhesion. In the second stage, SRB aggregate due to colonization and they are protected by EPS. In the third stage, EPS proliferation occurs, and leads to fully developed biofilm in the subsequent stage. Finally, some SRB get detached from the biofilm when there is a nutrient lack in the media, after which SRB regains the biofilm forming capacity. Thus, from the preceding literature review, sulfidogenic reactors are extensively being studied at the laboratory scale and some of these being adequately applied at full scale for heavy metal removal and recovery especially from industrial effluents.

Compared with the conventional chemical treatment process, SRB based bioprocess is economical, aid in the recovery of metals as metal sulfides, form stable metal precipitates with high metal and sulfate removal efficiencies. Passive treatment systems have no or minimum energy requirement, are accessible at remote locations, operates at low temperatures and involve low operation and maintenance costs. On the other hand, active treatment systems are compact in design and easy to operate and offer good process control for achieving the best results. In addition to the elimination of metal toxicity from wastewater, SRB based bioprocess aids in recovering the metals as their sulfide salts (Buisman et al., 2011). Hence, it is recognized as a resourceful method to both remove and recover metals from contaminated wastewater (Cabrera et al., 2006). However, for a successful application of this method, the choice of a suitable reactor system is essential.

Although the SRB based treatment system is effective and can be applied on a large-scale, it involves certain limitations. One such limitation is the inherent toxicity of the metals on SRB growth and activity (Min et al., 2008). For instance, high metal concentrations have been shown to be inhibitory towards growth and activity of freely suspended SRB (Sani et al., 2001; Utgikar et al., 2001; Utgikar et al., 2002). The use of such freely suspended SRB further results in activity loss due to cell washout in continuously operated reactors (Baskaran and Nemati, 2006), thereby, necessitating extended HRT for operating the system (Neculita et al., 2007). In order to overcome the drawbacks associated with the use of free/suspended systems, bio-film development using suitable bio-support material and active cell immobilization using suitable naturally available polymeric matrix are proposed.

Among the several bioreactor systems to treat wastewater, rotating biological contactor (RBC) reactor is well-known for treating various types of effluents (Alemzadeh et al., 2002; Kapdan and Kargi, 2002; Axelsson et al., 2006) as it offers a high interfacial area on its rotating discs for biomass growth and attachment, which enables sufficient contact between the microorganisms and contaminants present in the wastewater (Pakshirajan and Kheria, 2012).

Furthermore, RBC reactor is highly suitable for treating industrial wastewaters and sewage (Banerjee, 1997) owing to the combined action of both suspended growth and fixed film microorganisms present in this reactor system. Altough RBC reactors involve high initial investment cost, but simple process control, low operation and maintenance costs make these reactors technically feasible for large-scale applications (Borchardt, 1971; Wu and Smith, 1982; Wheatley, 1984). The utility of RBC reactors has been examined at the laboratory scale for removal of COD from textile dying and synthetic wastewater (Pakshirajan et al., 2009). Active immobilization method is becoming popular for preventing biomass washout from such bioreactors. Although a wide range of materials has been tested for active immobilization, the naturally available polymer sodium alginate has received significant interest owing to its easy availability and low-cost (Chen and Lin, 1994; Lozinsky and Plieva, 1998).

From the afore-mentioned literature, it is clear that the main focus has been to treat heavy metal and sulfate containing wastewater using different bioreactor configurations by employing SRB; however, RBC reactor system or sodium alginate immobilized SRB reactor system has not been investigated for batch/continuous heavy metal removal from wastewater. Moreover, the mechanism of simultaneous sulfate and metal removal by SRB needs to be detailed further for a better applicability of such reactor treatment system. The research work presented in this thesis therefore, aims to address all these research gaps. It mainly focuses on the potential application of anaerobic RBC reactor system immobilized with SRB for treating synthetic wastewater containing sulfate and different heavy metals in single and mixture component system.

It also compares the results of heavy metal removal both under batch and continuous mode of operation with that of a simple downflow column reactor packed with SRB immobilized sodium alginate beads. Furthermore, in order to carry out the heavy metal removal experiments involving mixture component system, statistically valid experimental design, viz Plackett Burman and factorial designs were applied. A brief introduction about statistical design of experiments is given in Section 2.11

2.11 Statistical Design of Experiments

Experiments are always considered the main tool for practically validating a theoretical hypothesis about scientific knowhow of a process or phenomenon, where the observation is expected to be correlated with some known associated process parameters. In order to identify important factors and their contribution towards an observed response, normal approach of using one-variable-at-a-time (OVAT) involves a large number of experiments to be carried out which often cannot elucidate any significant interaction among the process variables (Ryan et al., 2007). Statistical experimental designs are potent tools for improving the efficiency of experimentation and they facilitate a better understanding of the system being investigated with a minimum number of experiments. Inclusion of replicate test conditions allows the estimation of random, experimental variation. Statistical analysis of data generated from such experiments clearly establishes the relationship between the measured parameter of interest (response) and the process parameters (input factors or factors) being studied. Details of statistical design of experiments are presented in Appendix A.

2.12 Aim and Objectives

The present research work is aimed at the removal of metal ions by biogenic sulfide precipitation using two different sulfidogenic bioreactor systems with immobilized SRB: anaerobic rotating biological contactor (An-RBC) reactor and downflow column reactor (DFCR). To achieve this aim, the following investigations were carried out:

Screening of anaerobic biomass for metal removal by sulfide precipitation

Batch heavy metal removal from both single and multi-metal solution by free/suspended system

Heavy metal removal by immobilized SRB

Batch heavy metal removal using immobilized SRB in the form of beads

Continuous heavy metal removal

- Performance evaluation of DFCR packed with immobilized SRB for continuous heavy metal removal from single and multi-component systems
- Performance evaluation of the An-RBC reactor with immobilized SRB for continuous heavy metal removal from single and multi-component systems
- Identification and characterization of SRB

2.13 Presentation and Layout of the Thesis

The present thesis covers five chapters with appropriate sections and subsections and also contains references and visible research outputs. A brief description of these chapters is mentioned as follows:

Chapter 1 details introduction of the present work. The potential application of SRB using various types of bioreactors for treating both sulfate and heavy metal containing wastewater is detailed in **Chapter 2**. This chapter also deals with various factors that affect the performance of different sulfidogenic bioreactors and details the various reactors applied for treating metallic wastewater using SRB and also presents the objectives and scope of the present work. Materials and methods followed in the research work are described in **Chapter 3**. It details the methodology followed for screening different anaerobic biomass from different sources for heavy metal removal from single and multi-component systems and its characterization. Further, details of heavy metal removal using two different continuously operated laboratory scale sulfidogenic bioreactor systems with immobilized SRB and analytical methods followed in this study are mentioned.

Chapter 4 mainly presents and discusses the results of batch and continuous heavy metal removal obtained from single and multi-component systems and the metal removal mechanism involved. This chapter thoroughly describes the metal removal results obtained using the two continuously operated laboratory scale sulfidogenic bioreactor systems. Summary and conclusions drawn from this study are presented in **Chapter 5**. Some useful recommendations and scope for future work are also provided in this chapter.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

This section describes different techniques and methods followed in the present research. All chemicals and reagents used in this study were of analytical grade and supplied by Hi-Media Pvt. Ltd., India, LOBA Chemie Pvt. Ltd., India, SRL Chemicals Pvt. Ltd., India, Merck India Ltd., and CDH Pvt. Ltd., India. Membrane filtered water (reverse osmosis (RO)) was used for carrying out all experiments in the study (Sartorious, Arium 61316RO & 611UF, Germany).

3.2 Source of Anaerobic Biomass and Growth Conditions

Anaerobic biomass obtained from different sources was screened in this study for heavy metal removal from aqueous solution by sulfate reduction. The sources included, wastewater treatment plant (WWTP) located at Indian Institute of Technology Guwahati, Guwahati, Assam, India, a lab-scale upflow anaerobic packed bed reactor (UFAR) treating sulfate rich wastewater and another lab-scale anaerobic packed bed reactor (APR) treating sulfate rich water containing arsenic.

For anaerobic growth of the biomass, medium with the following composition was used (g/L): 0.5 KH_2PO_4, 1 NH_4Cl, 1.47 Na_2SO_4, 0.1 $CaCl_2 \cdot 2H_2O$, 0.1 ascorbic acid, 0.2 tri-sodium citrate, 0.2 ethylene diamine tetraacetic acid (EDTA) (Meers et al., 2009), 0.15 $FeSO_4 \cdot 7H_2O$, 0.2 bromo ethane sulfonic acid (BESA) (Jin et al., 2007) and 1 yeast extract (modified Postgate medium) and pH of the solution was adjusted to 7 using 1N NaOH (Postgate, 1984). 10% v/v of the respective anaerobic biomass from the different sources was added to an aspirator bottle containing 1 L of the pH adjusted medium. The bottle was purged with nitrogen gas (before and after inoculation) followed by incubation for seven days at 30 °C (JEIO TECH, SI-300R, Korea). Sodium lactate (60% v/v) was used as the carbon source for culturing the biomass. Such freshly grown biomass was designated as the maintenance culture for carrying out subsequent metal removal experiments.

3.3 Batch Heavy Metal Removal

3.3.1 Screening of heavy metals and anaerobic biomass

Heavy metals and anaerobic biomass (sulfate reducing bacteria (SRB)) obtained from three different sources were screened in this study for metal removal from aqueous solution *via* sulfate reduction. The biomass sources include WWTP, UFAR and APR. Simultaneous sulfate reduction and heavy metal removal experiments were carried out using 100 mL serum bottles fitted with a rubber stopper and aluminum crimp seal. Individual metal stock solutions of Cu(II), Cd(II), Ni(II), Fe(III), Pb(II) and Zn(II) of 10,000 mg/L concentration each were prepared using $CuCl_2{\cdot}2H_2O$, $Cd(NO_3)_2{\cdot}4H_2O$, $NiCl_2{\cdot}6H_2O$, $FeCl_3{\cdot}6H_2O$, $PbNO_3$ and $Zn(NO_3)_2{\cdot}6H_2O$, respectively.

Serum bottles containing the medium as mentioned earlier were added with the individual metal containing stock solution yielding 10 and 50 mg/L initial concentration of the respective metals. These bottles were purged with nitrogen gas before and after inoculation with 10% v/v biomass, as mentioned earlier. During the heavy metal removal experiments both under batch and continuous mode of operation, sulfate and COD concentration in the influent were adjusted to obtain a COD/SO_4^{2-} ratio of 0.67 ± 0.08 as this is the ideal stoichiometric proportion for complete sulfate reduction and degradation of organic substrates (Rinzema and Lettinga, 1988). The bottles were then agitated on an orbital shaking incubator set at 30 °C temperature and 120 rpm shaking speed (Lab Tech, LSI-3016R, Korea). Bottles with medium containing only the carbon source and biomass, but without any added metal, served as the control in these experiments. Samples were taken at regular intervals during the experiments to determine conductivity, pH, sulfate, COD, metal and sulfide concentrations. Mixed liquor volatile suspended solids (MLVSS) value in the samples was quantified using muffle furnace (Lab Tech, LEF-115P-2, Korea) (APHA, 2005). All these batch experiments were conducted in duplicate and the results presented are average of duplicate sample analysis.

3.3.1.1 Characterization of the metal precipitates

Characterization of the metal precipitates formed due to the anaerobic biomass collected from the UFAR which proved to be the best for metal removal among the three sources of biomass was carried out using Fourier transforms infrared (FTIR) spectrometer, transmission electron microscopy equipped with energy dispersive spectroscopy (TEM-EDS) and field emission scanning electron microscopy equipped with energy dispersive X-ray spectroscopy (FESEM-EDX). Fourier transforms infrared spectra were obtained to explain the changes in the functional groups of the biomass due to sulfate reduction for heavy metal removal. For FTIR analysis, control and metal loaded biomass were centrifuged (8000×*g*) for 5 min (Sigma, Sigma 1-15, Germany), washed twice with RO water and the pellets obtained were vacuum dried and analyzed using a FTIR spectroscope

(IR Affinity-1, SHIMADZU, Japan) (Singh et al., 2011). Similarly, for TEM analysis, copper loaded biomass was used as it showed the best removal efficiency among all the metals in the batch study. The pellet obtained was loaded on copper grid coated with carbon (Pacifi-Tech Grid, Cu-300CK, USA) for observation under TEM (JOEL, JEM2100, Japan) at 200 kV integrated with EDS (Jalali and Susan, 2000).

For FESEM analysis, the precipitates obtained by centrifuging the samples were oven-dried at 80 °C (Tanco, OVEN PLT-125, India) for 2 h and gold-coated in a sputter coater (Quorum, SC7620, UK and Edwards, RV3, Czech Republic) (Cao et al., 2013). The precipitates were then analyzed for morphology and elemental composition using FESEM-EDX (Zeiss, Sigma, Germany). Metal removal results obtained using the SRB were compared with the metal removal by chemical precipitation using sodium sulfide. To determine the influence of externally sulfide on heavy metal removal, different concentrations of sulfide (5, 10, 15 and 25 mg/L) were tested with two different concentrations (10 and 50 mg/L) of the heavy metals (Cd(II), Cu(II), Ni(II), Fe(III), Pb(II) and Zn(II) (The deatils of this study are presented in Appendix B).

3.3.2 Metal removal from multi-component system

Based on the results obtained from the previous study on metal removal from single component system using anaerobic biomass from three different sources, biomass that showed the best metal removal was chosen for studying the metal removal from multi-component system. In this study, different combinations of high and low concentration levels of six heavy metals, *viz.* Cd(II), Cu(II), Ni(II), Fe(III), Pb(II) and Zn(II), were chosen using the Plackett-Burman screening design. Analysis of variance (ANOVA) and student's *t* test were then applied for statistical analysis of the results to interpret the significance and effect of these metals on each other removal as well as on sulfate and COD removal in the study. The Plackett-Burman design consisted of twelve experimental runs with different combination levels of Cd(II), Cu(II), Ni(II), Fe(III), Pb(II) and Zn(II) (Table 3.1). The low and high concentration levels of each of Cd(II), Ni(II), Pb(II) and Zn(II) were chosen as 5 and 10 mg/L, respectively. Whereas, for Cu(II), these were 25 and 50 mg/L; in the case of Fe(III), 10 and 25 mg/L were chosen as the low and high initial levels, respectively.

All these initial levels of the heavy metals were based on the results obtained from the results of previous single component study using the anaerobic biomass collected from

UFAR. Input metal concentration levels used in Table 3.1, i.e., +1 and -1 indicate the high and low level of the metals, whereas 0 indicate centre point level of the metals. Individual metal stock solutions of different heavy metals used in this study were prepared as described earlier in Section 3.3.1. The modified Postgate medium as mentioned before was added with the corresponding metal stock solution so as to obtain a desired concentration of the heavy metals in each of the experimental runs. The statistical software Minitab (Version 16, PA, USA) was used for statistical analysis of the results obtained.

Table 3. 1 Plackett-Burman experimental design matrix showing different combination levels of the heavy metals in the multi-component study

Exp Run	**Different combination levels of the heavy metals (mg/L)**					
	Cd	**Cu**	**Ni**	**Fe**	**Pb**	**Zn**
1	-1(5)	-1(25)	-1(5)	-1(10)	-1(5)	-1(5)
2	+1(10)	-1(25)	+1(10)	-1(10)	-1(5)	-1(5)
3	-1(5)	-1(25)	+1(10)	+1(25)	+1(10)	-1(5)
4	-1(5)	+1(50)	-1(5)	-1(10)	-1(5)	+1(10)
5	-1(5)	+1(50)	+1(10)	+1(25)	-1(5)	+1(10)
6	-1(5)	+1(50)	+1(10)	-1(10)	+1(10)	-1(5)
7	+1(10)	+1(50)	-1(5)	+1(25)	+1(10)	-1(5)
8	+1(10)	-1(25)	-1(5)	-1(10)	+1(10)	+1(10)
9	+1(10)	-1(25)	+1(10)	+1(25)	-1(5)	+1(10)
10	+1(10)	+1(50)	-1(5)	+1(25)	-1(5)	-1(5)
11	-1(5)	-1(25)	-1(5)	+1(25)	+1(10)	+1(10)
12	+1(10)	+1(50)	+1(10)	-1(10)	+1(10)	+1(10)

All the experiments in this study were performed using 100 mL serum bottles. These bottles were purged with nitrogen gas before and after inoculation with 10% v/v anaerobic biomass (measured as MLVSS). The bottles were then incubated on an orbital shaker set at 30 °C temperature and 120 rpm agitation speed (Lab Tech, LSI-3016R, Korea). Bottles without any added metals but containing only the media, carbon source and the biomass, served as the control in these experiments. Liquid samples were taken from the bottles at regular intervals during the experiments to determine conductivity, pH, sulfate, COD, MLVSS, metal and sulfide concentrations in the samples. All these experiments were conducted in duplicate and the results presented are average of duplicate sample analysis.

3.3.2.1 Characterization of the metal bio-precipitates

Characterization of the metal bio-precipitates formed due to the SRB was carried out by FTIR spectroscopy, TEM-EDS and FESEM-EDX. For FTIR, TEM-EDS and FESEM-EDX analyses, samples from control experiment and experimental run 1 were prepared as described earlier in Section 3.3.1.1. Biomass sample from the experimental run 1 (Table 3.1) was chosen as it yielded a very high metal removal efficiency in the study. The precipitates were then analyzed for morphology and elemental composition using TEM and FESEM-EDX.

3.4 Batch Heavy Metal Removal Using Immobilized SRB Beads

3.4.1 Preparation and characterization of immobilized SRB beads

For preparing immobilized SRB beads, suspension containing the SRB culture (anaerobic biomass collected from UFAR; Section 3.3.1) was centrifuged (8000 × g for 5 min) (Remi, C24-L or R-24, India) and the obtained pellet was washed thrice with RO water before suspending in phosphate buffer saline solution (pH 7.4). The suspension was mixed with a solution containing sodium alginate at different compositions (1–5.5% w/v) for choosing the optimum concentration required (Sarma and Pakshirajn, 2011). The above mixture was then added drop-wise into chilled calcium chloride solution with stirring, which resulted in the formation of spherical shaped beads. The average size of the beads was found to be 2-3 mm. Immobilized SRB beads were then washed thrice with distilled water and tested for mechanical stability. Figure 3.1 shows the images of blank beads without SRB, SRB immobilized beads prior to their use in the experiment and SRB immobilized beads taken during the experiments at fifth day.

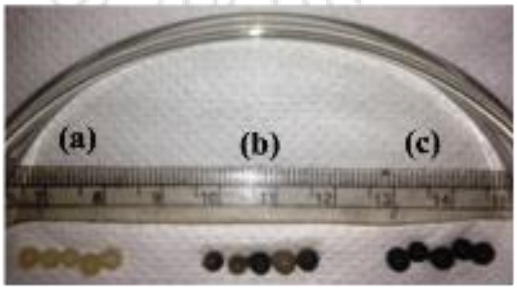


Figure 3. 1 Photograph of (a) blank beads without SRB, (b) SRB immobilized beads prior to use in the experiments (0th day) and (c) SRB immobilized beads during the experimental period at fifth day.

For the determination of mechanical stability of the beads, two grams of each type of the beads were taken in a serum bottle containing 100 mL distilled water and subjected to continuous agitation in a shaking incubator set at 30 °C and 120 rpm (Lab Tech, LSI-3016R, Korea) (Sarma and Pakshirajn, 2010). At fixed time intervals, the beads were collected and dried at ambient conditions before determining the final weight. Figure 3.2 shows the stability of the beads, which was estimated from the weight loss of the beads due to the mechanical stress induced as a result of continuous agitation.

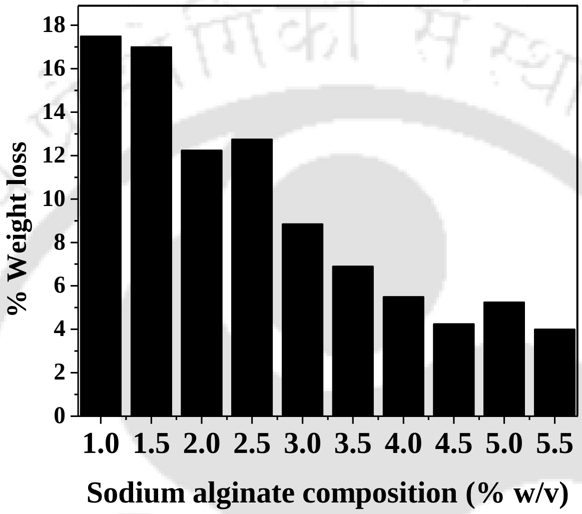


Figure 3. 2 Effect of sodium alginate on the stability of immobilized SRB beads.

3.4.2 Batch heavy metal removal

Batch metal removal experiments using the immobilized SRB beads were performed using 100 mL serum bottles with rubber stopper and aluminum crimp seal. Metal stock solutions were prepared as per the procedure described earlier in Section 3.3.1. Serum bottles filled with the SRB growth medium were added with the individual metal stock solution so as to yield a desired initial concentration (10 or 50 mg/L) of the corresponding metal. The initial metal concentrations were chosen based on heavy metal removal results obtained earlier using the free/suspended SRB described in Section 3.3.1. Prior to the metal removal experiments, immobilized SRB beads were activated by incubation under anaerobic condition at 30 °C temperature and 120 rpm agitation speed (Lab Tech, LSI-3016R, Korea) for seven days. Following activation, the immobilized SRB beads were initially washed with distilled water and two grams each of the beads (5%) were used as the inoculum source in the metal removal experiments. The bottles were then purged with nitrogen gas and incubated on a shaker incubator set at 30 °C temperature and 120 rpm shaking speed (Lab Tech, LSI-3016R, Korea).

3.4.3 Reusability and durability of the immobilized SRB beads

In order to ensure the reuse potential of the immobilized SRB beads for heavy metal removal, batch experiments were conducted with the beads using 25 mg/L initial concentrations of Ni(II), Zn(II), Pb(II), Cd(II), Fe(III) and 50 mg/L of Cu(II), respectively. Bottles with medium containing only the carbon source and the beads, but without any added metal served as control in these experiments. All these batch experiments were conducted in duplicate and the results reported are average of duplicate analysis of the samples.

3.4.4 Characterization of the immobilized SRB beads

The immobilized SRB beads prepared in this study were characterized using FESEM-EDX analysis. For FESEM analysis, the beads were washed thrice with distilled water and dried at 30 °C (Scigenics, ORBITEK^{R} LETTO, India) for 8 h and gold coated using a sputter coater prior to the analysis. In order to characterize the interior of the immobilized SRB beads, a single bead was cut into two halves and oven dried prior to FESEM-EDX analysis. Blank beads prepared using only sodium alginate and no immobilized SRB were also tested for metal removal and the results were compared with those obtained using the immobilized SRB beads.

3.5 Continuous Heavy Metal Removal Using Downflow Column Reactor (DFCR) With Immobilized SRB Beads

Continuous heavy metal removal experiments were conducted using downflow column reactor (DFCR) with immobilized SRB beads.

3.5.1 DFCR setup

The DFCR used in this study was made from a perspex tube of inner diameter (ID) 25.4 mm and effective length (L) 304.8 mm. Two sampling ports were provided at a distance of 10 cm each along the column length. The reactor was packed with immobilized SRB beads following its activation and washing with distilled water as mentioned earlier in Section 3.4.1. A schematic of the DFCR setup is shown in Fig. 3.3. The reactor was initially operated at an ambient temperature of 25 ± 2 °C for few days to attain steady state condition prior to the continuous metal removal experiments. Photograph of the DFCR packed with immobilized SRB beads is shown in Fig. 3.4. Pressure drop along the reactor was calculated as 0.83 psi or 0.0583 Kg/cm^2.

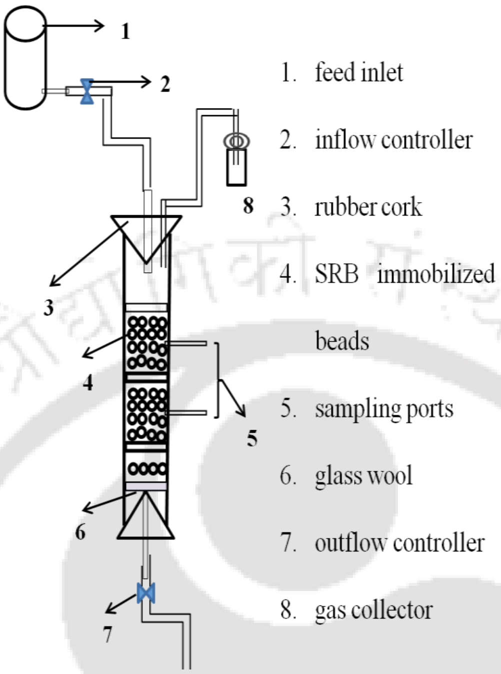


Figure 3. 3 Schematic of the DFCR with immobilized SRB beads.

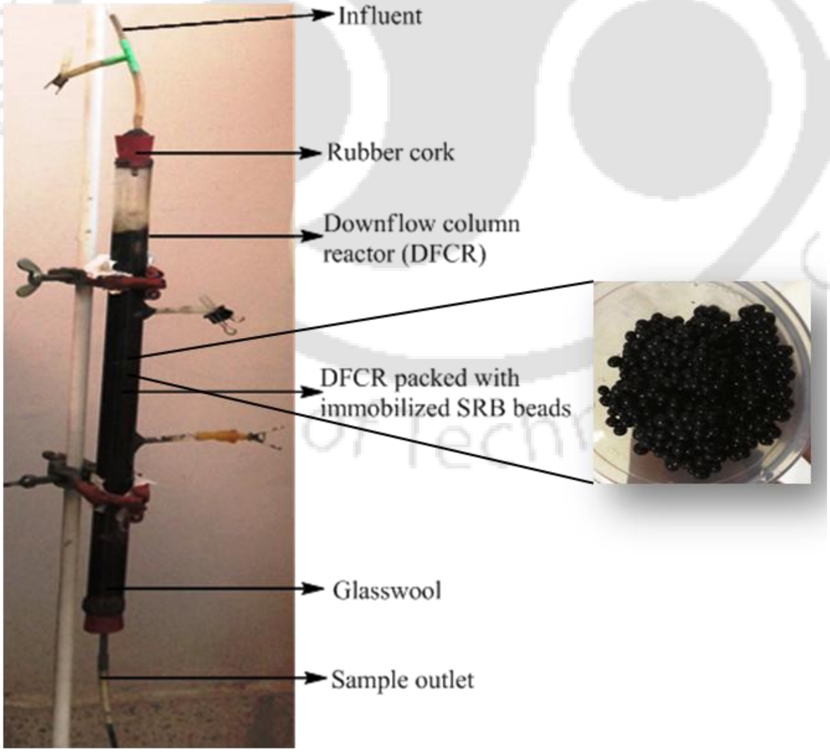


Figure 3. 4 Photograph of experimental setup showing downflow column reactor with immobilized SRB beads for heavy metal removal under continuous operation mode.

3.5.2 Single component system

3.5.2.1 Metal removal experiments

For continuous metal removal using the DFCR, medium supplemented with the respective metals was fed into the reactor at a constant flow rate. The input metal concentration levels of Ni(II), Zn(II), Pb(II), Fe(III) and Cd(II) were chosen as 50, 75 and 90 mg/L. In the case of Cu(II), these values were 100, 150 and 175 mg/L. All these initial levels of the heavy metals were chosen based on the results obtained from previous batch heavy metal removal study using the immobilized SRB beads. As metal precipitation by SRB occurs in the range of within a few days to 5 days (Kuyucak and St-Germain, 2006), two different hydraulic retention time (HRT) values were chosen for this continuous metal removal study (24 h and 48 h). Phase wise operational conditions followed for continuous metal removal using the DFCR are presented in Table 3.2. The reactor was operated at each experimental condition for a period until three steady state values of effluent heavy metal concentration at the respective HRT were obtained (Villa-Gomez et al., 2015).

Table 3. 2 Operational conditions followed with the DFCR for continuous metal removal experiments

Parameter	**Experimental phase**					
	I		II		III	
HRT (h)	24	48	24	48	24	48
Inlet metal concentration (mg/L)						
Cd(II), Ni(II), Fe(III), Pb(II) and Zn(II)	50	50	75	75	90	90
Cu(II)	100	100	150	150	175	175

Effect of metal loading on the performance of the DFCR reactor

The combined effect of inlet metal concentration and HRT on metal removal was examined by calculating the inlet metal loading rate (ILR) (mg/L·h) and the corresponding metal removal rate (mg/L·h) as per the following equations:

$$\text{Inlet metal loading rate (ILR)} = \frac{QC_i}{V} \tag{3.1}$$

$$\text{Removal rate (RR)} = \frac{Q(C_i - C_o)}{V} \tag{3.2}$$

$$\%\ \text{Metal removal} = \frac{(C_i - C_o)}{C_i} \times 100 \tag{3.3}$$

where, Q is the inlet flow rate (L/h), C_i and C_o are the inlet and outlet metal concentrations (mg/L), respectively, and V is the working volume of the reactor.

Samples collected at regular intervals of time were centrifuged at 8000 × *g* for 5 min (Remi, C24-L or R-24, India), and the supernatant obtained was analyzed for COD, sulfate, metal and sulfide concentrations.

3.5.3 Multi-component system

All experiments in this mixture study were carried out as per the statistically valid fractional factorial design (FFD) consisting of nineteen experimental runs with different combination levels of Ni(II), Cd(II), Fe(III), Zn(II), Pb(II) and Cu(II). Input metal concentration levels used in FFD of experiments are presented in Table 3.3 and these concentration levels shown are chosen based on the results obtained from batch study and the results obtained from single component system using the same bioreactor system.

Table 3. 3 Input concentration levels used in fractional factorial design of experiments

Heavy metal	**Range and levels of metals**		
	Low (-1)	Centre point (0)	High (+1)
Cd(II)	50	70	90
Cu(II)	100	137.5	175
Ni(II)	50	70	90
Fe(III)	75	82.5	90
Pb(II)	50	62.5	75
Zn(II)	50	62.5	75

Individual metal stock solutions of Fe(III), Ni(II), Cu(II), Zn(II), Pb(II) and Cd(II) of 100 g/L concentration each were prepared using $FeCl_3{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, $PbNO_3$ and $Cd(NO_3)_2{\cdot}4H_2O$, respectively. Desired metal concentration of the heavy metals in each of the experimental runs was obtained by adding corresponding metal stock solution to the modified Postgate medium as described earlier. All the experiments in this study were performed at 48 h HRT. The reactors were operated under continuous mode by supplying the medium at a constant flow rate at an ambient temperature of 25 ± 2 °C. The reactor was operated for a period until three steady state values of effluent heavy metal concentration. Samples collected at regular time intervals were centrifuged (8000 × g) for 5 min (Remi, C24-L or R-24, India), and the supernatant obtained was analyzed for sulfate, COD, metal and sulfide concentrations. The experimental design followed in this study with different metal combination levels is presented in Table 3.4, in which +1 and -1 indicate the high and low level of the metals, whereas 0 indicate centre point level of the metals.

Table 3. 4 Fractional factorial experimental design matrix showing different combination levels of the heavy metals in mixture using the DFCR

Experimental runs	**Cd**	**Cu**	**Ni**	**Fe**	**Pb**	**Zn**
1	+1	-1	-1	-1	+1	-1
2	+1	-1	+1	+1	-1	-1
3	-1	+1	-1	+1	+1	-1
4	+1	+1	+1	+1	+1	+1
5	-1	-1	-1	+1	-1	+1
6	+1	-1	-1	+1	+1	+1
7	-1	+1	-1	-1	+1	+1
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	+1	+1	-1	-1	-1	+1
11	-1	+1	+1	+1	-1	+1
12	-1	+1	+1	-1	-1	-1
13	+1	-1	+1	-1	-1	+1
14	+1	+1	-1	+1	-1	-1
15	-1	-1	+1	+1	+1	-1
16	0	0	0	0	0	0
17	+1	+1	+1	-1	+1	-1
18	-1	-1	-1	-1	-1	-1
19	-1	-1	+1	-1	+1	+1

Precipitates obtained from experimental run 18 performed using the DFCR which yielded a maximum heavy metal removal efficiency in this study was analyzed for elemental composition and morphology by means of FESEM-EDX (Zeiss, Sigma, Germany) as per the procedure described earlier in the Section 3.3.1.1. Results reported are average of three steady state values. For the assessment of individual and collective effect of the metals, the results obtained from the fractional factorial design were analyzed in the form of student's *t* test and ANOVA. The statistical software, Minitab (Version16, PA, USA) was used for designing the fractional factorial experiments and for statistical analysis of the results obtained. The effect due to a particular factor was defined as either reduction (negative) or improvement (positive) in the responses.

3.6 Continuous Heavy Metal Removal Using The Anaerobic Rotating Biological Contactor Reactor

Continuous heavy metal removal experiments were conducted using SRB immobilized anaerobic rotating biological contactor (An-RBC) reactor.

3.6.1 An-RBC reactor setup

A laboratory scale An-RBC reactor with a total working volume of 3 L was fabricated using polymethyl methacrylate (PMMA) cylinder material of length 70 cm and inner diameter 9 cm. The cylinder was cut transversely into two halves such that the upper half was used as the lid and the lower half served as the reactor vessel. Both the ends of the vessel were sealed using semicircular plates. For sample collection, four sample ports were provided at the bottom of the vessel with a spacing of 2 cm between each (Pakshirajan and Kheria, 2012).

The reactor was divided into two compartments (stages) each with seven PMMA discs (8 cm in diameter) by fitting a semicircular plate in the middle of the vessel. A nylon rod connected to a variable speed motor located outside the working unit passed through central hole in the discs and was attached firmly by means of screws to enable rotation of the discs inside the vessel (Pakshirajan and Kheria, 2012). Polyurethane foam (PUF) of 5 mm thickness and polystyrene mesh (0.6 mm thick) covering the discs were used as the bio-support medium for immobilizing the SRB biomass in the reactor. With 40% disc submergence in the liquid portion of the reactor, immobilization of the SRB biomass was initiated by adding freshly prepared medium (Pakshirajan and Kheria, 2012). Table 3.5 presents the design specifications of the An-RBC reactor used in this study. A schematic of the reactor is shown in Fig. 3.5.

Table 3. 5 Design specification of the An-RBC reactor

Specifications	
Number of stages	2
Number of disks in each stage	7
Diameter of each disk	16 cm
Spacing between each stage	2 cm
Total working volume	3 L
Disc Submergence	40%

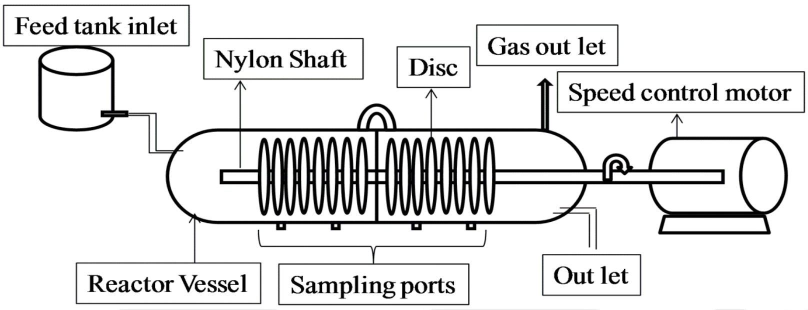


Figure 3. 5 Schematic of the An-RBC reactor setup.

3.6.2 Biofilm development

Sulfate reducing bacteria present in the anaerobic biomass collected from UFAR, which showed maximum metal removal efficiency in the batch study was used in this continuous An-RBC reactor study. For SRB immobilization in the reactor, the culture was allowed to grow onto the reactor discs covered with polystyrene mesh and PUF, which served as the bio-support material. For SRB growth during the reactor startup phase, modified Postgate medium at pH 7 and with the SRB biomass, but without any added metal were supplied to the reactor. The An-RBC reactor was operated at an ambient temperature of 25 ± 2 °C in batch mode and the reactor was recharged with a fresh medium after every five days for over three months during this startup phase. Change in color of the input medium from colorless to black at the outlet due to the formation of FeS and generation of hydrogen sulfide during this startup phase confirmed SRB growth and activity in the reactor (Herbert and Gilbert, 1984; Hamilton, 1994; Singh et al., 2011). Photographs of the An-RBC reactor before and after immobilization with SRB are shown in Figs. 3.6 and 3.7. However, no efforts were made to measure the amount of biomass immobilized in the reactor. Later, the An-RBC reactor was operated under continuous mode by supplying the medium at a constant flow rate for performing the heavy metal removal experiments.

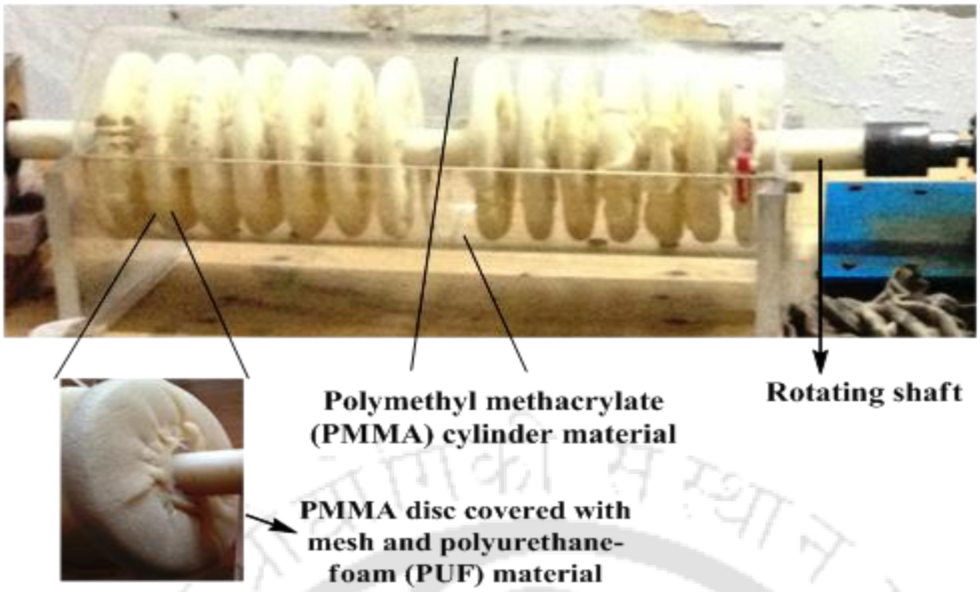


Figure 3. 6 Photograph of the experimental setup showing the rotating biological contactor (RBC) reactor prior to immobilization with SRB.

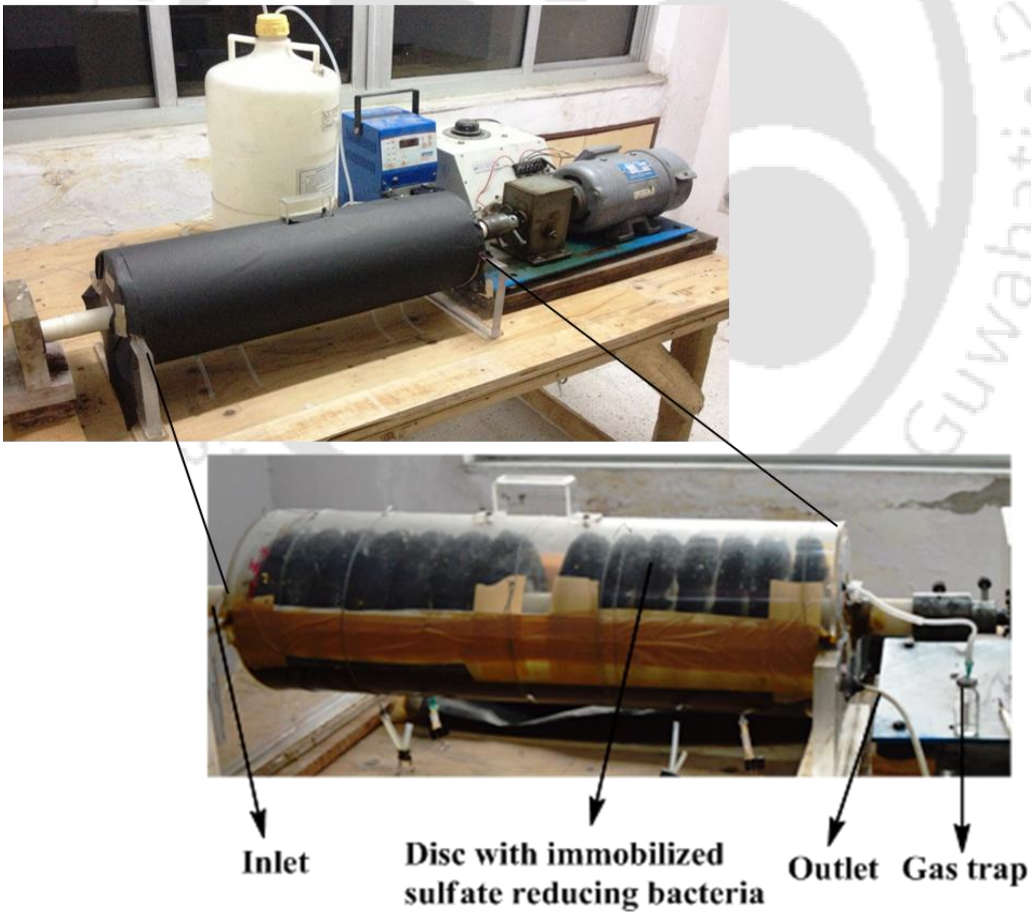


Figure 3. 7 Photograph of experimental setup showing the rotating biological contactor (RBC) reactor with the immobilized SRB on its discs.

3.6.3 Characterization of the bio-support material

Characterization of the bio-support material utilized for immobilizing the SRB anaerobic biomass was carried out by FESEM analysis, for which the SRB immobilized bio-support material was fixed using 3% glutaraldehyde for 2 h followed by dehydration using different concentrations of ethanol and by drying at 30 °C (Scigenics, ORBITEK^R LETTO, India) (Utgikar et al., 2002). The finally prepared sample was then observed using a FESEM.

3.6.4 Heavy metal removal experiments

3.6.4.1 Single component system

Experiments for studying heavy metal removal from single component system using the An-RBC reactor were carried out under continuous mode of operation. Individual metal stock solutions were prepared as per the procedure described in Section 3.3.1. The modified Postgate medium as described earlier was added with the corresponding metal stock solution so as to obtain a desired concentration of the heavy metals in each of the experimental phases. The initial concentration for each of the metals Fe(III), Pb(II), Ni(II), Zn(II) and Cd(II) were chosen as 50, 75 and 90 mg/L. Whereas, in case of Cu(II), these values were 100, 150 and 175 mg/L. All these initial levels of the heavy metals were chosen based on the results of batch heavy metal removal using the SRB (Section 3.3.1). Phase wise operational conditions followed with the An-RBC reactor are presented in Table 3.6.

Table 3. 6 Operational conditions followed with the An-RBC reactor for continuous metal removal experiments

Parameter	**Different experimental phases**					
	I		II		III	
HRT (h)	48	24	48	24	48	24
Inlet metal concentration (mg/L)						
Cd(II), Ni(II), Fe(III), Pb(II) and Zn(II)	50	50	75	75	90	90
Cu(II)	100	100	150	150	175	175

The reactor performance in terms of heavy metal removal efficiency was evaluated at two different HRT values (24 h and 48 h) as described earlier (Table 3.6). Each experiment was carried out for a period until three steady state values of effluent heavy metal concentration at the respective HRT were obtained (Villa-Gomez et al., 2015).

Effect of metal loading on the performance of the An-RBC reactor

The combined effect of inlet metal concentration and HRT on metal removal was examined by calculating the ILR (mg/L·h) and the corresponding metal removal rate (mg/L·h) as per the equations 3.1 - 3.3 mentioned earlier (Section 3.5.2.1). Samples collected at regular time intervals were centrifuged at 8000 × *g* for 5 min (Remi, C24-L or R-24, India), and the supernatant obtained was analyzed for metal, sulfate, sulfide and COD concentrations. Each sample analysis was carried out in duplicate and the results presented are average of duplicate sample analysis.

3.6.4.3 Identification and characterization of SRB

Sample preparation for V3-V4 Metagenomics Sequencing and Analysis

For SRB characterization, the bacterial culture from the An-RBC reactor was collected after completing the three phases of continuous operation of the reactor (Table 3.6) and grown in the presence of modified Postgate medium using serum bottles at 30 °C temperature under continuous agitation (120 rpm) (Scigenics, ORBITEK^{R} LETTO, India) for seven days. The culture was then sent to AgriGenome Labs Pvt Ltd, India for V3-V4 metagenomics sequencing and analysis for its detailed characterization. Complete details of V3-V4 metagenomics sequencing and analysis are presented in Appendix C.

3.7. Multi-component System

All experiments in this mixture study were carried out as per the statistically valid fractional factorial design consisting of nineteen experimental runs with different combination levels of Ni(II), Cd(II), Fe(III), Zn(II), Pb(II) and Cu(II). Input metal concentration levels used in FFD of experiments are presented in Table 3.7, which are almost similar to those presented in Table 3.3. Metal concentration levels shown in Table 3.7 are based on the results obtained from single component system using the An-RBC reactor. Individual metal stock solutions were prepared as per the procedure described in Section 3.5.3. Desired metal concentration of the heavy metals in each of the experimental runs was obtained by adding corresponding metal stock solution to the modified Postgate medium as described earlier. All the experiments in this study were performed at 48 h HRT based on the results obtained from single component system. The experimental design followed using the An-RBC reactor with different metal combination levels is presented in Table 3.8, which closely resembles the details presented in Table 3.4.

Table 3. 7 Input concentration levels used in fractional factorial design of experiments

Input variables	**Range and levels of variables**		
	Low (-1)	**Centre point (0)**	**High (+1)**
Cd(II)	50	70	90
Cu(II)	100	137.5	175
Ni(II)	50	70	90
Fe(III)	50	70	90
Pb(II)	50	70	90
Zn(II)	50	62.5	75

Table 3. 8 Fractional factorial design of experiments presenting different heavy metal combination levels tested in the mixture using the An-RBC reactor

Experimental runs	**Cd**	**Cu**	**Ni**	**Fe**	**Pb**	**Zn**
1	+	+	+	-	+	-
2	-	+	+	-	-	-
3	-	-	+	-	+	+
4	-	-	+	+	+	-
5	-	-	-	-	-	-
6	-	+	-	+	+	-
7	+	-	+	+	-	-
8	+	-	-	+	+	+
9	0	0	0	0	0	0
10	-	-	-	+	-	+
11	+	-	-	-	+	-
12	-	+	+	+	-	+
13	-	+	-	-	+	+
14	+	-	+	-	-	+
15	+	+	+	+	+	+
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	+	+	-	+	-	-
19	+	+	-	-	-	+

The reactor was operated under continuous mode by supplying the medium at a constant flow rate at an ambient temperature of 25 ± 2 °C. Samples collected at regular time intervals were centrifuged (8000 × g) for 5 min (Remi, C24-L or R-24, India), and the supernatant obtained was analyzed for sulfate, COD, metal and sulfide concentrations.

FEEM-EDX analysis of the bio-precipitates acquired from experimental run 5 which yielded a maximum heavy metal removal efficiency using the An-RBC reactor was carried out as per the procedure described earlier in the Section 3.3.1.1. The reactor was operated for a period until three steady state values of effluent heavy metal concentration. Results reported are average of three steady state values.

3.8 Analytical Methods

Heavy metals

Metal concentration in the samples was determined using a flame emission atomic absorption spectrometer (AAS) (Varian, AA240, The Netherlands) as per the methods described in American Public Health Association (APHA) (APHA, 2005). The standard solutions of these metals were prepared using the same stock solutions as mentioned earlier (Section 3.3.1). Metal concentration of the samples was estimated using a standard plot prepared for each of the metals tested in the study.

Chemical oxygen demand

Chemical oxygen demand in the samples was determined following the closed reflux method (APHA, 2005). In this method, 2.5 ml of a suitably diluted sample taken in COD vials was 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 using a COD digester (Hach, DRB 200, USA) at 150 °C for 2 hours. Later, the solution mixture was 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 was considered as the end point. Reverse osmosis water was used as the blank in the COD analysis. The FAS solution was standardized against standard 0.1 M $K_2Cr_2O_7$ digestion solution regularly. COD in the sample was thus calculated using the following equation 3.4 and was expressed as mg O_2/L.

$$\text{Chemical oxygen demand (COD) (mg O}_2\text{/L)} = \frac{(\text{A - B}) \times 8000 \times \text{M}}{\text{V mL (Sample)}} \qquad (3.4)$$

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

Sulfate and sulfide

Sulfate concentration in the liquid samples was measured by the standard turbidimetric method (APHA, 2005). The samples prior to the determination of sulfate were pretreated with $ZnCH_3COONa$ (1N) and NaOH (6N) in order to fix the sulfide present (Sabumon, 2008) and centrifuged at 8000 × g for 5 minutes (Remi, C24-L or R-24, India).

Buffer solution prepared using RO water containing KNO_3, $MgCl_2 \cdot 6H_2O$, CH_3COONa and 99% glacial acetic acid was added to a suitable portion of the diluted supernatant solution along with 1 mL of 0.5 M $BaCl_2$ (Garcia et al., 2001). The mixture was continuously stirred before measuring its turbidity using a digital nephelo-turbidity meter (Systronics, DNTM 132, India). Sulfate concentration in the samples was determined from a calibration curve prepared using different concentrations of standard sulfate versus absorbance in terms of Nephelometric turbidity units (NTU), as shown in Fig. 3.8.

The dissolved sulfide concentration in the liquid samples was determined as per the method described by Cord Ruwish (Cord Ruwish, 1985). For this analysis, supernatant (50 μL) obtained by centrifuging the sample was added with copper reagent solution, prepared using 50 mmol/L HCl and 5 mmol/L $CuSO_4$ under continuous stirring condition. The absorbance of the resulting mixture was measured at 480 nm using a Ultraviolet (UV)–visible spectrophotometer (Cary 100, Agilent, Singapore). A mixture of 50 μL culture liquid and 1.95 mL HCl (50 mmol/L) served as the blank. Sulfide concentration in the samples was determined from a calibration curve prepared using different concentrations of standard sulfide versus absorbance at 480 nm, as shown in Fig. 3.9.

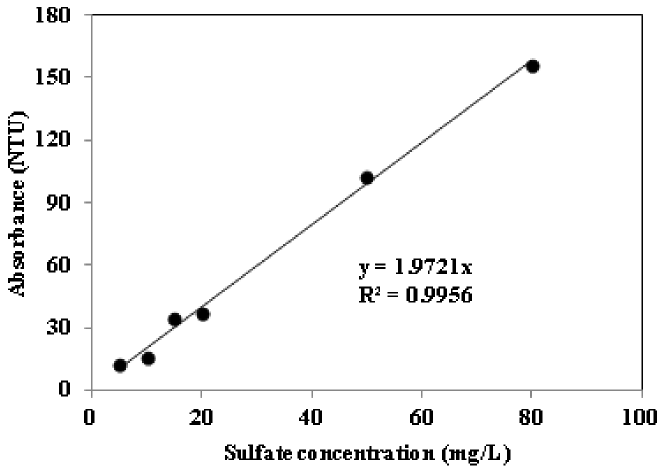


Figure 3. 8 Standard curve of sulfate concentration vs. absorbance (NTU) used in the estimation of sulfate content in the samples.

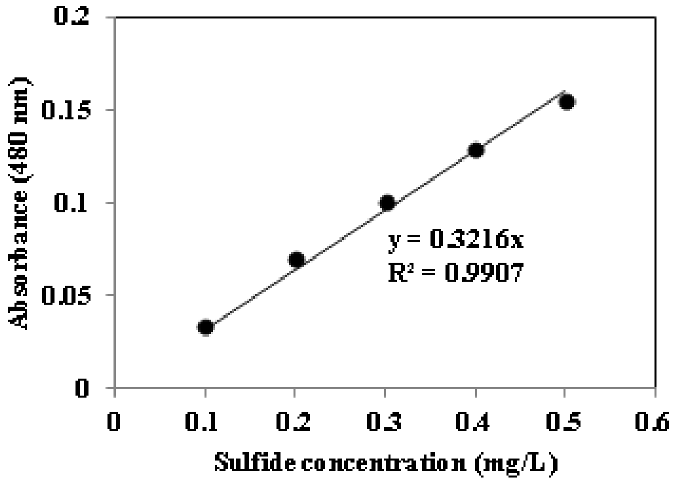


Figure 3. 9 Standard curve of sulfide concentration vs. absorbance at 480 nm used in the estimation of soluble sulfide content in the samples.

CHAPTER 4

RESULTS AND DISCUSSION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The key highlight of the present study as outlined in the objectives was to develop a suitable continuous reactor system for toxic heavy metal removal using mixed consortia of sulfate reducing bacteria (SRB). In this chapter, results of batch and continuous reactor studies carried out on the removal of cadmium (Cd(II)), copper (Cu(II)), nickel (Ni(II)), iron (Fe(III)), lead (Pb(II)) and zinc (Zn(II)) from synthetic wastewater using SRB are presented and discussed.

4.1 Batch Heavy Metal Removal From Single Component System

Batch experiments were performed to select the best source of SRB biomass for heavy metal removal.

4.1.1 Screening of biomass sources for heavy metal removal

4.1.1.1 Effect of initial metal concentration

Three anaerobic biomass samples were screened on the basis of their sulfate reduction, metal and chemical oxygen demand (COD) removal efficiency. Anaerobic biomass was collected from three different sources, namely wastewater treatment plant (WWTP) located at Indian Institute of Technology Guwahati, Guwahati, Assam, India, a lab-scale upflow anaerobic packed bed reactor (UFAR) and another lab-scale anaerobic packed bed reactor (APR) treating sulfate rich water containing arsenic.

The effect of the metal addition on sulfate along with COD reduction was evaluated during the experiments. Figure 4.1 shows the metal removal efficiency obtained using anaerobic biomass from the three sources at 10 mg/L initial metal concentration. More than 95% removal was achieved for all the metals using the anaerobic biomass from UFAR (Fig. 4.1b). In the case of biomass from WWTP and APR, more than 90% removal was achieved for all the metals, but for zinc, the removal values were slightly low (85% and 88%, respectively) (Fig. 4.1a-4.1c).

At 50 mg/L initial concentration, the metal removal efficiency was, however, reduced using the three biomass sources (Fig. 4.2). At 50 mg/L initial metal concentration, more than 65% removal of copper, iron, lead and zinc was obtained using the WWTP biomass, whereas cadmium removal was only 46%. In the case of nickel, it was 60% (Fig. 4.2a). Over 77% removal was achieved for all the metals using the UFAR biomass (Fig. 4.2b). Biomass obtained from APR resulted in 60% cadmium removal and more than 70% removal with the other metals (Fig. 4.2c).

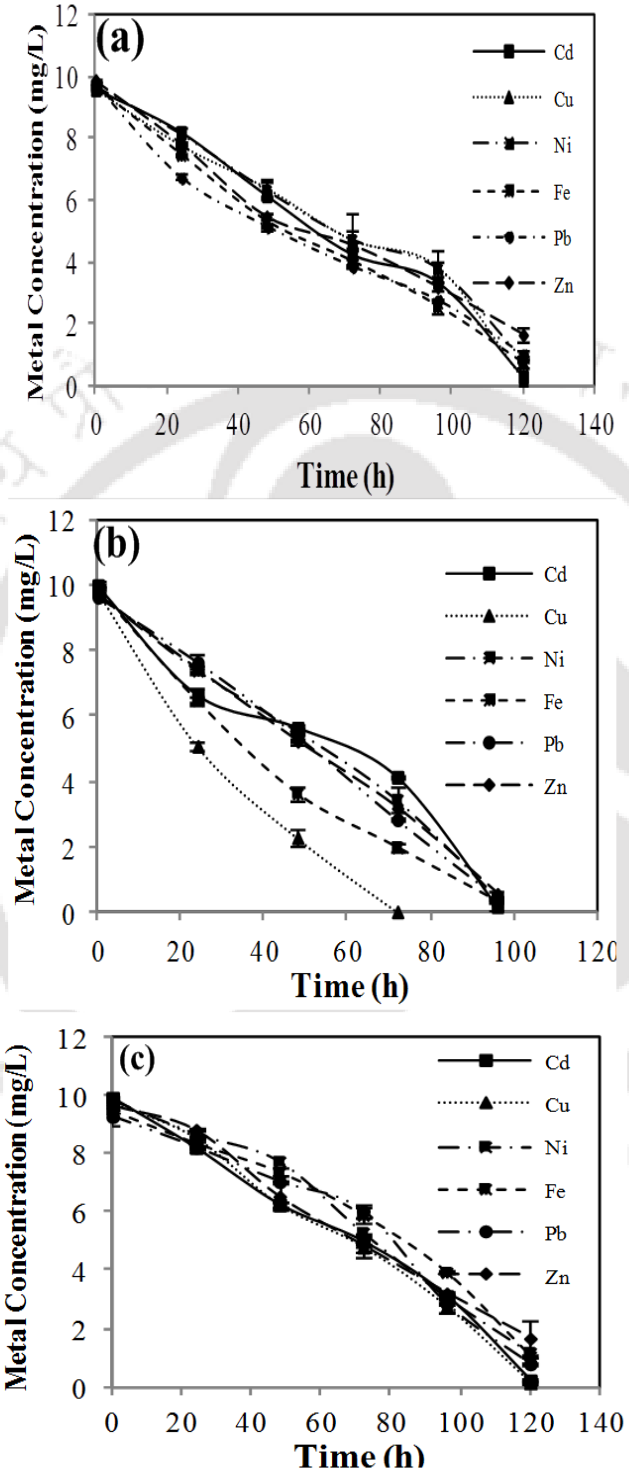


Figure 4. 1 Heavy metal removal using biomass from different sources: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 10 mg/L.

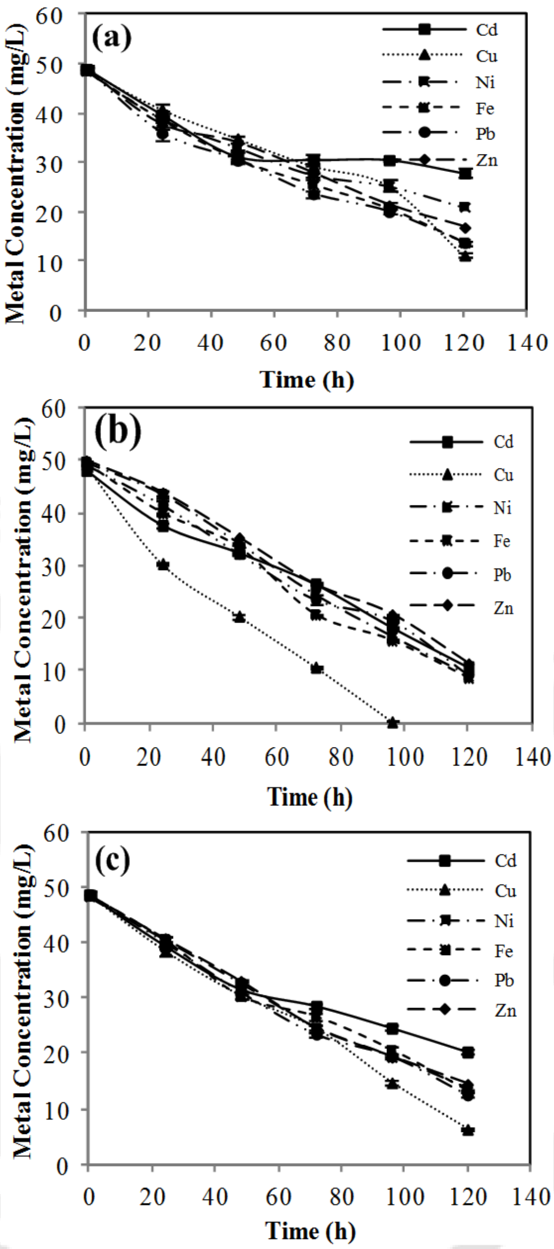


Figure 4. 2 Heavy metal removal using biomass from different sources: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 50 mg/L.

The results of sulfate reduction at 10 mg/L and 50 mg/L initial concentrations by the biomass from different sources are depicted in Figs. 4.3-4.4, respectively. At 10 mg/L initial metal concentration, the biomass from WWTP resulted in a low sulfate reduction of 55% in the presence of cadmium, copper, nickel and lead. In the presence of iron and zinc, the sulfate reduction was even less at 51% (Fig. 4.3a). Biomass from UFAR yielded 98% sulfate reduction except with nickel, in which case, it was slightly less at 94% (Fig. 4.3b). More than 85% sulfate reduction was achieved using the APR biomass with the different metals (Fig. 4.3c).

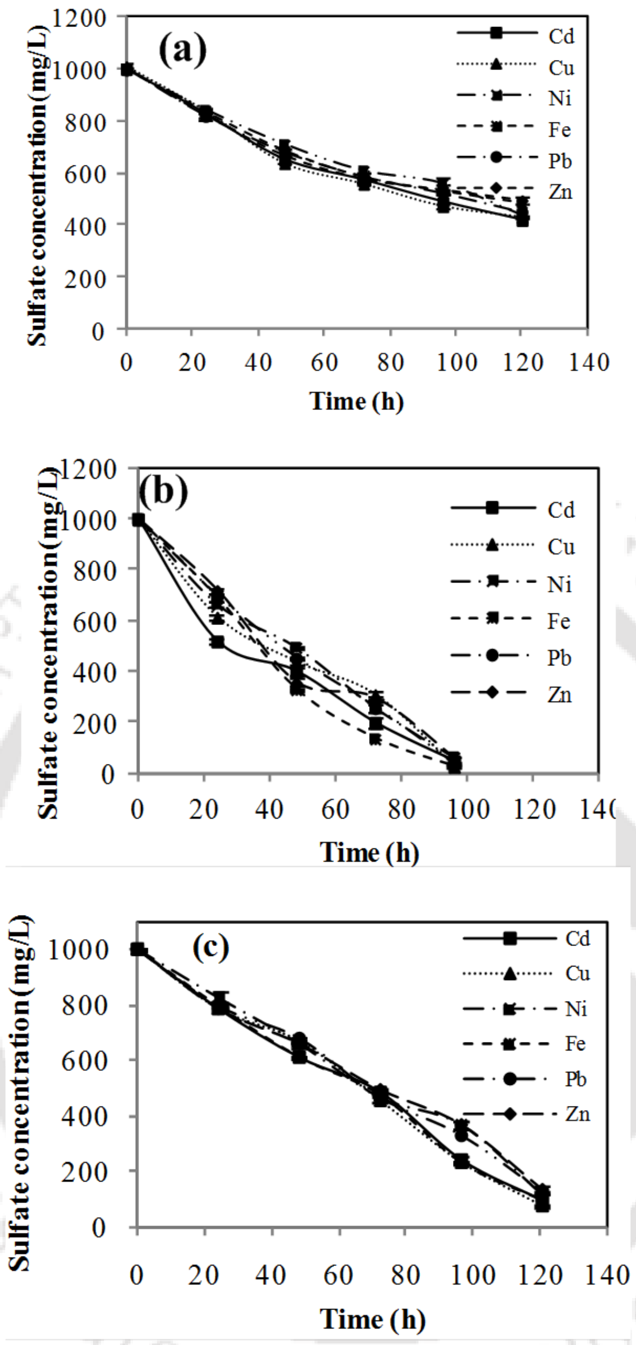


Figure 4. 3 Sulfate reduction in the presence of different heavy metals by biomass from: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 10 mg/L.

The biomass from WWTP yielded 50% sulfate reduction in the presence of the individual metals at 50 mg/L initial concentration, except with cadmium, which resulted in only 28.73% reduction (Fig. 4.4a). More than 91% sulfate reduction was achieved using the UFAR biomass (Fig. 4.4b). The APR biomass resulted in more than 70% sulfate reduction in the presence of copper, nickel, iron and lead, whereas in the presence of cadmium and zinc, the sulfate reduction was 68% and 66%, respectively (Fig. 4.4c).

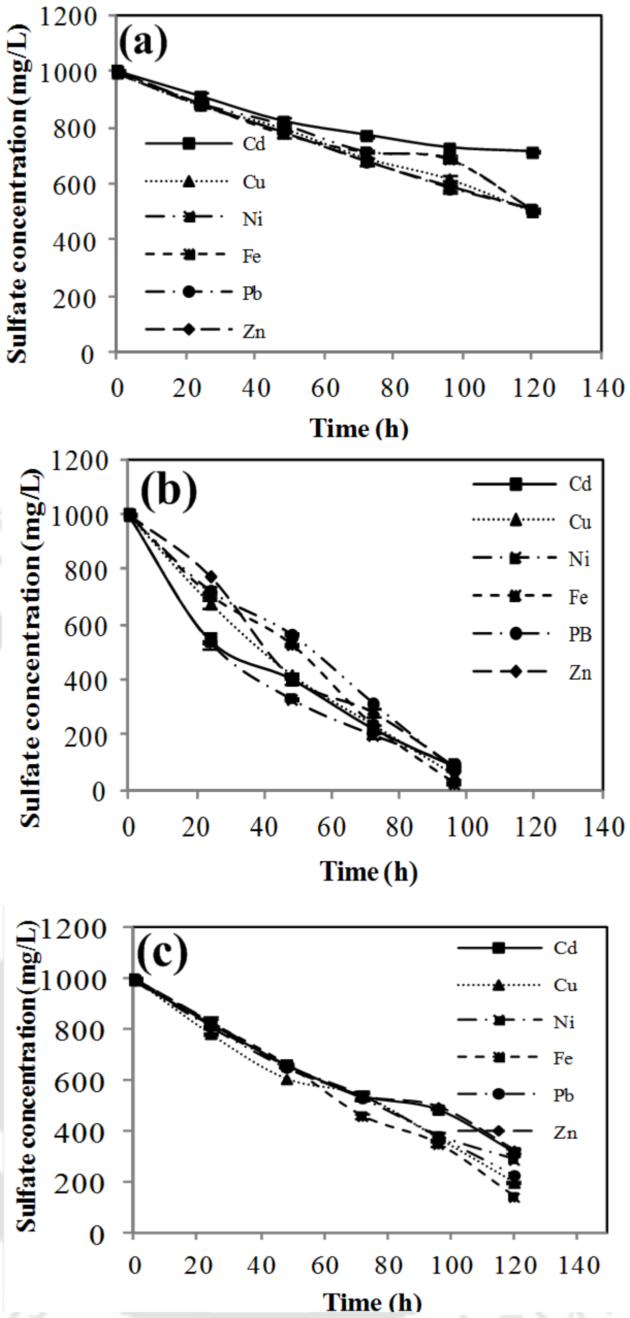


Figure 4. 4 Sulfate reduction in the presence of different heavy metals by biomass from: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 50 mg/L.

Figures 4.5 and 4.6 depict COD removal in the presence of different metals at 10 and 50 mg/L initial concentration by biomass from three sources. More than 75% COD removal was achieved in the presence of iron, nickel, lead, cadmium and zinc at 10 mg/L initial concentration by WWTP biomass (Fig. 4.5a). This value was higher (81%) in case of copper. Over 85% COD removal was achieved in presence of all the metals at an initial concentration of 10 mg/L using the biomass from UFAR and APR (Fig. 4.5b-4.5c).

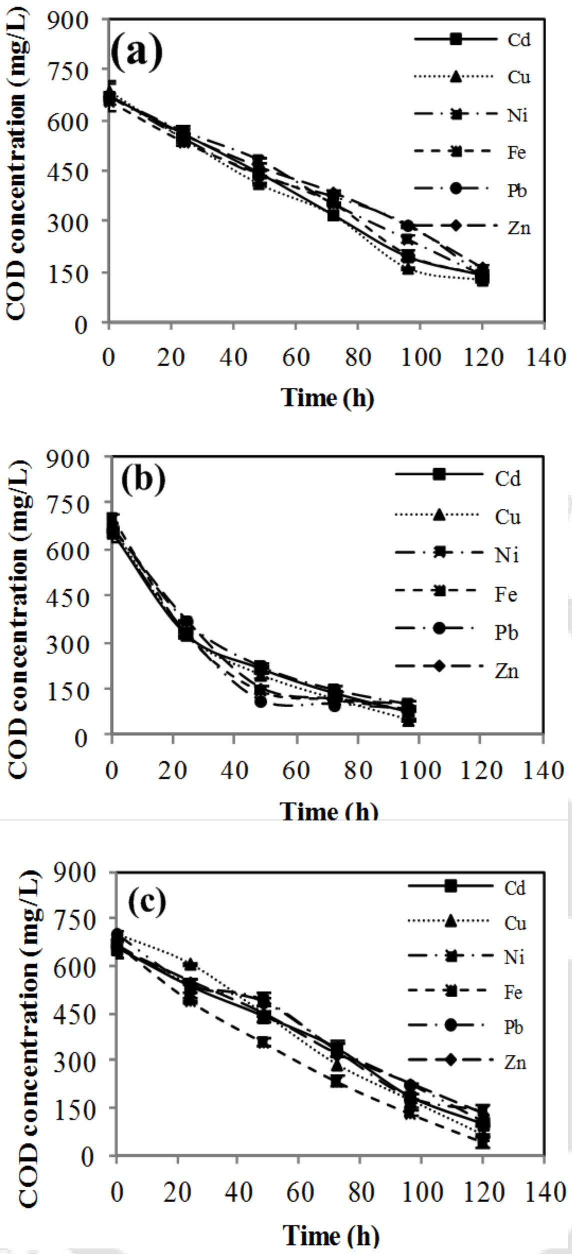


Figure 4. 5 COD removal in the presence of different heavy metals by biomass from: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 10 mg/L.

Removal of COD in the presence of metals at 50 mg/L initial concentration was lower than that obtained at 10 mg/L initial metal concentration. Biomass from WWTP resulted in more than 65% COD removal in the presence of metals, except for zinc (64% removal) (Fig. 4.6a). More than 78.5% COD removal was achieved for all the metals using the UFAR biomass (Fig. 4.6b). Over 70% COD removal was obtained in the presence of copper, nickel, iron and lead; these values were 52.3%, 57% for cadmium and zinc, respectively using the APR biomass (Fig. 4.6c).

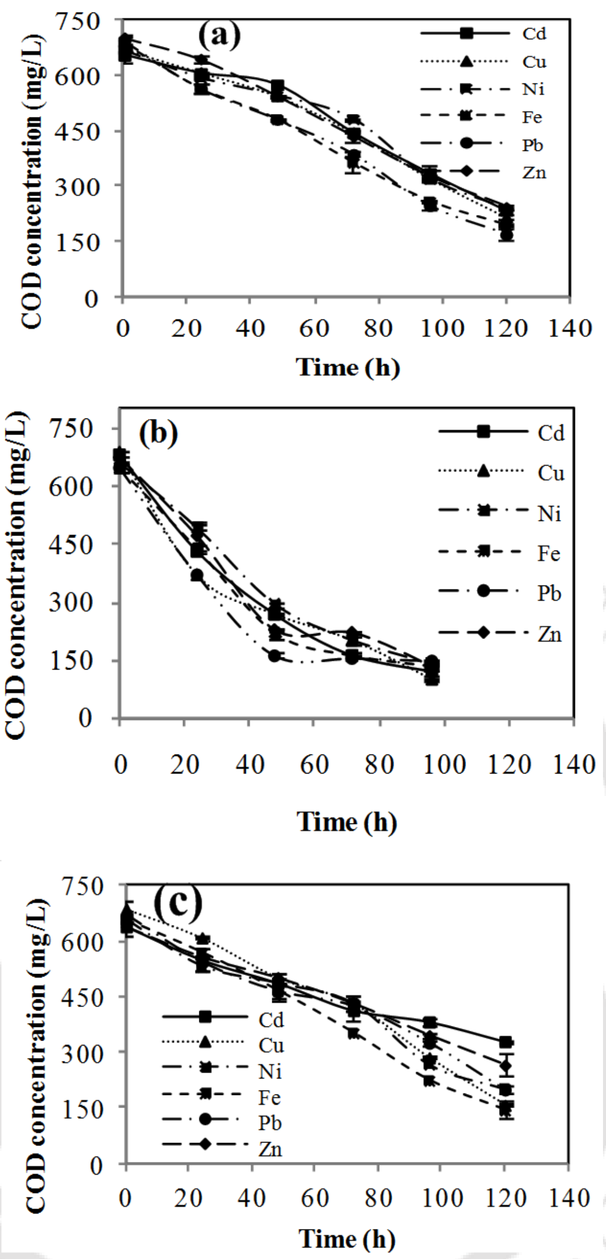


Figure 4. 6 COD removal in the presence of different heavy metals by biomass from: (a) WWTP, (b) UFAR and (c) APR. Initial metal concentration = 50 mg/L.

4.1.1.2 Heavy metal removal mechanism

The results of heavy metal removal shown in Figs. 4.1-4.2 and the sulfate reduction results shown in Figs. 4.3-4.4 correlated well with each other, suggesting sulfate reduction by SRB present in the different biomass sources for heavy metal removal. Among the various heavy metals tested, copper was removed to the maximum extent due to its low solubility product value with sulfide. Similar observation can be made on the removal of the other metals by the three different biomass sources. The solubility product values of CuS, PbS, NiS, ZnS, FeS and CdS are 6×10^{-37}, 3×10^{-28}, 3×10^{-19}, 2×10^{-25}, 6×10^{-19} and 8×10^{-28}, respectively (de Vegt et al., 1998; Hil et al., 2005; Johnson and Hallberg, 2005). Thus, the order of removal of these metals as sulfide salts easily correlates with their solubility product values of the corresponding salts. The results shown in Figs. 4.1 and 4.3 further suggest that metals at 10 mg/L initial concentration did not inhibit the growth of SRB present in the three biomass sources. However, an increase in the initial metal concentration from 10 mg/L to 50 mg/L in the medium resulted in a reduced activity of the biomass and, therefore, its sulfate reduction efficiency, which negatively influenced the heavy metal removal efficiency (Figs. 4.4 and 4.2). Among the three biomass sources, the biomass from UFAR and APR resulted in a better metal removal compared with the WWTP biomass (Figs. 4.1 and 4.2), probably due to its prior acclimatization to sulfate reduction.

Toxic levels of the metals on SRB growth and its activity are presented in Table 4.1 (Azabou et al., 2007). Low concentrations of heavy metals are able to substitute for essential ions necessary for proper buildup of the biomass structure. At high concentrations, these metals are toxic to microorganisms as they lead to deactivation of enzymes and may also damage the cellular membrane and cause protein denaturation (Kikot et al., 2010).

Table 4. 1 Minimum inhibitory concentration (MIC) range of different heavy metals on mixed consortia of SRB

Metals	**Cd**	**Cu**	**Ni**	**Fe**	**Pb**	**Zn**
MIC[a] (mg/L)	4-54	2-50	10-20	>60	75-125	13-40

[a] Minimum inhibitory concentration

Among the different biomass sources, the UFAR biomass showed the maximum sulfate reduction in the presence of the individual metals (Fig. 4.3b). This can be easily attributed to the earlier acclimatization of the biomass to sulfate reduction in the UFAR and APR, which was not the case with the biomass from WWTP. However, at 50 mg/L initial metal concentration, sulfate reduction efficiency was reduced (Fig. 4.4b), probably due to the toxicity induced by the metals (Jong and Parry, 2003).

The decrease in sulfate reduction efficiency at a high initial metal concentration in the medium can also be attributed to the hindrance due to the precipitated metal sulfides in the media (Utgikar et al., 2002; Jong and Parry, 2003). The existence of the fine solid precipitates in the vicinity of the SRB cells can act as a physical barrier, thus partially blocking the transport of sulfate ions and other substrates into the cell cytoplasm (Barbosa et al., 2014). Though the sulfate reduction was slightly low at a high metal concentration, the amount of sulfate reduced was sufficient to precipitate the metals as sulfide, which is evident from Figs. 4.1 and 4.2. COD reduction results shown in Figs. 4.5 and 4.6 revealed a strong effect of the metals on the activity of SRB, particularly the presence of metal at a high initial concentration.

Figure 4.1 showed that the removal efficiency of all the metals was maximum at a low initial concentration of 10 mg/L. At high initial concentration (50 mg/L), the removal efficiency was slightly less. These can be attributed to the fact that the metal removal by sulfate reduction and therefore, metal sulfide precipitation is mainly dependent on M/S^{2-} ratio (Villa-Gomez et al., 2015), where M is the initial metal concentration and S^{2-} is the dissolved sulfide concentration in the liquid phase. At a low M/S^{2-} ratio (<1), sulfide produced is high, thereby ensuring metal sulfide precipitation and also any toxic effect due to very low residual metal ions in solution on the sulfate reduction process becomes insignificant.

At M/S^{2-} ratio greater than 1, the residual metal concentration exerts significant toxic effect on the organism, thereby lowering the sulfate reduction efficiency and, therefore, its own removal (Fig. 4.4) (Villa-Gomez et al., 2015). Among the three biomass types investigated in this study, the anaerobic biomass collected from UFAR showed very high removal of different metals at both low and high initial concentrations. Further, characterization of the metal precipitates formed due to the anaerobic biomass collected from the UFAR, which proved to be the best among the three biomass sources, was carried out using different techniques.

4.1.1.3 Characterization of metal precipitates

The anaerobic biomass from UFAR, which showed maximum metal removal as well as sulfate reduction was characterized using Fourier transforms infrared spectrometer (FTIR), transmission electron microscopy integrated with energy dispersive spectroscopy (TEM-EDS), field emission scanning electron microscopy equipped with energy dispersive X-ray spectroscopy (FESEM-EDX) analyses in order to further probe into the sulfate reduction mechanism for heavy metal removal. The FTIR spectra of the anaerobic biomass from

UFAR were obtained to determine the functional groups responsible for heavy metal removal by sulfate reduction. Figure 4.7 shows that the major stretching occurred in the range 500-4000 cm^{-1}. It further indicates the appearance of sharp peaks in the region from 522 cm^{-1} to 1558 cm^{-1}. Presence of neutral C=O complex in the control biomass is indicated by their stretching modes at 1665 cm^{-1}.

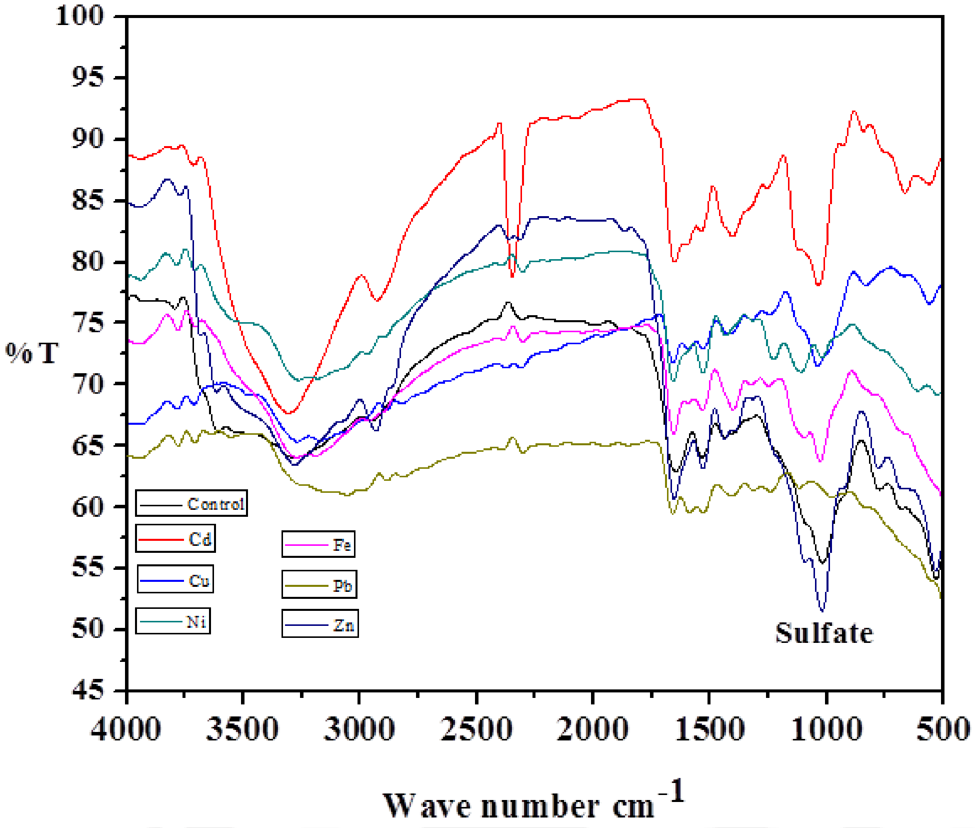


Figure 4. 7 FTIR spectra of the UFAR biomass obtained at the end of the metal removal experiments.

Bands corresponding to the stretching mode from 1525 to 1558 cm^{-1} are mainly due to -NH stretching and can be attributed to protein amide I and amide II bands (Quan et al., 2013). Bands corresponding to the range from 3748 to 3764 cm^{-1} indicate either N-H stretching of amine group or O-H stretching of hydroxyl group. Similar studies on spectroscopic investigations from the literature report that sulfate ions exhibit five bands centered at 1230 cm^{-1}, 1130 cm^{-1}, 1070 cm^{-1}, 1000 cm^{-1} and 610 cm^{-1} (Nakamoto, 1970; Feio Maria et al., 2004). The FTIR spectra (Fig. 4.7) of the metal laden biomass showed bands corresponding to the sulfate ions, which clearly indicate their utilization for its growth and metabolism (Singh et al., 2011).

To understand the morphology and elemental composition of the metal precipitates formed using the UFAR biomass, TEM-EDS, FESEM-EDX analyses of the precipitates were carried out. Figure 4.8 shows the TEM image of the copper laden biomass, which among the different metals showed maximum removal efficiency. Figure 4.8a displays clear image of a single bacterial cell associated with copper in the form of copper sulfide as confirmed by the EDS spectrum on the outer cell surface (Fig. 4.8b).

The TEM image (Fig. 4.8a) confirmed the ability of these SRB to grow in presence of copper in the media. The image also revealed the presence of a layer or shell like structure on cell wall of the bacteria. It has been reported that these naturally produced polymers bind the bacterial cells physically or electrostatically and help in sequestering of other constituents in the medium as co-aggregates (Geesey and Jang, 1989). The presence of precipitated copper sulfide on outer surface of the bacteria was confirmed by EDS indicating sulfide and copper peaks (Fig. 4.8b).

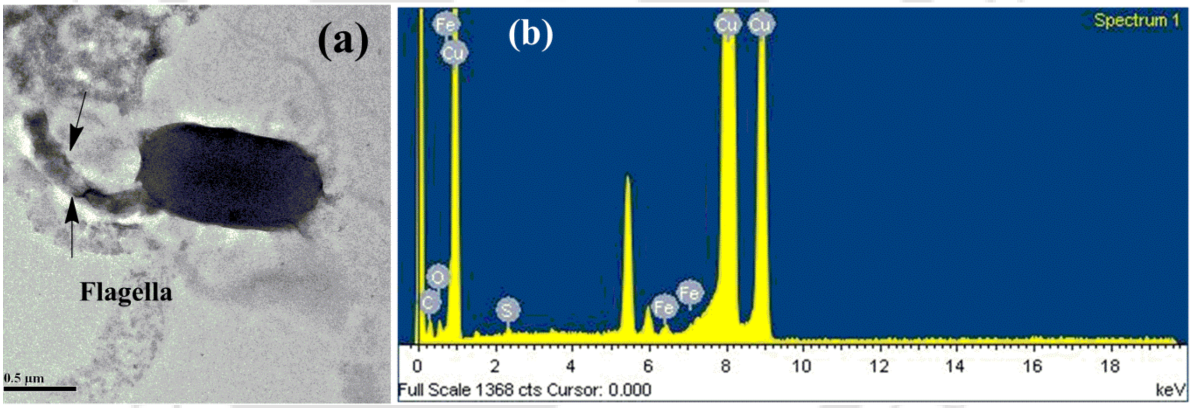


Figure 4. 8 (a) TEM image of the SRB grown in copper loaded UFAR biomass, (b) EDS spectrum from a spot on cell wall surface indicated by arrows.

Figure 4.9a depicts EDX spectrum along with the FESEM image of the bioprecipitate from control biomass in which the solid biomass appears as a coalescent material. Similarly, Figs. 4.9b to 4.9f show the EDX spectrum of the metal loaded biomass along with FESEM image of the corresponding biomass. All these figures indicate the presence of individual metals along with other elements, such as carbon, oxygen, nitrogen, sodium, phosphorous, chlorine, calcium, potassium and iron in the precipitates, which constituted the modified Postgate medium. From FESEM-EDX spectra of the precipitates (Fig. 4.9), it is clear that metals are precipitated as metal sulfides (Azabou et al., 2007). The UFAR biomass is mainly composed of SRB that are curved and oval shaped rods (White, 2000; Madigan et al., 2003). Figure 4.10 shows a schematic of the mechanism involved in heavy metal removal by SRB.

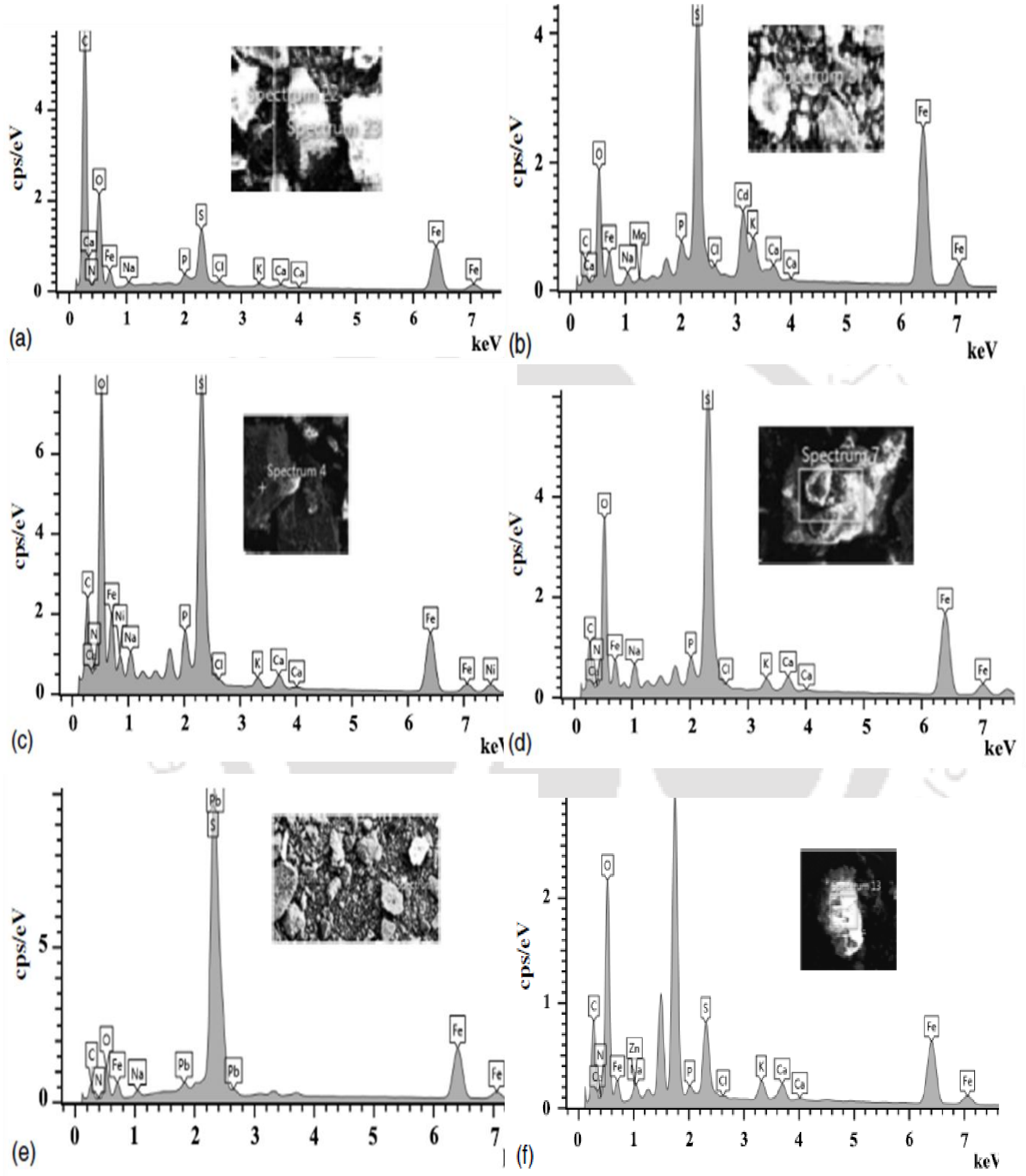


Figure 4. 9 EDX spectra of the bio-precipitates with images (insert) of the corresponding bio-precipitates from, a) control biomass, b) cadmium loaded biomass, c) nickel loaded biomass, d) iron loaded biomass, e) lead loaded biomass, f) zinc loaded biomass.

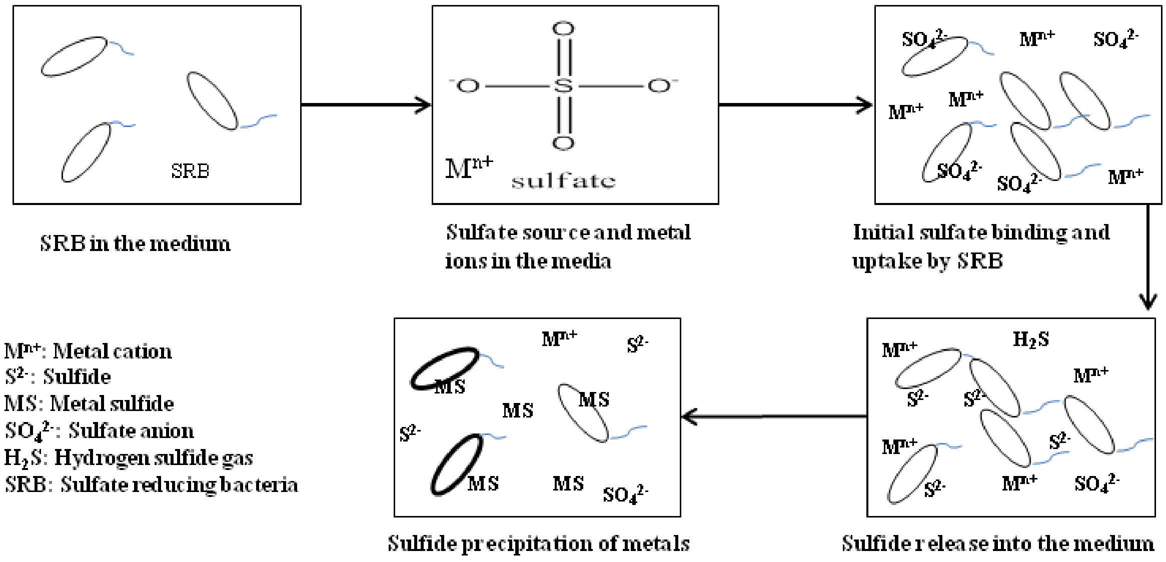


Figure 4. 10 Schematic showing the steps involved in metal removal by SRB.

4.1.2 Batch heavy metal removal from multi-component system

Pollutants, such as heavy metals never exist single in wastewater; they rather coexist with other inorganic species. Industrial effluents, e.g. acid mine drainage (AMD), usually contain more than one metal along with high sulfate content. Several authors have reported the effect of individual heavy metals on the sulfate reduction by SRB (Luptakova and Kusnierova, 2005). However, there is very less understanding on the combined effect of more than one metal in mixture on simultaneous sulfate and COD reduction as well as on each other metal removal in multi-component system. Thus, it is important to analyze and characterize the metal removal from mixture by SRB.

Therefore, this study was aimed at investigating heavy metal precipitation from mixture and also their effect on sulfate and COD reduction by SRB using UFAR biomass which showed the best results among the various biomass sources tested for heavy metal removal from single component system. One way to study the combined effect of heavy metals and sulfate on cultures of SRB is by using statistically designed experiments. For instance, individual main effect of factors on a given response can be investigated employing Plackett-Burman design, which is an efficient statistical experimental design technique (Flouty and Estephane, 2012). Analysis of the results is accomplished through analysis of variance (ANOVA) and student’s *t* test.

4.1.2.1 Heavy metal and COD removal and sulfate reduction in the multi-component system

Table 4.2 presents the results of simultaneous removal of Cd(II), Cu(II), Ni(II), Fe(III), Pb(II) and Zn(II) by SRB in the multi-component system, which reveals that the removal efficiency of each of these metals varied depending upon their combination level in their respective mixtures. The results obtained shows a maximum removal for Cu(II) (98.9%), followed by Ni(II) (97%), Cd(II) (94.8%), Zn(II) (94.6%), Pb(II) (94.4%) and Fe(III) (93.9%). A maximum metal removal was achieved at a low concentration combination of these metals, i.e. at 5 mg/L each of Ni(II), Cd(II), Zn(II) and Pb(II), at 25 mg/L Cu(II) and 10 mg/L Fe(III) in the experimental run 1 (Table 4.2). These results clearly indicate the dependence of metal precipitation by SRB on the metals and their concentration combination in mixture. An overall removal efficiency of more than 75% for each metal was achieved except for nickel (72.4%) (Table 4.2).

Table 4. 2 Sulfate reduction, metal and COD removal from mixture by SRB

Exp Run	**Sulfate removal^a (%)**	**COD removal^a (%)**	**Metal removal (%)^a**					
			Cd	**Cu**	**Ni**	**Fe**	**Pb**	**Zn**
1	92.5	91.2	94.8	98.9	97	93.9	94.4	94.6
2	85.8	87.7	86.4	98.0	83.2	93.5	92.0	86.6
3	84.6	70.8	84.4	95.0	85	78.9	87.2	89.3
4	77.3	71.7	81.8	91.6	92.2	91.9	93.1	81.3
5	75.5	66.5	92.4	92.2	81.4	78.3	90.8	83.1
6	73.2	65	92.6	91.1	72.4	77.4	85.5	84
7	81.1	74.8	79.1	93.0	95.2	77.3	88.3	92.6
8	78.4	86	84	94.6	93.4	93.9	88.4	80.2
9	83.5	69	79	92.9	92.5	78	91.4	85.0
10	73.7	63	83.9	93.6	86.2	84.6	92.4	92
11	85.2	66.8	91.9	92.5	94	84.3	93.2	85.1
12	72.6	62.5	81.5	93.8	83.2	83.5	83.3	82.2

^{a}Values show are average of two sample analysis for sulfate reduction, metal and COD removal

Sulfate and COD reduction efficiencies corresponding to the different experimental runs are presented in Table 4.2. Experimental run 1 resulted in maximum sulfate reduction efficiency (92.5%), followed by experimental runs 2 and 11. Maximum COD reduction efficiency (91.2%) was observed in experimental run 1 followed by runs 2 and 8 (Table 4.2). Both sulfate and COD reduction efficiencies were minimum in the experimental run 12 with metals added at their respective high initial levels. It can be observed from Table 4.2 that the results corresponding to heavy metal removal and the sulfate reduction correlated well with each other, confirming heavy metal precipitation by SRB through sulfate reduction. Similar to the heavy metal removal from the previous single component system, among the different heavy metals in the multi-component system, copper removal was maximum (98.9%) due to its low solubility product value with sulfide.

Thus, the order of removal of these metals as sulfide salts is attributed to their respective solubility product constant values (Hill et al., 2005). The results shown in Table 4.2 further confirmed that metals at a low initial concentration level (experimental run 1) in the multi-component system did not inhibit the activity of SRB. However, a high initial metal concentration level (experimental run 12) resulted in a reduced activity of the SRB and, therefore, its sulfate reduction efficiency (72.6%). Due to the low sulfate reduction efficiency, metal removal was also less compared to those in the other experimental runs (Table 4.2).

At a low initial metal concentration, metal precipitation due to sulfide produced by SRB avoids any toxic effect of the metal on SRB owing to its reduced bioavailability. This could be the major mechanism for metal precipitation and metal tolerance by SRB (White and Gadd, 2000). On the other hand, at an elevated concentration, these metals tend to be toxic to microorganisms due to their easy bioavailability, thus resulting in denaturation and deactivation of enzymes, rupture of cell organelles and membrane integrity, etc. (Alexandrinoa et al., 2011). Such toxic effects of copper on SRB has been reported by Kadukova and Vircikova (2005). The heavy metal resistance due to SRB also varied with respect to the different metal species, as presented earlier in Table 4.1 (Azabou et al., 2007).

4.1.2.2 Statistical analysis

For a better interpretation and assessment of the significance of the individual heavy metals on each other removal as well as on the sulfate and COD reduction by SRB, the results obtained were analyzed statistically in terms of student's *t* test and ANOVA. Student *t* test, which is used as a common tool to verify the significance of the coefficients of the regression model parameters on a given response, was applied to understand the effect of these metals on each other removal in the multi-component system. Tables 4.3 and 4.4 present student *t* test of heavy metal removal, sulfate reduction and COD removal from multi-component system. The estimated coefficients of individual effect of these metals are described in Tables 4.3 and 4.4 in which the associated T and P values were used to specify their significance.

Table 4. 3 Student *t* test of heavy metal removal from multi-component system

Term	**Cd(II)**	**Cu(II)**	**Ni(II)**	**Fe(III)**	**Pb(II)**	**Zn(II)**
[a]Effect	-7.3	-1.5	0.118	-1.7	-0.785	-1.7
Coef	-3.6	-.076	0.059	-0.85	-0.39	-0.89
T	-2.29	-0.48	0.04	-0.53	-0.25	-0.56
P	0.07	0.652	0.972	0.617	0.816	0.601
[b]Effect	0.77	-2.7	-0.18	-1.45	-1.19	-2.01
Coef	0.385	-1.3	-0.09	-0.72	-0.59	-1.00
T	0.75	-2.68	-1.42	-1.42	-1.17	-1.19
P	0.48	**0.044**	0.216	0.216	0.29	0.107
[c]Effect	1.95	-5.7	-10.0	2.15	-1.55	2.9
Coef	0.97	-2.8	-5.0	1.0	-0.7	1.4
T	0.69	-2.03	-3.55	0.76	-0.5	1.04
P	0.522	0.098	**0.016**	0.48	0.6	0.3
[d]Effect	1.1	-5.0	-5.8	-8.9	-4.28	0.52
Coef	0.5	-2.5	-2.9	-4.4	-2.1	0.26
T	0.5	-2.46	-2.8	-4.33	-2.0	0.25
P	0.5	0.057	**0.036**	**0.008**	0.093	0.81
[e]Effect	-1.4	-2.1	-3.2	1.1	-4.7	0.06
Coef	-0.7	-1.0	-1.6	0.55	-2.3	0.03
T	-1.9	-3.0	-4.5	1.5	-6.6	0.09
P	0.1	**0.02**	**0.006**	0.18	**0.001**	0.9
[f]Effect	0.20	-0.9	-2.6	3.0	-1.5	-7.0
Coef	0.102	-0.4	-1.3	1.5	-0.7	-3.5
T	0.1	-0.5	-1.5	1.8	-0.9	-4.2
P	0.9	0.5	0.17	0.12	0.4	**0.008**

[a](For Cd(II) removal); [b](For Cu(II) removal); [c](For Ni(II) removal); [d](For Fe(III) removal); [e](For Pb(II) removal); [f](For Zn(II) removal); Effect; Coeff: Coefficient; T: T value; P: probability

Table 4. 4 Student *t* test of sulfate and COD reduction in the presence of different heavy metals in multi-component system

Term	Effect	Coef	T	P
Sulfate reduction				
Constant	80.307	1.169	0	
Cd	-2.217	-1.109	-0.95	0.386
Cu	-9.452	-4.726	-4.04	**0.01**
Ni	-2.166	-1.083	-0.93	0.397
Fe	0.65	0.325	0.28	0.792
Pb	-2.182	-1.091	-0.93	0.393
Zn	-3.071	-1.536	-1.31	0.246
COD reduction				
Constant	72.917	2.084	0	
Cd	1.833	0.917	0.44	0.678
Cu	-11.33	-5.667	-2.72	**0.042**
Ni	-5.333	-2.667	-1.28	0.257
Fe	-8.867	-4.433	-2.13	0.087
Pb	-3.867	-1.933	-0.93	0.396
Zn	-5	-2.5	-1.2	0.284

Effect; Coeff: Coefficient; T: T value; P: probability

Table 4.5 presents the ANOVA of metal removal (Appendix D.1); ANOVA of sulfate and COD removal is presented in Table 4.6 (Appendix D.2). In these ANOVA tables, a high Fisher's value (F) and a low probability value (P) of the regression model indicate the model precision in explaining the variations in the results. Values of the statistical parameters F, P, standard error (S), coefficient of determination (R^2) and adjusted R^2, collectively illustrate whether the level means are significantly different from each other or not.

These values also clearly show goodness of fit of the respective regression models used to explain the experimental results (Haider and Pakshirajan, 2007). A low value of the parameter S represents an accurate prediction capability of these models. R^2 and adjusted R^2 values describe the amount of variation in the observed response values. Hence, a minimum value of S and a maximum value of R^2 represent an accurate prediction capability of the model (Montgomery, 2004). Tables 4.5 and 4.6 also present the accuracy and precision of the models in terms of R^2, adjusted R^2, S and predicted residual error sum of squares (PRESS). These values suggest that the models were accurate in predicting the results. Best results were, however, obtained for the models used to describe Fe(III), Pb(II) and Zn(II) removal by SRB (Table 4.5). All these effects of different heavy metals on each other removal in the multi-component system are better illustrated in the form of Pareto charts, depicted in Fig. 4.11.

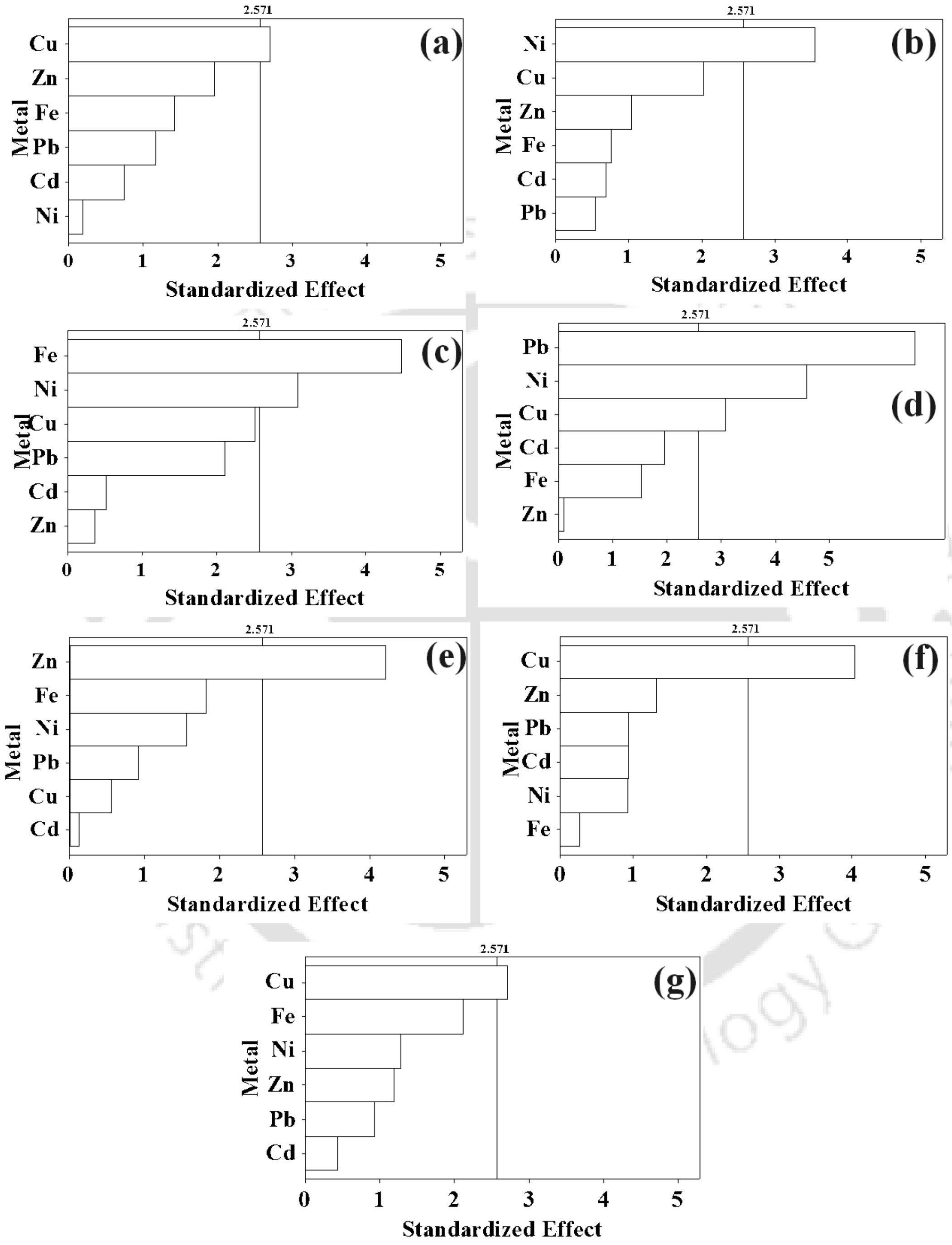


Figure 4. 11 Pareto chart showing the effect of different heavy metals on each other removal, sulfate and COD reduction by SRB: a) Cu(II) removal, b) Ni(II) removal, c) Fe(III) removal, d) Pb(II) removal, e) Zn(II) removal, f) sulfate reduction and g) COD reduction (vertical line shows significance cutoff at P value less than 0.05).

Horizontal bars in these charts represent the effect due to the individual metals, and those extending past the reference line (vertical line on the chart) represent the significant ones ($\alpha=0.05$). In summary, an increase in Cu(II), Ni(II) and Zn(II) concentration level in the mixture was inhibitory to their own removal (Fig. 4.11a, 4.11b and 4.11e). Fe(III) and Ni(II) inhibited Fe(III) removal (Fig. 4.11c), whereas Pb(II), Ni(II) and Cu(II) showed inhibitory effect on Pb(II) removal in the multi-component system (Fig. 4.11d). Pareto chart results matched well with the student *t* test and all these effects of heavy metals on each other removal from mixture can be attributed to the solubility product constant value of the corresponding metal sulfide salts (Hill et al., 2005). Among the different metals, Cu(II) inhibited both sulfate and COD reduction by SRB (Figs. 4.11f and 4.11g). This can be attributed to the formation of high sulfide precipitates which may result in diffusion limitation of the substrates (COD and sulfate) (Kieu et al., 2011).

4.1.2.3 Characterization of metal bioprecipitates

Characterization of the bio-precipitates (biomass from control and experimental run 1) using TEM-EDS, FESEM-EDX and FTIR was carried out to understand the morphology and elemental composition of the metal precipitates formed by SRB in this multi-component study. Figures 4.12a and 4.13a display TEM images of the bacterial cell with metal sulfides precipitated on its surface. The presence of different metals as sulfides is confirmed by the EDS spectrum taken from a spot on the outer cell surface of the bacteria (Figs. 4.12b and 4.13b). Metal precipitates in the vicinity of the bacterial cell surface are clearly visible from Figs. 4.12 and 4.13.

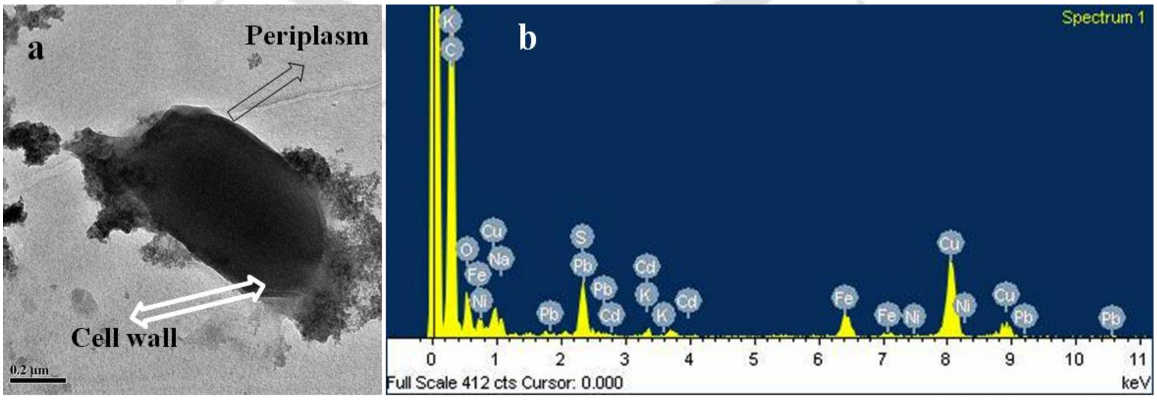


Figure 4. 12 (a) TEM image of a metal loaded bacterial cell from experimental run #1, (b) EDS spectrum from a spot on the bacterial cell surface.

The TEM images (Figs. 4.12a and 4.13a) confirmed the ability of these SRB to grow in the presence of metals in mixture which is also confirmed by color change of the input medium from colorless to black due to the formation of FeS and generation of hydrogen sulfide owing to SRB growth and activity (Herbert and Gilbert, 1984; Hamilton, 1994; Singh et al., 2011). The images also reveal the presence of a layer or shell like structure on cell wall of the bacteria, confirming that metal sulfide is associated with outer layer of the bacterial cell surface (Figs. 4.12b and 4.13b).

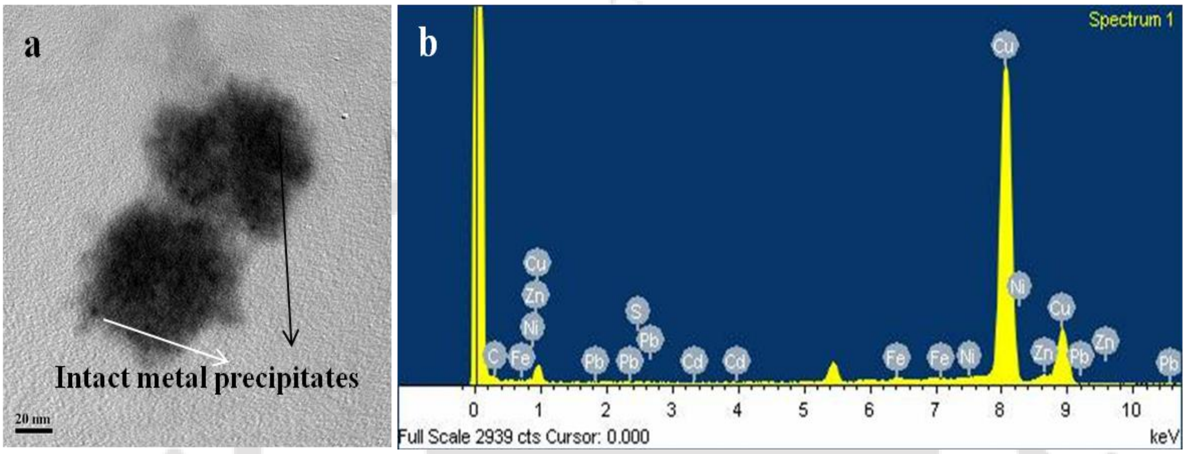


Figure 4. 13 (a) TEM image of a metal loaded bacterial cell from experimental run #1 showing intact metal precipitate on SRB cell surface, (b) EDS spectrum from a spot on the bacterial cell surface.

Elemental composition of the control biomass was confirmed by EDX spectrum shown in Fig. 4.14a; insert to Fig. 4.14a represents the FESEM image of the control biomass which appears as a coalescent material. Similarly, elemental composition of the biomass taken from experimental run 1 was confirmed by EDX spectrum (Fig. 4.14b); insert to Fig. 4.14b represents the FESEM image of the same biomass. A comparison between these figures confirms metal sulfide precipitation by SRB together with the presence of other elements that constituted the modified Postgate medium, which is similar to the findings obtained in the previous study (Fig. 4.9). The presence of sulfur peak in the spectra (Figs. 4.12b-4.14b) is attributed to the metal sulfide precipitation as a result of SRB activity. The precipitates formed are predominantly amorphous form of sulfide salts corresponding to different metals added in mixture (Fig. 4.14). Sulfate and COD reduction along with metal sulfide formation confirmed that sulfidogenesis is the governing mechanism for heavy metal removal by SRB (Jin et al., 2007).

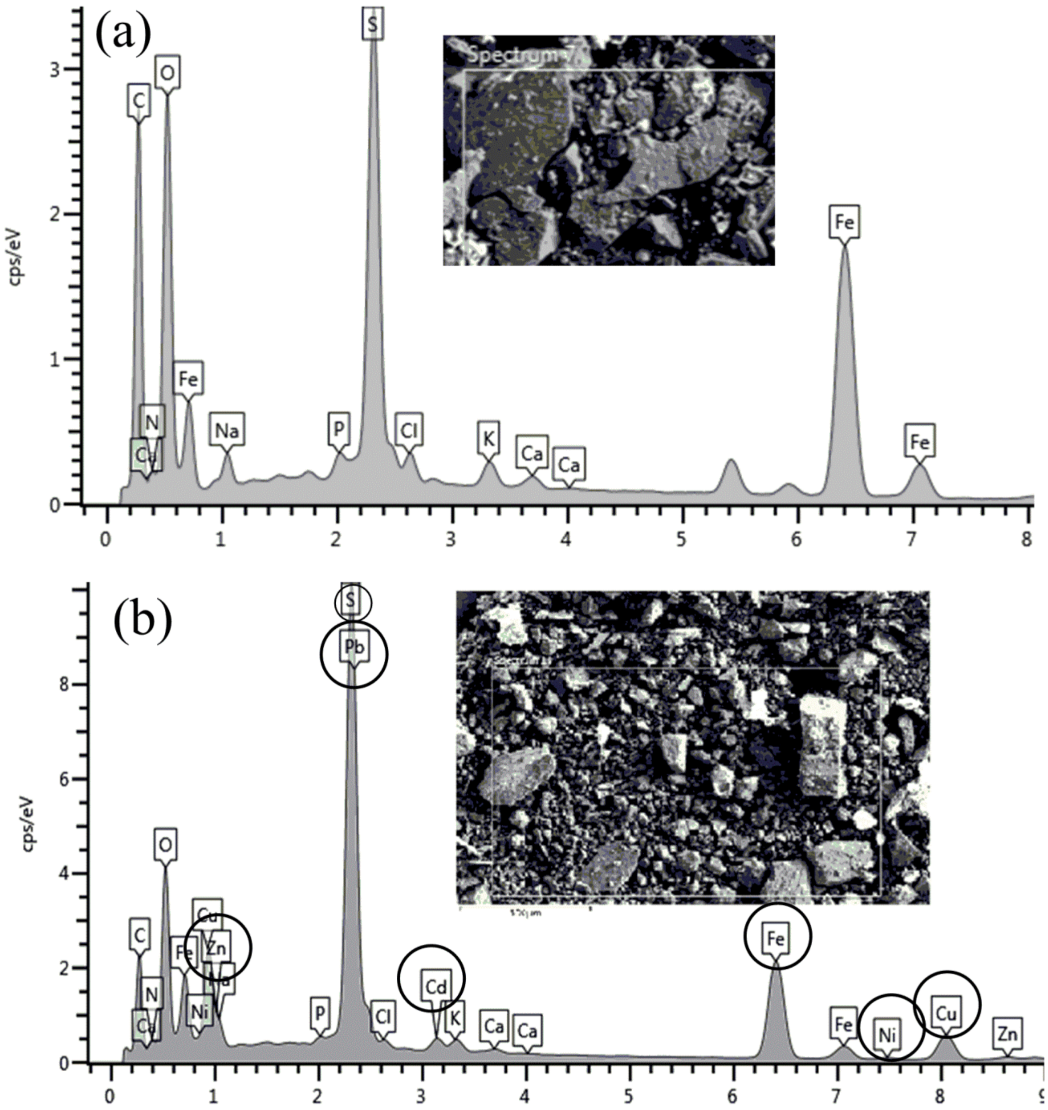


Figure 4. 14 EDX spectrum of (a) control biomass, (b) metal loaded biomass from experimental run #1. Insert to these figures show the image of the respective biomass.

Overall, the results from TEM and FESEM revealed that the metals were mainly removed by sulfide precipitation. From FESEM-EDX spectra of the precipitates (Fig. 4.14), it is clear that the metals were precipitated as metal sulfides (Azabou et al., 2007). Different metals precipitated in the experimental run 1 are highlighted with circle in Fig. 4.14b. Among the peaks due to the different elements, only the peak due to sulfide is significant indicating that the metals were present as sulfides in the precipitate (Figs. 4.12-4.14). All other forms, such as $M(OH)_x$, MCO_3, etc., were not significant.

Figure 4.15 shows the FTIR spectra of the heavy metal laden anaerobic biomass from the experiments which was obtained to verify the interaction between the metal ions and the functional groups present on the bacterial surface. The major stretching in the spectrum was in the range 468-3696 cm^{-1} for the control biomass and 467-3468 cm^{-1} for biomass obtained from experimental run 1. The spectra show characteristic sharp peaks in the different wave regions 599-618 cm^{-1}, 1018-1043 cm^{-1}, 1535-1544 cm^{-1} and 1638-1642 cm^{-1}, respectively. Occurrence of neutral C=O complex was indicated by the stretching at 1644 cm^{-1} in control biomass and a minor shift to 1642 cm^{-1} in the heavy metal laden biomass.

Bands in the range 1199-800 cm^{-1} are associated with C–O–C and C–O–P stretching. These stretching vibrations involve oligo and polysaccharides present in the bacteria (Rubio et al., 2006). Bands corresponding to the stretching from 1525 to 1558 cm^{-1} are mainly due to -NH stretching and can be attributed to protein amide I and amide II bands (Quan et al., 2013). Bands corresponding to the range from 3748 to 3764 cm^{-1} indicate either N-H stretching of amine group or O-H stretching of hydroxyl group. An earlier study on the FTIR spectra of SRB has reported that sulfate ions exhibit five bands centered around 1230 cm^{-1}, 1130 cm^{-1}, 1070 cm^{-1}, 1000 cm^{-1} and 610 cm^{-1} (Nakamoto, 1970). Similar observation was made in the results obtained from single component system (Fig. 4.7).

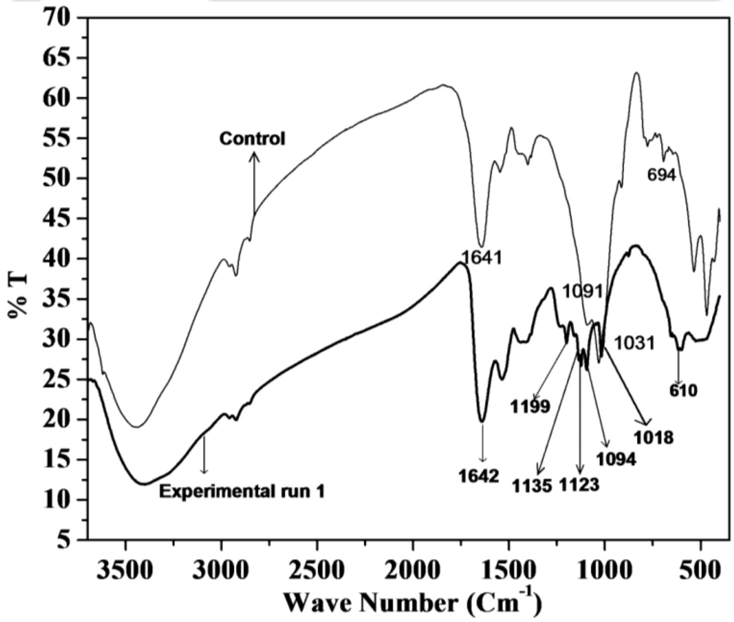


Figure 4. 15 FTIR spectra of control biomass and heavy metal loaded biomass from experimental run # 1 in the study.

The FTIR spectra (Fig. 4.15) of the biomass used in this multi-component system showed bands corresponding to the sulfate ions, which clearly indicate sulfate reduction for its growth and metabolism (Singh et al., 2011; Zaina et al., 2011). FTIR analysis confirmed a high degree of similarity of the functional groups corresponding to SRB in the present study with several other reported species of SRB, such as *Desulfovibrio vietnamensis* DSM 10520, *Desulfovibrio gigas* ATCC 19364, *Desulfovibrio gabonensis* DSM 10636 and *Desulfovibrio indonesiensis* NCIMB 13468 (Feio Maria et al., 2004).

4.1.3 Sulfate reduction, metal and COD removal using immobilized SRB

There is no doubt that SRB based treatment system is effective and can be applied on a large-scale, but it involves certain limitations. One such limitation is the inherent toxicity of the metals on SRB growth and activity (Min et al., 2008). For instance, high metal concentrations have been shown to be inhibitory towards growth and activity of free/suspended SRB (Sani et al., 2001; Utgikar et al., 2001; Utgikar et al., 2002).

Although a wide range of materials has been tested for active immobilization, the naturally available polymer sodium alginate has received significant interest owing to its easy availability, low-cost (Chen and Lin, 1994; Lozinsky and Plieva, 1998). However, the applicability of sodium alginate immobilized SRB for heavy metal removal from wastewater and the mechanism involved are very limited. Therefore, this study was aimed at investigating the performance of sodium alginate immobilized SRB beads for sulfate and heavy metal removal and study the mechanism involved in metal removal.

4.1.3.1 Heavy metal and COD removal and sulfate reduction

Figure 4.16 illustrates the metal removal efficiency obtained using the immobilized SRB beads at 10 mg/L and 50 mg/L initial metal concentration. At 10 mg/L initial metal concentration, more than 95% removal was achieved for all the metals; at 50 mg/L, more than 85% metal removal was obtained for all the metals, but in case of nickel the removal was less (75%) (Fig. 4.16c). These results were slightly better than the metal removal results obtained previously using free/suspended SRB biomass (Figs. 4.1-4.2). The immobilized SRB showed very good tolerance towards the different heavy metals even at a high initial concentration (Fig. 4.16). In general, heavy metals are reported to be toxic to free/suspended SRB activity, particularly at a high initial concentration, often due to enzyme deactivation, protein denaturation and cell membrane damage (Kikot et al., 2010). The capability of the SRB immobilized beads to withstand the toxic effect of heavy metals as compared with free/suspended SRB biomass can be attributed to metal precipitation outside the bead surface, which prevented any direct contact between the metals and the SRB.

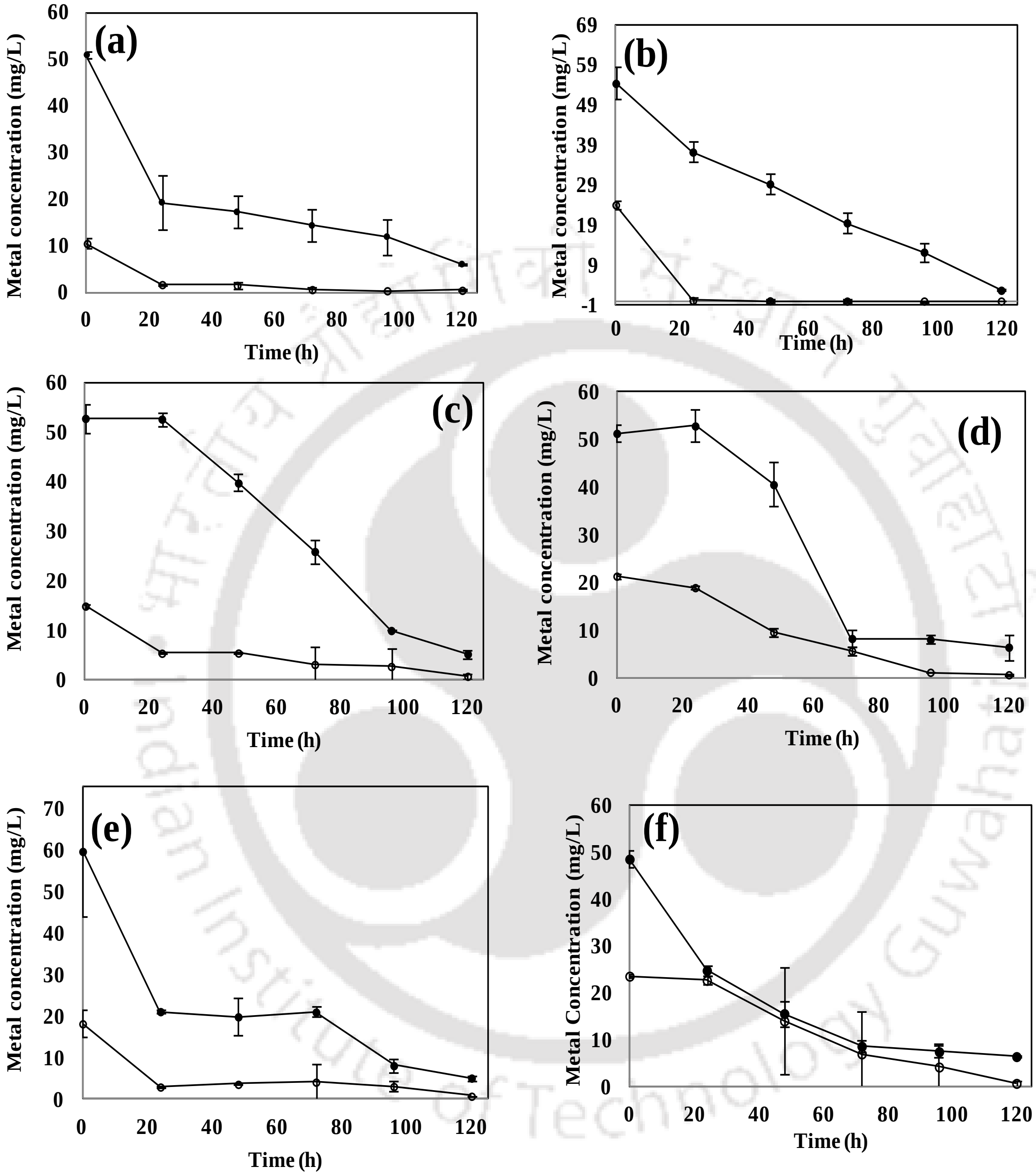


Figure 4. 16 Heavy metal removal by immobilized SRB beads at 10 and 50 mg/L initial metal concentration: (a) Cd(II), (b) Cu(II), (c) Ni(II), (d) Fe(III), (e) Pb(II) and (f) Zn(II); (○:10 mg/L, ●: 50 mg/L).

Sulfate reduction and COD removal by immobilized SRB beads in the presence of different metals at 10 and 50 mg/L initial concentrations are presented in Fig. 4.17. At 10 mg/L initial metal concentration, more than 82% sulfate removal was obtained (Fig. 4.17). At 50 mg/L initial metal concentration, more than 75% sulfate removal was achieved, except with nickel, and in which case the removal was slightly less at 71% (Fig. 4.17c).

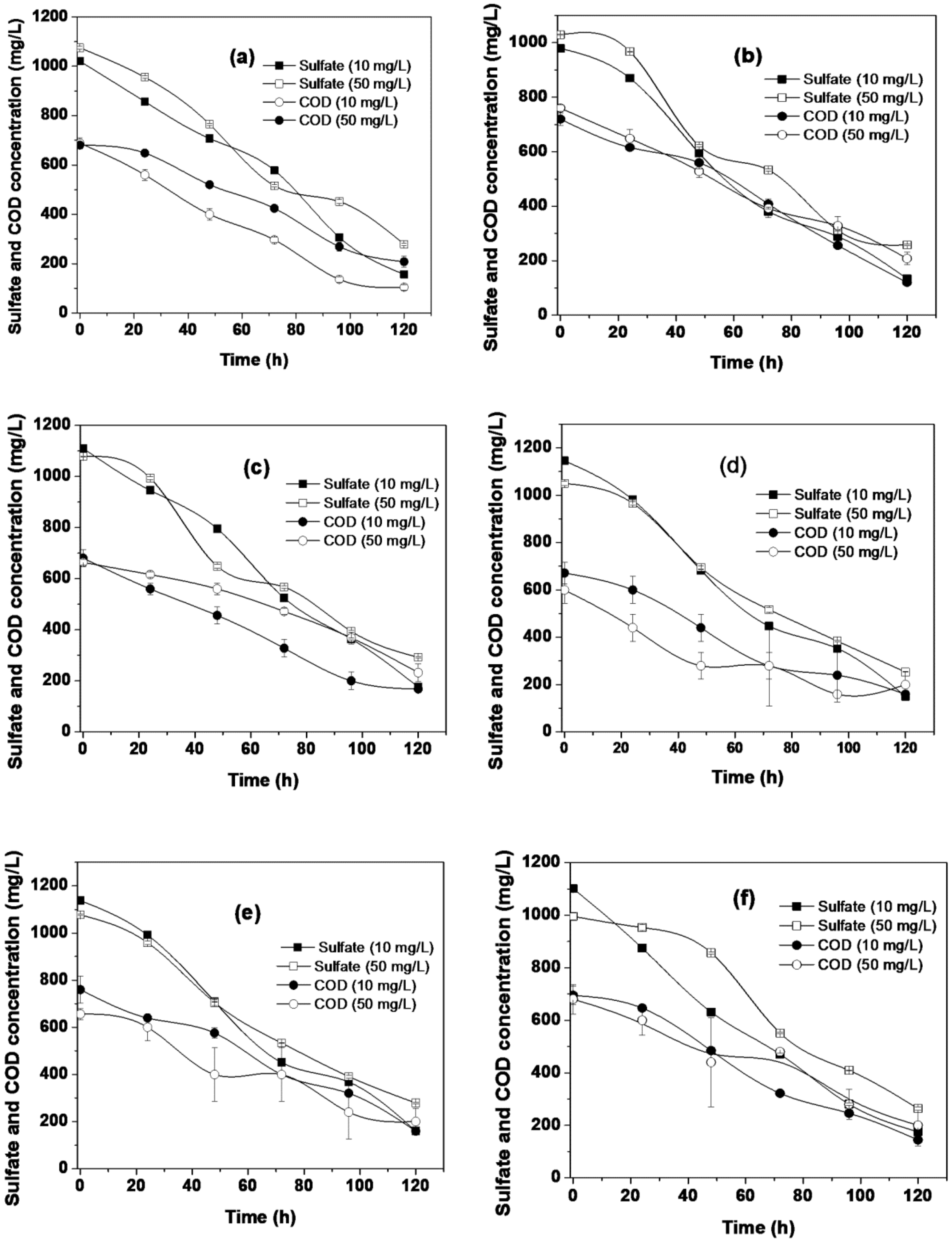


Figure 4. 17 Sulfate and COD removal by immobilized SRB at 10 and 50 mg/L of initial metal concentration: (a) Cd(II), (b) Cu(II), (c) Ni(II)), (d) Fe(III), (e) Pb(II) and (f) Zn(II).

In an earlier study using the free/suspended biomass, more than 90% sulfate reduction efficiency was achieved for both the initial metal concentrations (Figs. 4.3-4.4), which is slightly higher than that obtained using the immobilized SRB (Fig. 4.17). Figure 4.17 also depicts the COD removal in the presence of different metals. Over 75% COD removal was achieved using the immobilized SRB at 10 mg/L initial metal concentration. At 50 mg/L initial metal concentration, more than 65% COD removal was achieved with Cd(II), Cu(II), Fe(III), Ni(II) and Pb(II), and 64% with Zn(II) (Fig. 4.17c). These results of COD removal (Fig. 4.17) were lower than that obtained previously using the free/suspended SRB biomass (Figs. 4.5-4.6).

Compared with the sulfate reduction by free/suspended SRB biomass, immobilized SRB yielded a low sulfate as well as COD reduction efficiency for both 10 and 50 mg/L initial metal concentrations (Fig. 4.17), which may be attributed to diffusion limitation of these substrates into the SRB immobilized beads (Utgikar et al., 2002; Jong and Parry, 2003; Min et al., 2008). Results of sulfate reduction and metal removal shown in Figs. 4.16-4.17 matched well with each other, indicating that sulfide generated through sulfate reduction by SRB was responsible for precipitating the metals. Among the different heavy metals tested, copper removal was maximum due to its low solubility product value with sulfide, which is in agreement with the results obtained previously.

4.1.3.2 Reusability and durability of immobilized SRB

Based on the results of metal removal efficiencies by immobilized SRB obtained at different initial metal concentrations (10 and 50 mg/L), reusability and durability of the immobilized biomass for heavy metal removal were tested with 25 mg/L initial concentration of Ni(II), Zn(II), Pb(II), Fe(III) and Cd(II); in case of Cu(II), 50 mg/L initial concentration was chosen. The results obtained from reusability and durability experiments are shown in Fig. 4.18. From Fig. 4.18, it can be seen that immobilized SRB beads yielded almost complete metal removal except in case of Ni(II) and Fe(III) for the first two cycles of reuse. The removal values remained stable with its further reuse in the experiments for all the metals but for Ni(II) and Fe(III), the removal was slightly high (Fig. 4.18). All these results clearly reveal that immobilized SRB beads can be reused several times for achieving the best metal removal results. Reusing capacity of SRB immobilized using a mixture of SiO_2, $CaCO_3$, activated carbon, polyvinyl alcohol (PVA) and sodium alginate was tested for zinc removal; 99% removal was reported with multiple use of the beads without any effect on the zinc removal rate (Min et al., 2008).

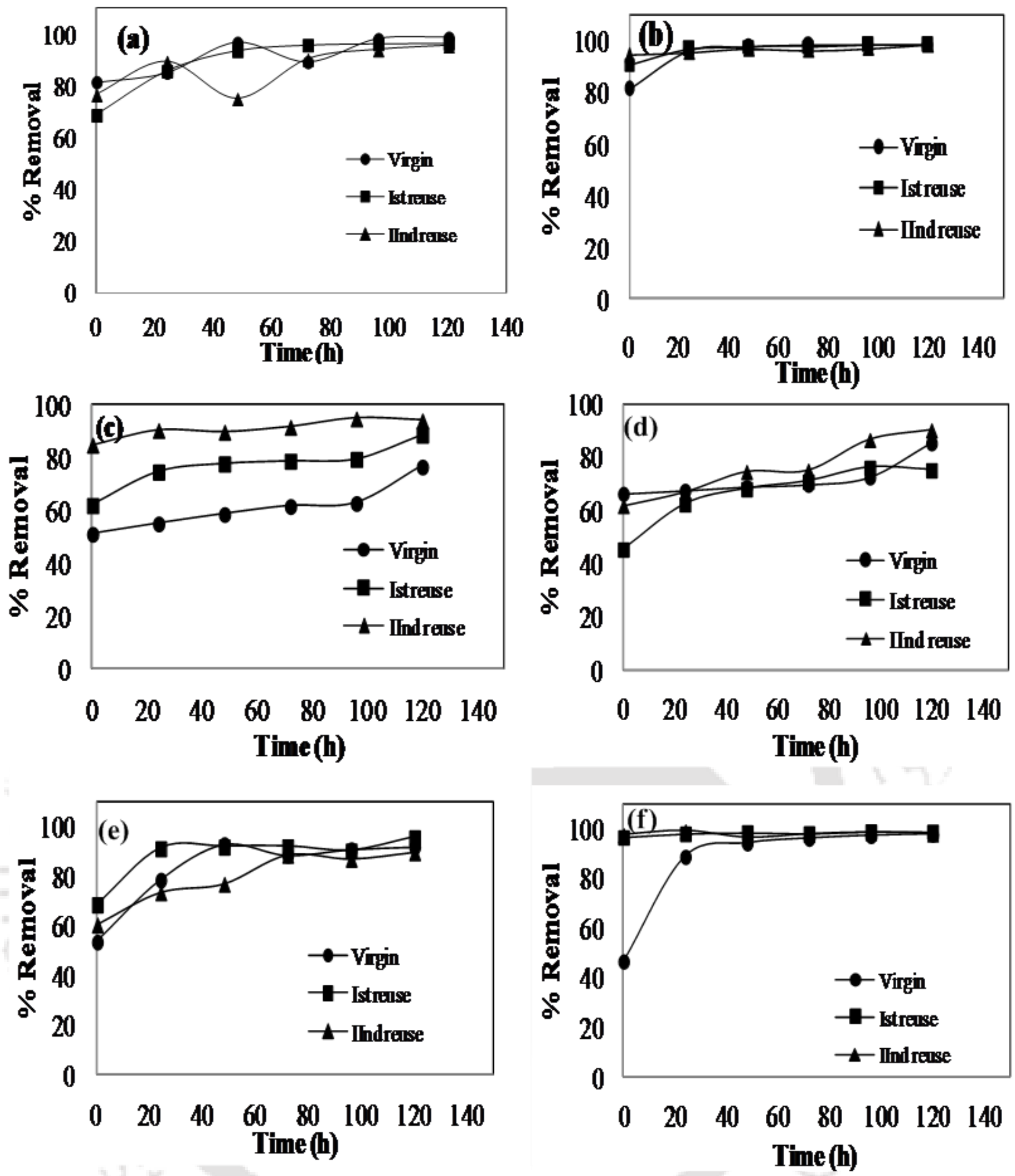


Figure 4. 18 Heavy metal removal by immobilized SRB for three consecutive cycles: (a) Cd(II), (b) Cu(II) and (c) Ni(II), (d) Fe(III), (e) Pb(II), and (f) Zn(II).

4.1.3.3 Characterization of immobilized beads

Characterization of the immobilized beads was carried out by FESEM-EDX to understand the bead morphology and the metal removal mechanism involved which is shown in Figs. 4.19 and 4.20. In order to understand the exact mechanism of metal precipitation by SRB immobilized beads, i.e., whether it occurs interior or exterior to the beads, FESEM-EDX analyses of one-half of the bead at its cross-section, whole cell immobilized bead and blank bead (without SRB) were carried out.

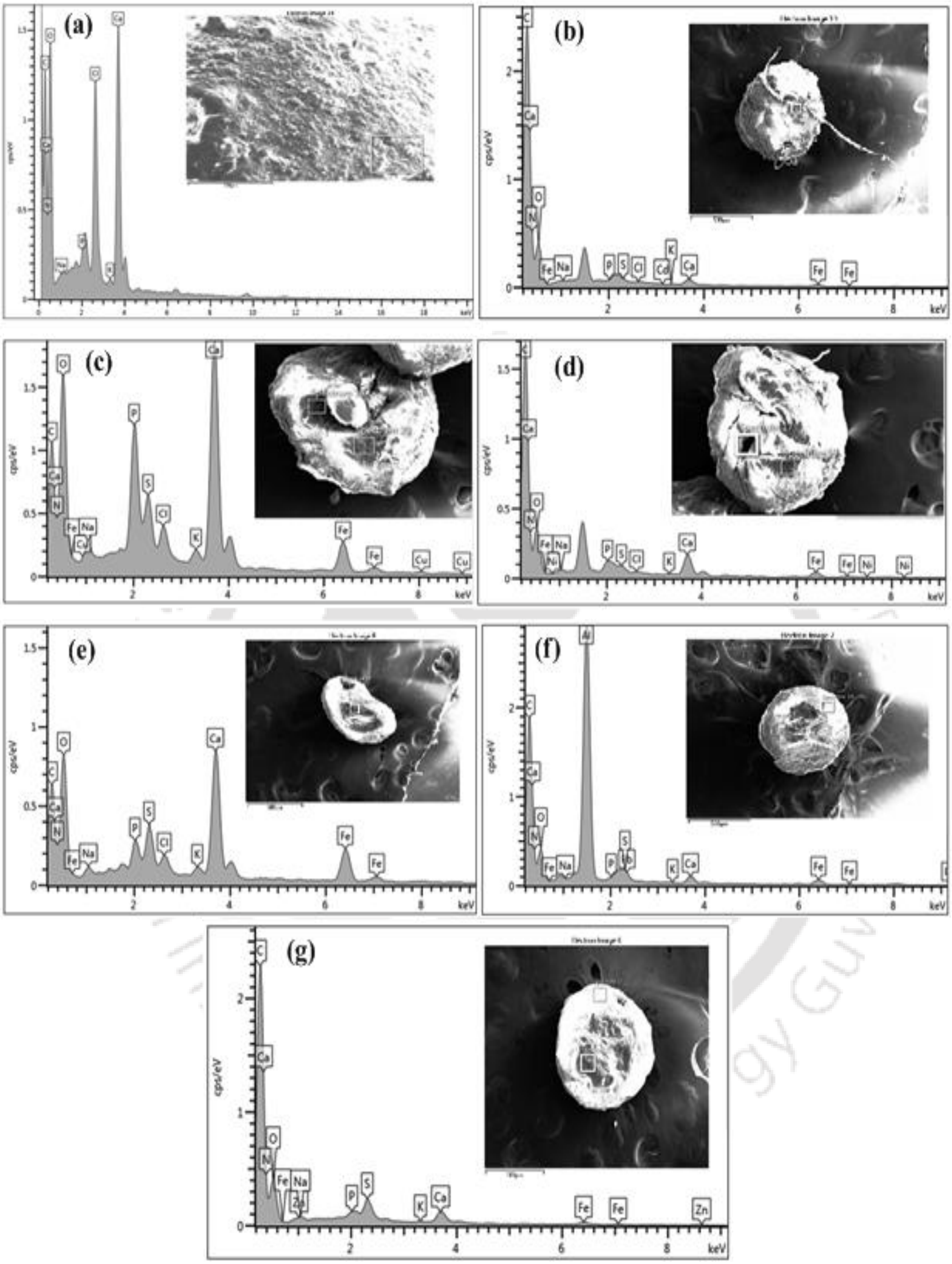


Figure 4. 19 EDX spectra of immobilized SRB containing beads: (a) control bead, (b) Cd(II) laden, (c) Cu(II) laden, (d) Ni(II) laden, (e) Fe(III) laden, (f) Pb(II) laden and (g) Zn(II) laden; insert show FESEM image of the corresponding beads.

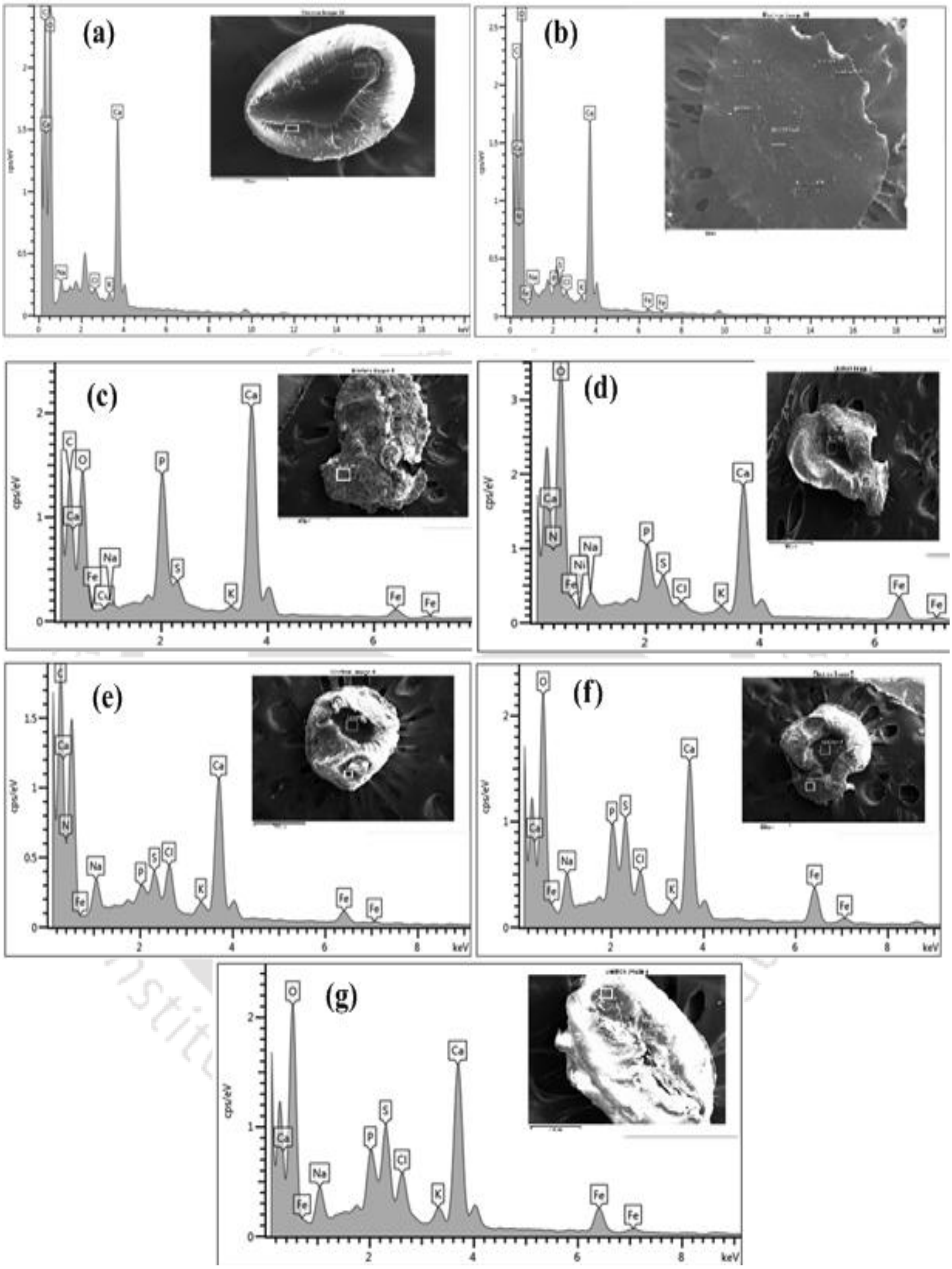
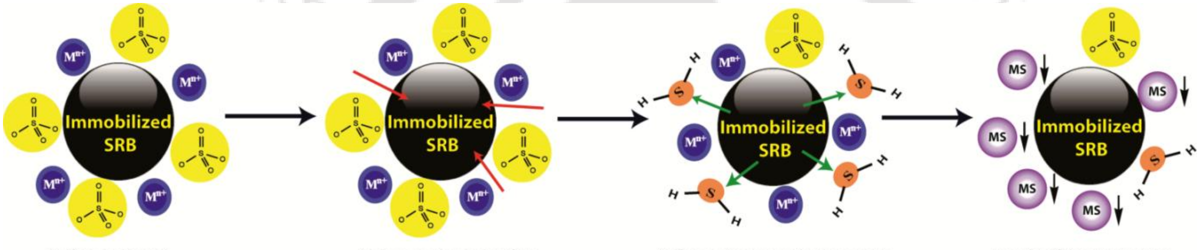


Figure 4. 20 EDX spectra of the middle section of the immobilized SRB containing beads: (a) control bead, (b) Cd(II) laden, (c) Cu(II) laden, (d) Ni(II) laden, (e) Fe(III) laden, (f) Pb(II) laden and (g) Zn(II) laden; insert show FESEM image of the corresponding beads.

Figure 4.19a represents EDX spectrum of the whole cell immobilized bead (control bead); insert shows FESEM image of the bead. Similarly, Fig. 4.19(b-g) illustrates EDX spectra of metal laden immobilized beads; insert show FESEM images of the corresponding beads. The EDX spectrum and FESEM image of immobilized SRB bead cut at its cross-section and metal laden bead are shown in Fig. 4.20. A comparison of FESEM-EDX profiles of these immobilized SRB containing beads (Figs. 4.19 and 4.20) confirms metal precipitation by SRB together with the presence of other elements that constituted the modified Postgate medium, similar to the findings obtained in the previous study (Figs. 4.9 and 4.14). Moreover, metal removal by the beads is attributed to their precipitation outside the beads (Figs. 4.19 and 4.20). Based on the results obtained, a schematic of metal removal mechanism by immobilized SRB beads is shown in Fig. 4.21.



Sulfate and metal ions in the media

Carbon source and sulfate diffusion into the bead

Sulfate reduction and subsequent sulfide release from the bead

Metal sulfide precipitation on the bead surface

Figure 4. 21 Schematic of metal removal mechanism by immobilized SRB beads.

4.2 Continuous Metal Removal Using DFCR With Immobilized SRB Biomass

The use of free/suspended SRB results in activity loss due to cell washout in continuously operated reactors (Baskaran and Nemati, 2006), and, therefore, require extended hydraulic retention time (HRT) for operating the system (Neculita et al., 2007). In order to overcome the drawbacks associated with the use of free/suspended SRB, active cell immobilization using suitable naturally available polymeric matrix is proposed. The applicability of sodium alginate immobilized SRB for continuous heavy metal removal from wastewater is very limited. Therefore, this study was aimed at investigating the performance of sodium alginate immobilized SRB beads for heavy metal removal from single and multi-component system using a downflow column reactor (DFCR) operated under continuous mode.

4.2.1 Single component system

4.2.1.1 Heavy metal and COD removal and sulfate reduction

Figure 4.22 shows the column reactor (DFCR) performance in terms of metal removal at 48 h and 24 h HRT. At all the tested inlet metal concentrations, more than 88% metal removal was obtained at 48 h HRT for all the metals, except for Fe(III), which showed nearly 75% (Fig. 4.22a). At 24 h HRT, more than 86% removal is obtained for Pb(II), Cd(II), Zn(II) and Cu(II), whereas in case of Fe(III) and Ni(II), more than 75% removal could be achieved at the respective inlet metal concentrations (Fig. 4.22b). Figures 4.23-4.24 present sulfate and COD reduction profiles obtained using the DFCR operated at different conditions. A low inlet metal loading (48 h HRT) yielded sulfate removal efficiency in the range 54-71% for all the metals (Fig. 4.23a).

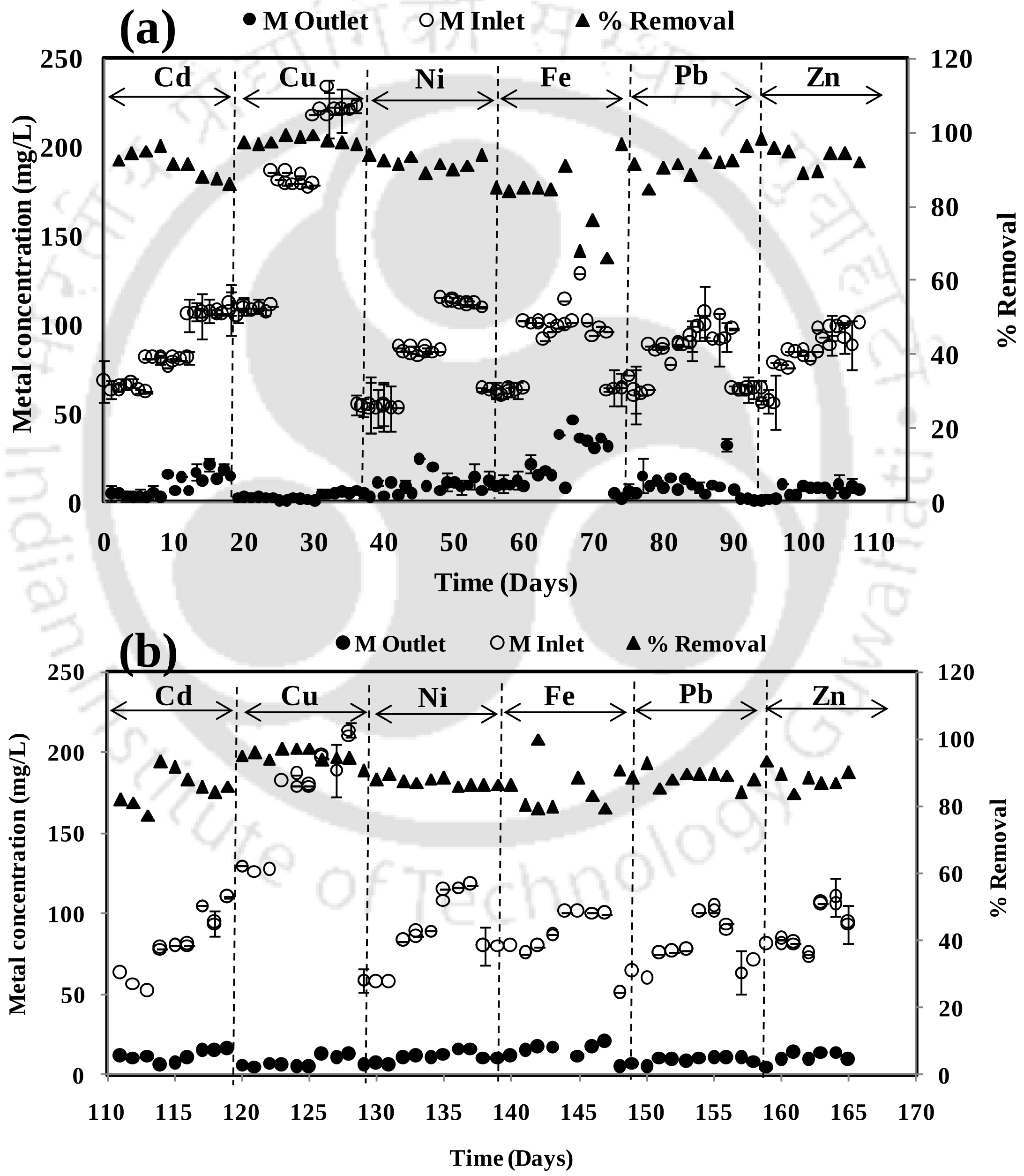


Figure 4. 22 Continuous removal of heavy metals using the DFCR with immobilized SRB beads: (a) 48 h HRT and (b) 24 h HRT (●: Metal outlet, ○: Metal inlet, ▲: % Metal removal).

However, these values were reduced (46-62%) at 24 h HRT (high inlet metal loading) (Fig. 4.23b). Figure 4.24a reveals that COD removal ranged from 57-73% for all the metals at a low inlet metal loading (48 h HRT). At a high metal loading (24 h HRT), COD removal was slightly low (Fig. 4.24b).

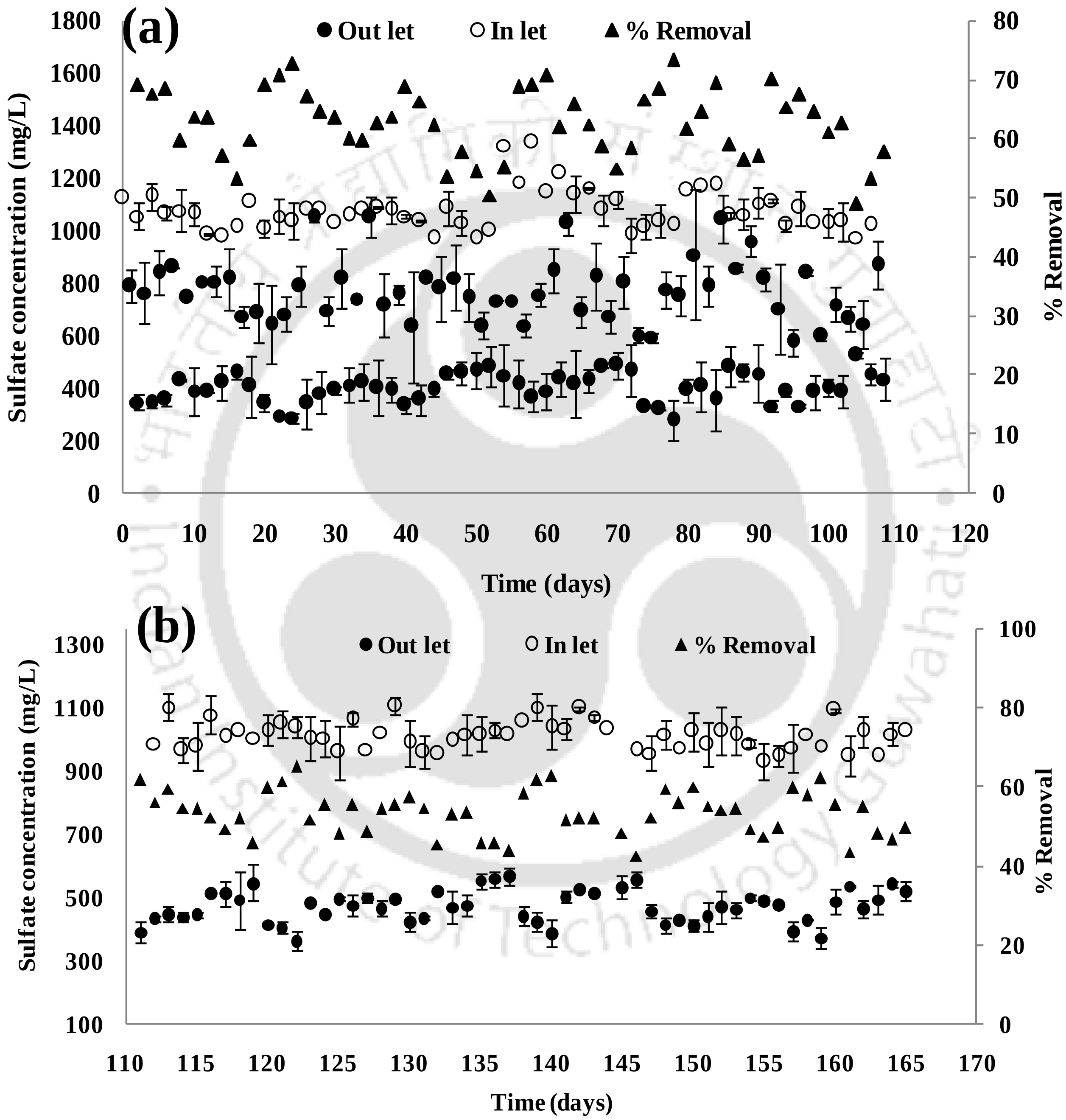


Figure 4. 23 Sulfate removal in the presence of different heavy metals using the DFCR: (a) 48 h HRT and (b) 24 h HRT (●: Sulfate outlet, ○: Sulfate inlet, ▲: % Sulfate removal).

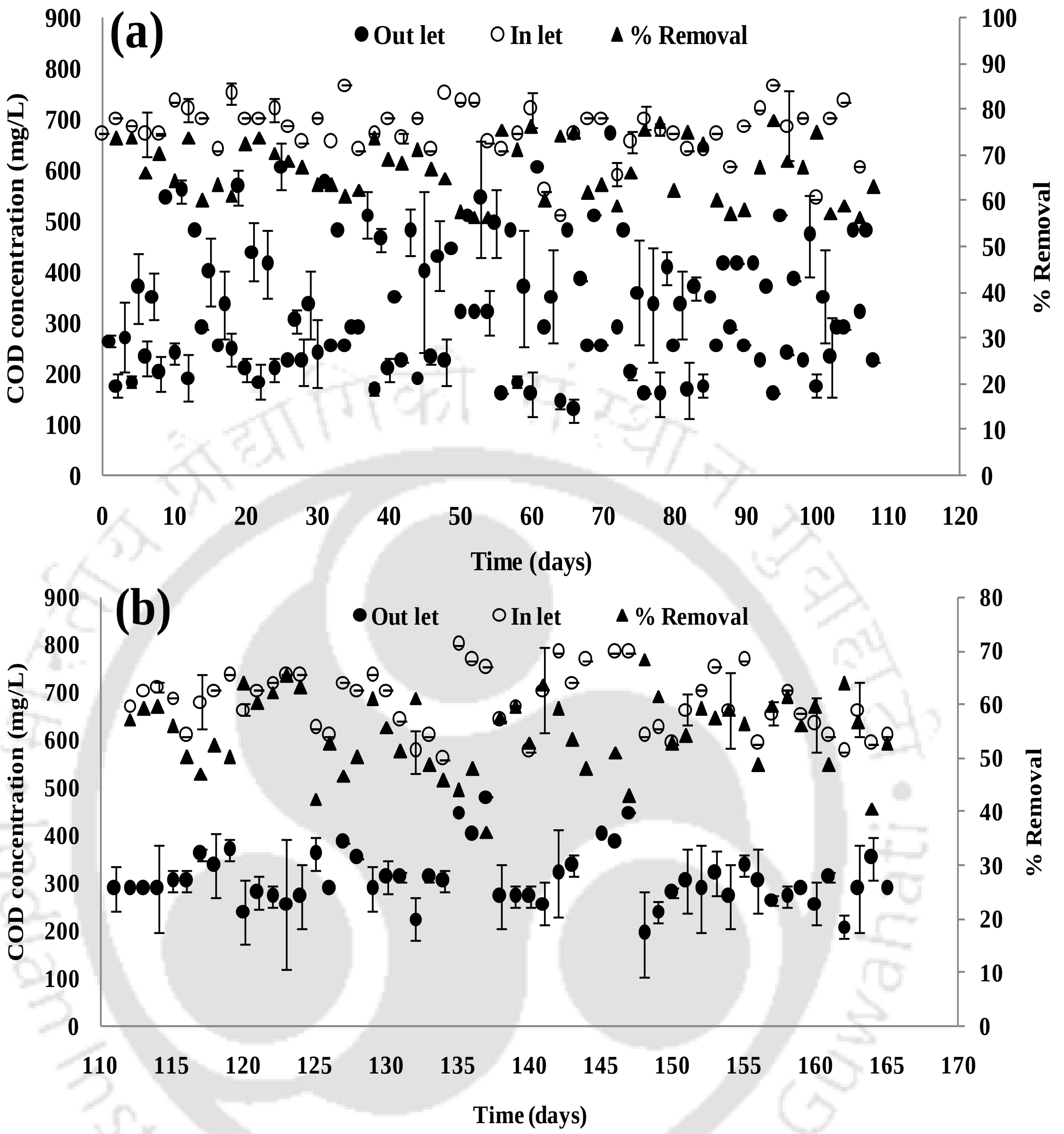


Figure 4. 24 COD removal in the presence of different heavy metals using the DFCR: (a) 48 h HRT and (b) 24 h HRT (●: COD outlet, ○: COD inlet, ▲: % COD removal).

A slight reduction in the metal removal at a high inlet metal concentration (Fig. 4.22) indicated inhibitory effect of the metals on SRB activity and growth (Hsu et al., 2010), which is also evident from the corresponding low sulfate and COD reduction values presented in Figs. 4.23-4.24. This small reduction in the continuous removal of heavy metals, sulfate and COD reduction at a high inlet metal concentration can be attributed to diffusion limitation of the substrates (sulfate and COD), as noted previously in the case of batch heavy metal removal using immobilized SRB (Figs. 4.16-4.17). Similar results of sulfate and COD reduction were reported by Tucker et al. (1998) in their study, but with formate and lactate as the electron donors. Results of continuous heavy metal removal using the DFCR packed with immobilized SRB beads (Fig. 4.22) reveal that the metal removal was maximum for both 24 h and 48 h HRT at their respective low inlet metal concentrations, which correlated well with SRB activity in terms of sulfate reduction, COD removal and sulfide formation (Figs. 4.23-4.25). Values of sulfide generated during the experiments are shown in Fig. 4.25 for 48 h and 24 h HRT, which correlated well with the sulfate reduction values observed earlier.

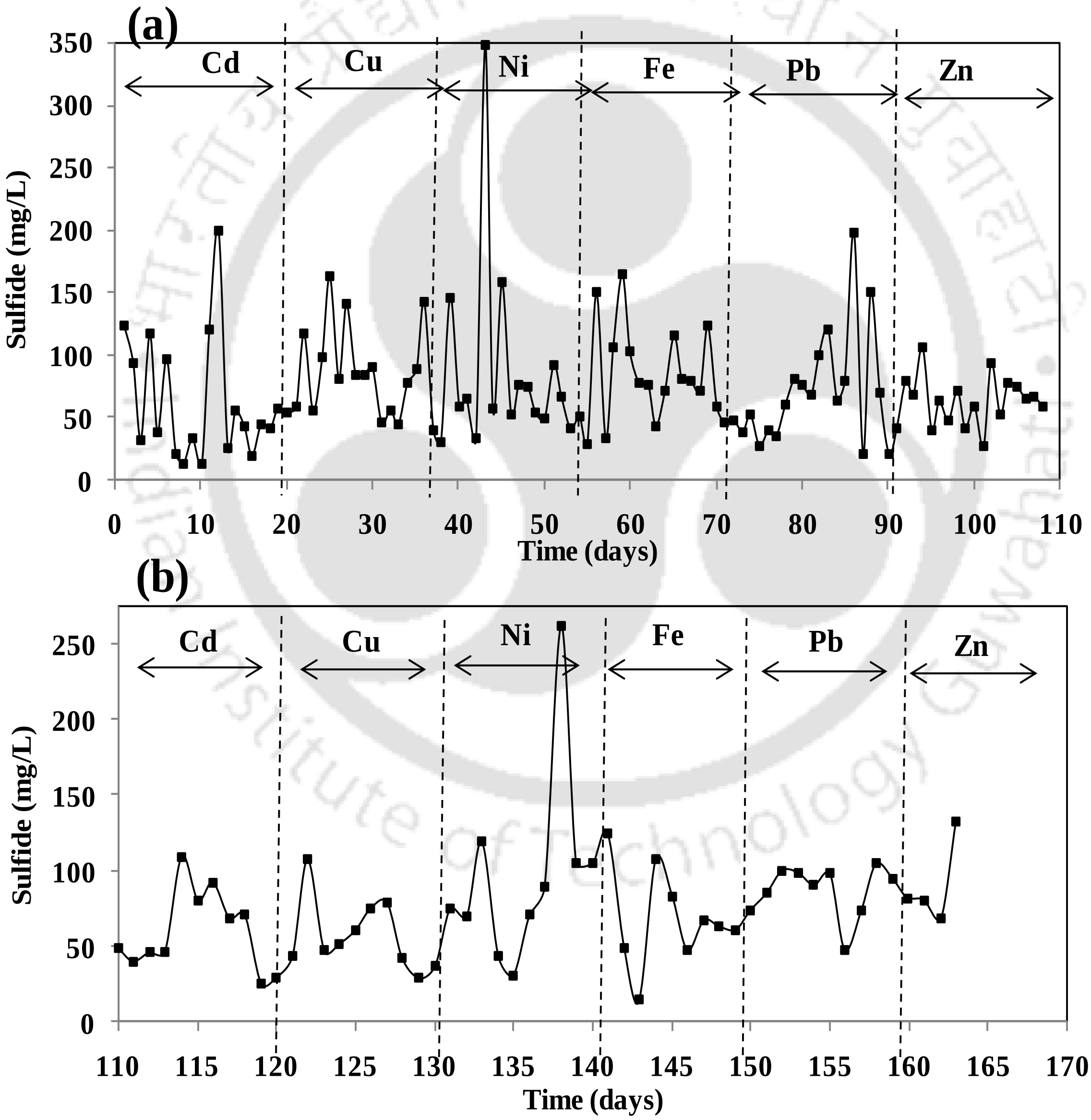


Figure 4. 25 Results of sulfide generated in the DFCR with immobilized SRB beads at (a) 48 h HRT and (b) 24 h HRT (■ Sulfide generated (mg/L)).

Tucker et al. (1998) investigated the potential of SRB immobilized polyacrylamide gel in column reactors for the removal of uranium and molybdenum, and achieved almost 99% metal removal without any inhibitory effect on SRB activity at a low inlet concentration in the range 5-10 mg/L. Zhang et al. (2016) achieved more than 99% metal removal at very high initial concentrations of Cd, Cu, Fe and Zn using upflow anaerobic packed bed reactor with immobilized SRB beads; the beads were prepared based on a mixture of PVA, sodium alginate, silicon sand, maize straw powder, zero valance iron and SRB sludge.

Compared with 24 h HRT, 48 h HRT yielded a maximum removal of metals, sulfate and COD reduction with the DFCR (Figs. 4.22a-4.24a). Effect of HRT on reactor performance is well reported in the literature; in general, metal precipitation by SRB occurs in the range of a few days to 5 days (Kuyucak and St-Germain, 2006). Moreover, it is usually observed that at short HRT values, sufficient time is unavailable for the SRB to reduce sulfate, thereby hindering metal precipitation as sulfide. Whereas, a long HRT offers a greater extent of sulfate reduction by using the carbon source more efficiently, thereby precipitating the metals as their respective sulfide salts (Dvorak et al., 1992).

Another reason for a low heavy metal removal efficiency at 24 h HRT may be due to substrate diffusion limitation and intermittent biomass washout from the reactor, which could be overcome by improving the mechanical stability and porosity of the bio-support material. Among the different metals tested in this study, Cu(II) removal was maximum at all inlet concentration levels owing to its low solubility product value with sulfide. Consequently, the metal removal order matched well with their solubility product values reported in the literature (Hill et al., 2005). The metal removal thus followed the order: Cu > Zn > Pb > Cd > Ni > Fe, which matches well with the metal removal obtained in the batch study (Fig. 4.16).

Performance of the DFCR in terms of metal removal rate with respect to inlet metal loading rate (ILR) is shown in Fig. 4.26, which reveals that the metal removal efficiency is maximum at a low ILR, indicating that SRB activity is unaffected due to the metals at a low inlet concentration (Fig. 4.26). However, with an increase in the ILR, the removal rate and removal efficiency decreased, indicating an inhibitory effect of the metals at a high inlet concentration (175 mg/L in case of Cu(II) and 90 mg/L for the other metals) on SRB activity (Fig. 4.26).

From Fig. 4.26, the line passing through the origin indicates a stable performance of the system at different input conditions; the removal rate values that are offset from the line passing through the origin indicate that ILR values beyond this point are unfavorable for achieving a high metal removal using the DFCR. Hence, metal loading rates greater than 4.29 mg/L·h in case of Cu(II) and 2.20 mg/L·h, in case of Ni(II), Zn(II), Pb(II), Fe(III) and Cd(II) are considered toxic and inhibitory to SRB activity and, therefore, detrimental to the reactor performance.

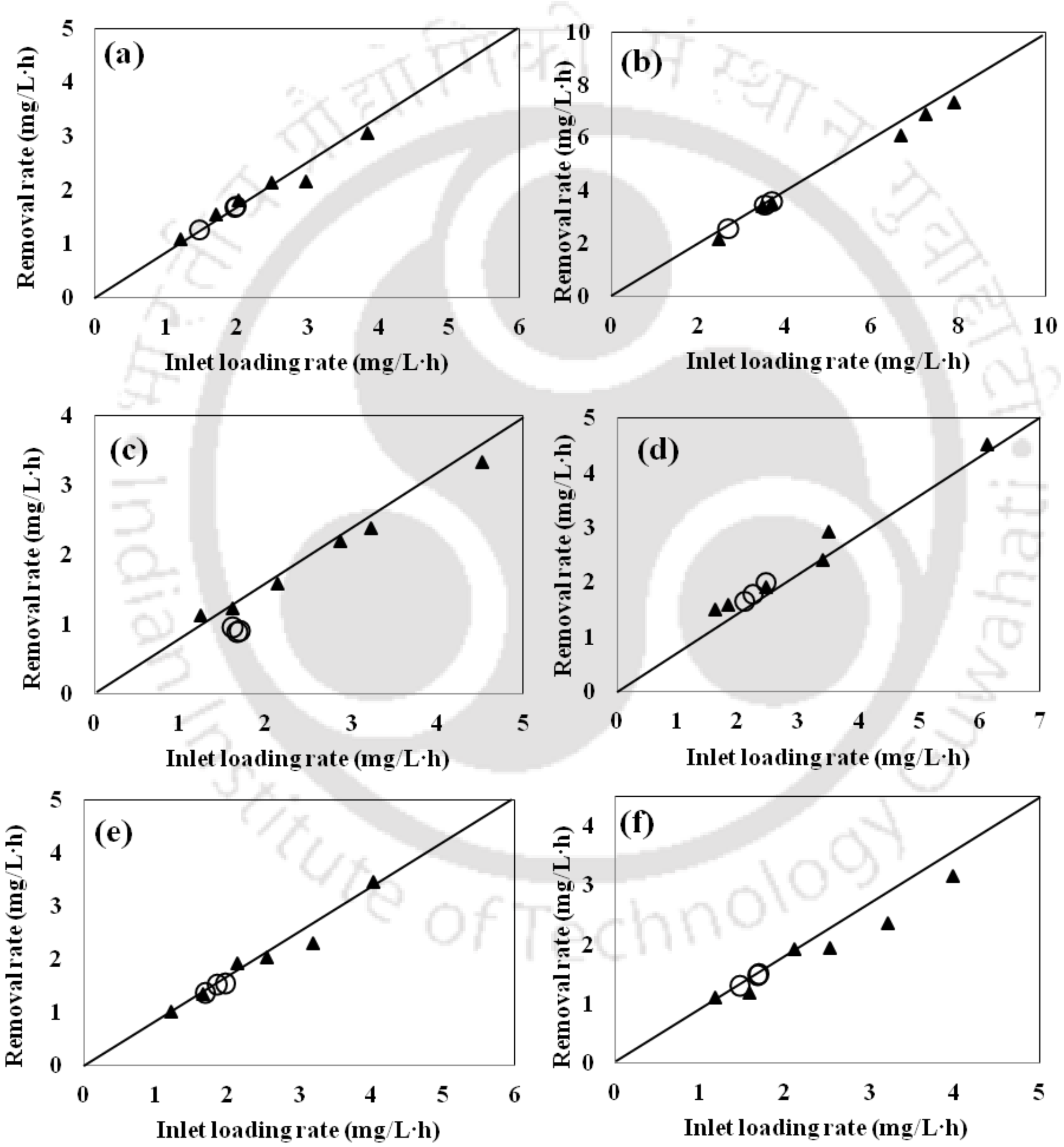


Figure 4. 26 Heavy metal removal rate as a function of inlet metal loading rate obtained using the continuously operated DFCR with immobilized SRB beads: (a) Cd(II), (b) Cu(II), (c) Ni(II), (d) Fe(III), (e) Pb(II) and (f) Zn(II) (▲: Metal removal rate from single component system; ○: Metal removal rate from multi-metal solution).

4.2.2 Heavy metal removal from multi-component system

The collective effect of more than one metal can be greater than the sum of each metal effect (synergy) or, sometimes, it can be equal to the summation of the effects of each individual metal (additive effect) (Utgikar et al., 2004; Gikas et al., 2007) or lower than their sum due to antagonistic effect (Utgikar et al., 2004). Hence, metals in mixture present a different effect on microorganisms in a biological system than the individual metals themselves (Sema et al., 2012). Furthermore, it has been well reported that results from batch experiments cannot be extrapolated to understand the combined effect of heavy metals on each other removal in continuously operated bioreactor systems (Chen et al., 1997; Hu et al., 2004; Sen et al., 2007). Very limited reports are available on studying the combined effect of metals on microbes in both batch and continuous systems using SRB.

Hence, this study was aimed at investigating the combined effect of different heavy metals on heavy metal removal, sulfate reduction and COD removal by SRB using the DFCR. The statistically valid fractional factorial design of experiments was employed in this study; student's *t* test and ANOVA were then applied for statistical analysis of the results to interpret the significance and effect of these metals on each other removal as well as on COD removal and sulfate reduction under continuous operation mode.

4.2.2.1 Heavy metal and COD removal and sulfate reduction

Metal removal from mixture using the DFCR is presented in Fig. 4.27a which shows that Cu(II) removal is maximum (99%), followed by Zn(II) (95.8%), Cd(II) (94%), Pb(II) (92%), Fe(III) (86%) and Ni(II) (70%) at their respective low inlet concentrations (experimental run 18, Table 3.4). A low inlet concentration of the different heavy metals i.e, 50 mg/L each of Ni(II), Pb(II), Fe(III), Cd(II) and Zn(II) and 100 mg/L of Cu(II) resulted in a maximum removal of the metals (Fig. 4.27a).

The metal removal values were low at their respective high inlet concentration combination in the mixture (Cu(II) 96.4%, Cd(II) 92%, Zn(II) 79.8%, Fe(III) 71%, Pb(II) 61.5% and Ni(II) 47.5%) (experimental run 4, Table 3.4). These values are slightly lower than the corresponding metal removal values obtained in case of the single metal solution (Fig. 4.22). These results confirm the dependence of metal and their initial concentration in the mixture on their removal by SRB. Figure 4.27b presents sulfate reduction and COD removal and sulfide generated consequent to the experimental runs using the DFCR.

Experimental run 18 (metals added at their respective low initial level combination) showed maximum sulfate reduction followed by experimental run 5; COD removal was maximum in experimental runs 2 and 18 (Fig. 4.27b).

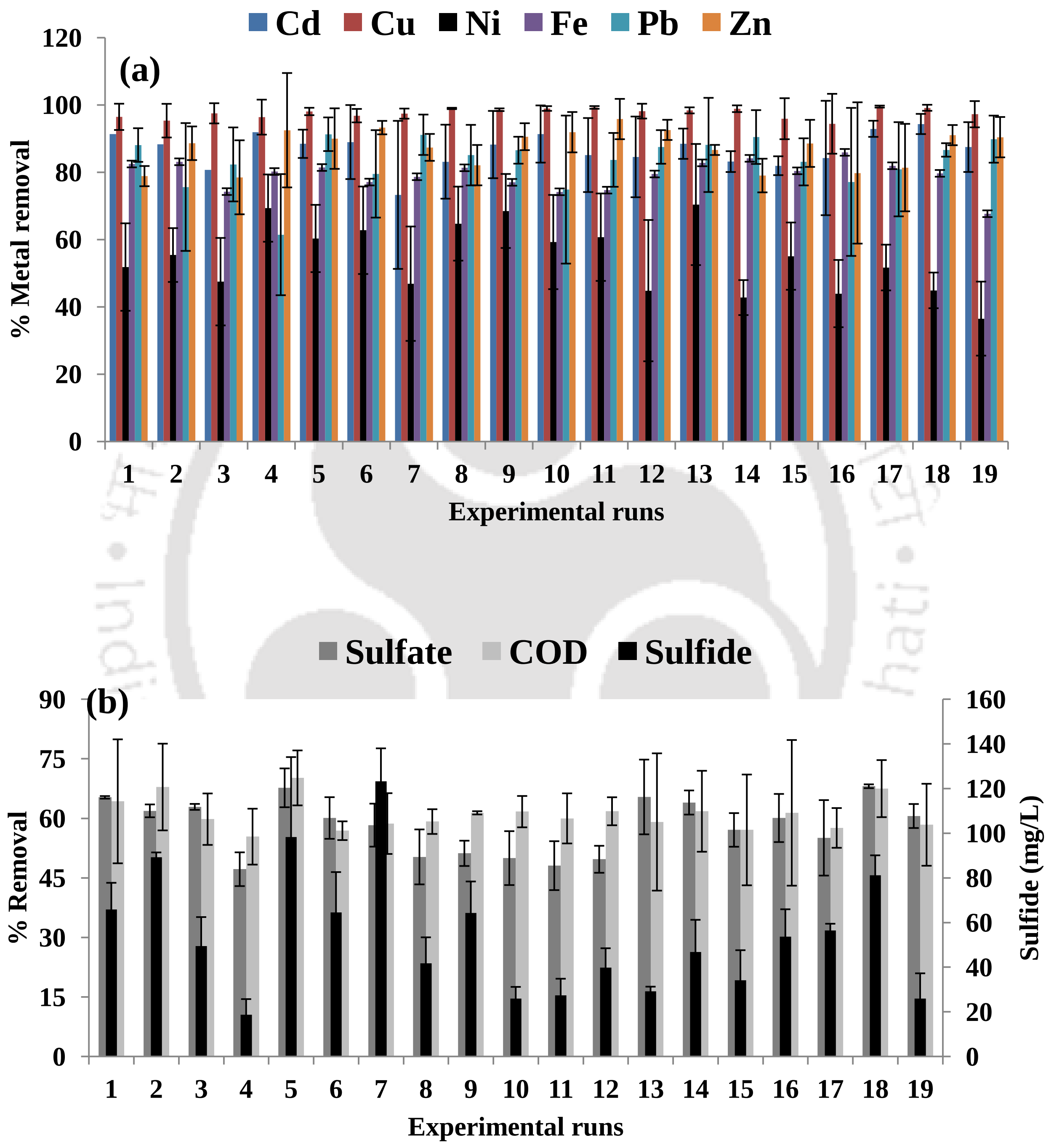


Figure 4. 27 (a) Continuous removal of heavy metal removal from mixture, (b) Continuous COD removal, sulfate reduction and sulfide generated in the mixture experiments using the DFCR.

Sulfide generated was maximum in experimental runs 7, 5, 2 and 18, respectively (Fig. 4.27b). Both COD removal and sulfate reduction efficiencies were minimum in experimental run 4 (metals added at their respective high initial level combination) using the DFCR. These values of COD removal, sulfate reduction and sulfide generated were less than that obtained in the single component system (Figs. 4.23-4.25). Thus, it is clear from Fig. 4.27 that the removal of different heavy metals correlated well with the sulfate removal results, confirming that the metal removal by SRB is due to sulfide precipitation.

These results further imply that low initial level concentration of the heavy metals in the multi-component system did not inhibit the SRB activity (Figs. 4.22a and 4.27a). Copper removal was maximum amongst all the heavy metals tested, which matched well with the results obtained from a previous study (Hill et al., 2005). Low values of COD removal and sulfate reduction obtained using the DFCR can be attributed to the diffusion limitation of these substrates in the DFCR packed with immobilized SRB beads (Min et al., 2008), which also matched with the results obtained in the single component system using the DFCR (Figs. 4.23 and 4.24). Biomass washout from the reactor could be another reason for low values of COD and sulfate reduction (Hsu et al., 2010). Figure 4.26 compares the removal rate of metals from single and multi-metal solution. It can be noted that the removal rate of Ni(II) from mixture was affected compared with its removal from single metal solution (Fig. 4.26c), whereas removal rates of all other metals were stable and unaffected in both single and mixture systems (Fig. 4.26).

4.2.2.2 Characterization of bio-precipitates formed in the mixture experiments

Bio-precipitate analysis using FESEM-EDX was performed to comprehend the elemental composition and morphology of the bio-precipitates produced in the reactor. Figure 4.28a illustrates the EDX spectrum of the precipitate collected from experimental run 18, FESEM image of the precipitate is shown in the same figure. Different metals precipitated in the experimental run 18 are highlighted with circle in Fig. 4.28a. X-ray dot mapping of the precipitates obtained from experimental run 18 collected from the DFCR is presented in Fig. 4.28b. From FESEM-EDX results (Fig. 4.28a), it is evident that sulfide peak is more prominent compared to the other peaks due to the media composition, revealing that metals were precipitated as metal sulfides, whereas the other forms, e.g., MCO_3, $M(OH)_x$, etc., are not considerable, which is in agreement with the metal removal from the previous batch study (Fig. 4.14). X-ray dot mapping of the precipitate (Fig. 4.28b) further reveal the presence and distribution of different metals in the bio-precipitates (Utgikar et al., 2002).

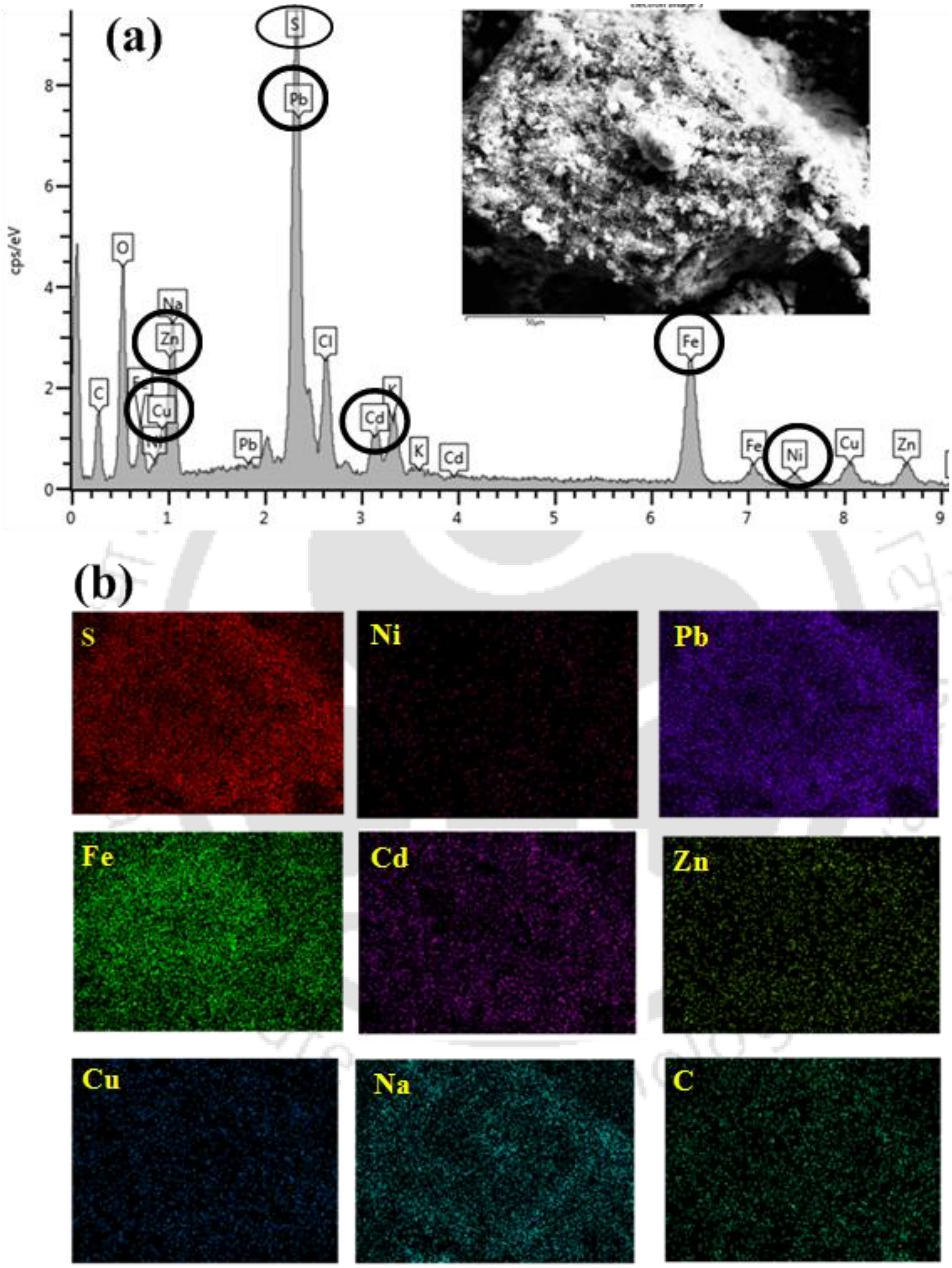


Figure 4. 28 (a) EDX spectrum with the image of the precipitate (experimental run # 18) collected from the DFCR and (b) X-ray dot mapping of the same precipitate.

4.2.2.3 Statistical analysis

Tables 4.7 and 4.8 (Appendix E.1 and E.2) present the results of ANOVA and student *t* test of heavy metal removal using the DFCR. In this study, a low P value ($P < 0.1$ or 0.25) for the factors and their effects is considered as significant (Madamba et al., 2001; Montgomery, 2004). Furthermore, from the results of ANOVA (Table 4.7) and student *t* test (Table 4.8) of metal removal from mixture using the DFCR, Cd(II) concentration inhibited ($P < 0.2$) Zn(II) removal efficiency owing to their similar solubility product constant values (Montgomery, 2004).

Zinc and nickel also showed inhibitory effect ($P < 0.09$; Table 4.8) toward Fe(III) removal due to their similar solubility product constant values with sulfide. Similarly, Pb(II) removal from mixture was inhibited due to the simultaneous presence of the other metals ($P < 0.2$; Table 4.8). These results of metal removal from mixture can be attributed to the competitive/interaction effect posed by simultaneous presence of different heavy metals on the sulfate reducing activity of the SRB which is the prevailing mechanism for metal removal. It is also reported that metals in mixtures exert more toxic effect than in single component systems (Utgikar et al., 2004).

4.3 Heavy Metal Removal Using The Anaerobic Rotating Biological Contactor (An-RBC) Reactor In Continuous Operation Mode

For a successful application of heavy metal removal by sulfide precipitation on large-scale, the choice of a suitable reactor system is essential. The previous section described the performance of actively immobilized SRB biomass based DFCR for heavy metal removal. In order to evaluate the performance of a bioreactor with passively immobilized biomass, anaerobic rotating biological contactor (An-RBC) reactor was chosen.

Among the several bioreactor systems to treat wastewater, RBC reactor is well known for treating various types of effluents (Alemzadeh et al., 2002; Kapdan and Kargi, 2002; Axelsson et al., 2006) as it offers a high interfacial area on its rotating discs for biomass growth and attachment, which enables sufficient contact between the microorganisms and contaminants present in the wastewater (Pakshirajan amd Kheria, 2012). Furthermore, RBC reactor is highly suitable for treating industrial wastewater and sewage (Banerjee, 1997) owing to the combined action of both suspended growth and fixed film microorganisms present in this reactor system.

The utility of RBC reactors has been examined at the laboratory scale for removal of COD from textile dying and synthetic wastewater (Pakshirajan et al., 2009). However, it has not been tested for the removal of heavy metals by biogenic sulfide precipitation. Hence, this study is focused on evaluating the performance of a laboratory scale An-RBC reactor in which the SRB exist mainly as passively attached biofilm onto its rotating discs for continuous metal removal from single and multi-component systems at different inlet metal concentration and at different HRT values.

4.3.1 Single component system

4.3.1.1 FSESM characterization of immobilized SRB onto the support material

Figure 4.29a compares the FESEM image of the bio-support material for morphology characteristics without the SRB biomass with that of the bio-support material with the immobilized SRB (Fig. 4.29b-f). These figures clearly reveal immobilization of the SRB biomass onto the support material. However, no efforts were made to measure the thickness or composition of the biofilm formed on each disk of the An-RBC reactor, which need more detailed characterization techniques for a better understanding. Black precipitate observed in the reactor during the startup phase is attributed to sulfide generation by SRB (Herbert and Gilbert, 1984; Hamilton, 1994; Singh et al., 2011).

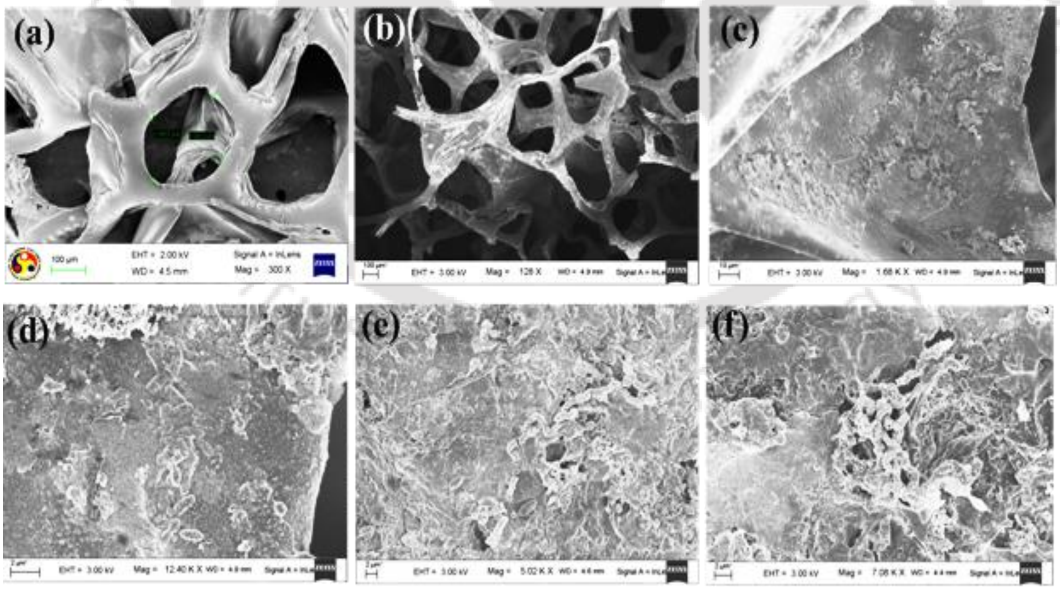


Figure 4. 29 FESEM image of the bio-support material used in the An-RBC reactor: (a) without and (b-f) with immobilized SRB at different positions on the bio-support.

4.3.1.2 Heavy metal and COD removal and sulfate reduction

Figure 4.30 shows the reactor performance in terms of metal removal at 48 h and 24 h

HRT, respectively. At all the inlet metal concentrations, more than 85% metal removal was obtained at 48 h HRT for all the metals except Ni(II), which showed nearly 77% (Fig. 4.30a). These removal values were, however, lower at 24 HRT for all the metals (Fig. 4.30b). More than 80% removal was obtained for Cd(II), Fe(III) and Pb(II), whereas in case of Cu(II), more than 93% removal could be achieved. In case of Ni(II) and Zn(II), the removal values were more than 73% and 75%, respectively. These results were slightly less than the metal removal results obtained previously using the DFCR (Fig. 4.22).

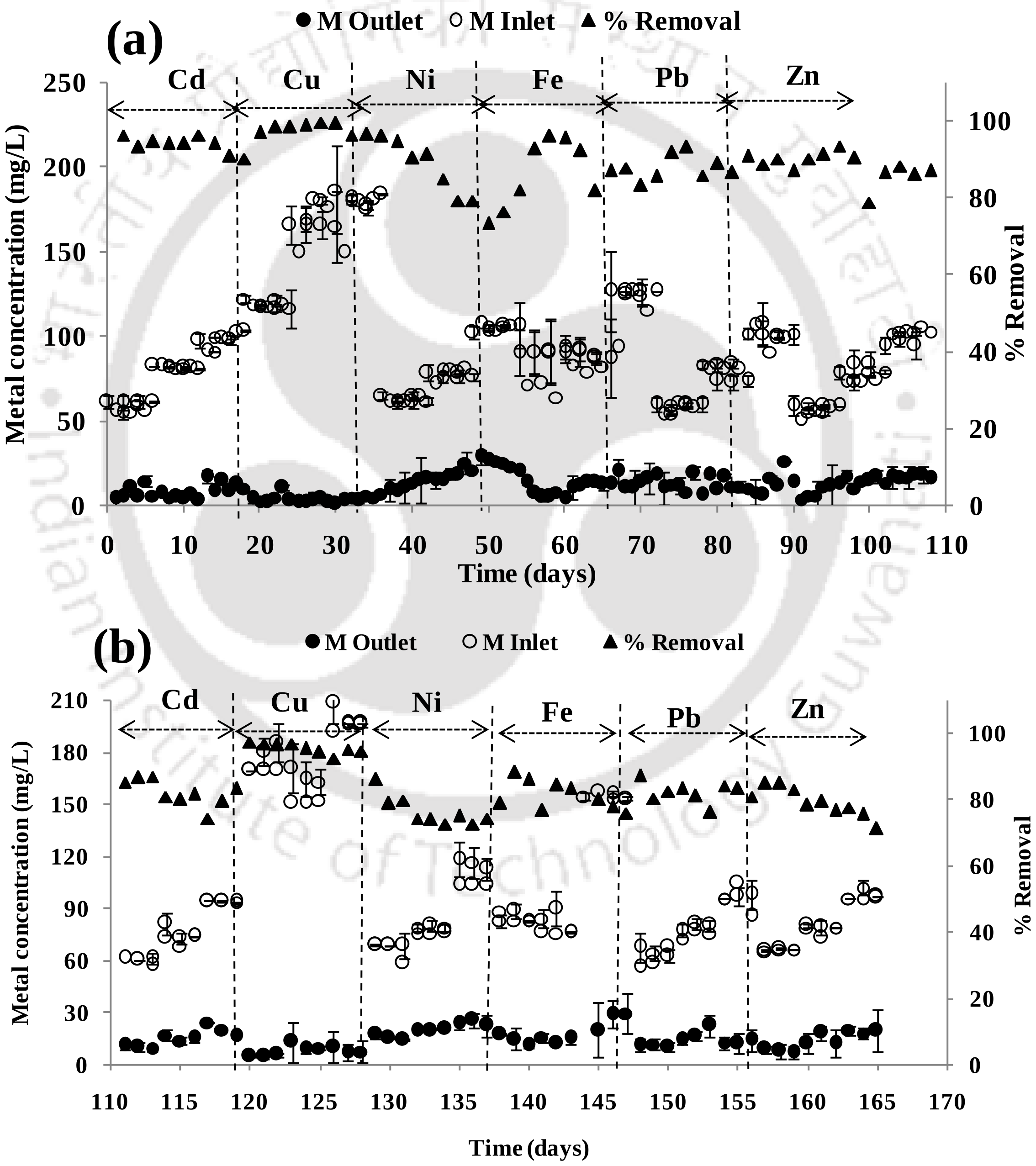


Figure 4. 30 Removal of different heavy metals using the An-RBC reactor: (a) 48 h RT and (b) 24 h RT (●: Metal outlet, ○: Metal inlet, ▲: % Metal removal).

Sulfate and COD reduction profiles obtained using the An-RBC reactor are depicted in Figs. 4.31-4.32. A low inlet metal loading into the system yielded sulfate reduction in the range 53 - 77% at 48 h HRT for all the metals (Fig. 4.31a). However, these values were reduced (49 - 64%) at 24 h HRT (high inlet loading of the metals) (Fig. 4.31b). Figure 4.32a reveals that COD removal ranged from 68 to 82% for all the metals at a low inlet metal loading (48 h HRT). Whereas, at a high metal loading (24 h HRT), COD removal was more than 62-75% (Fig. 4.32b). These results were slightly more than that obtained previously using the DFCR (Figs. 4.23-4.24).

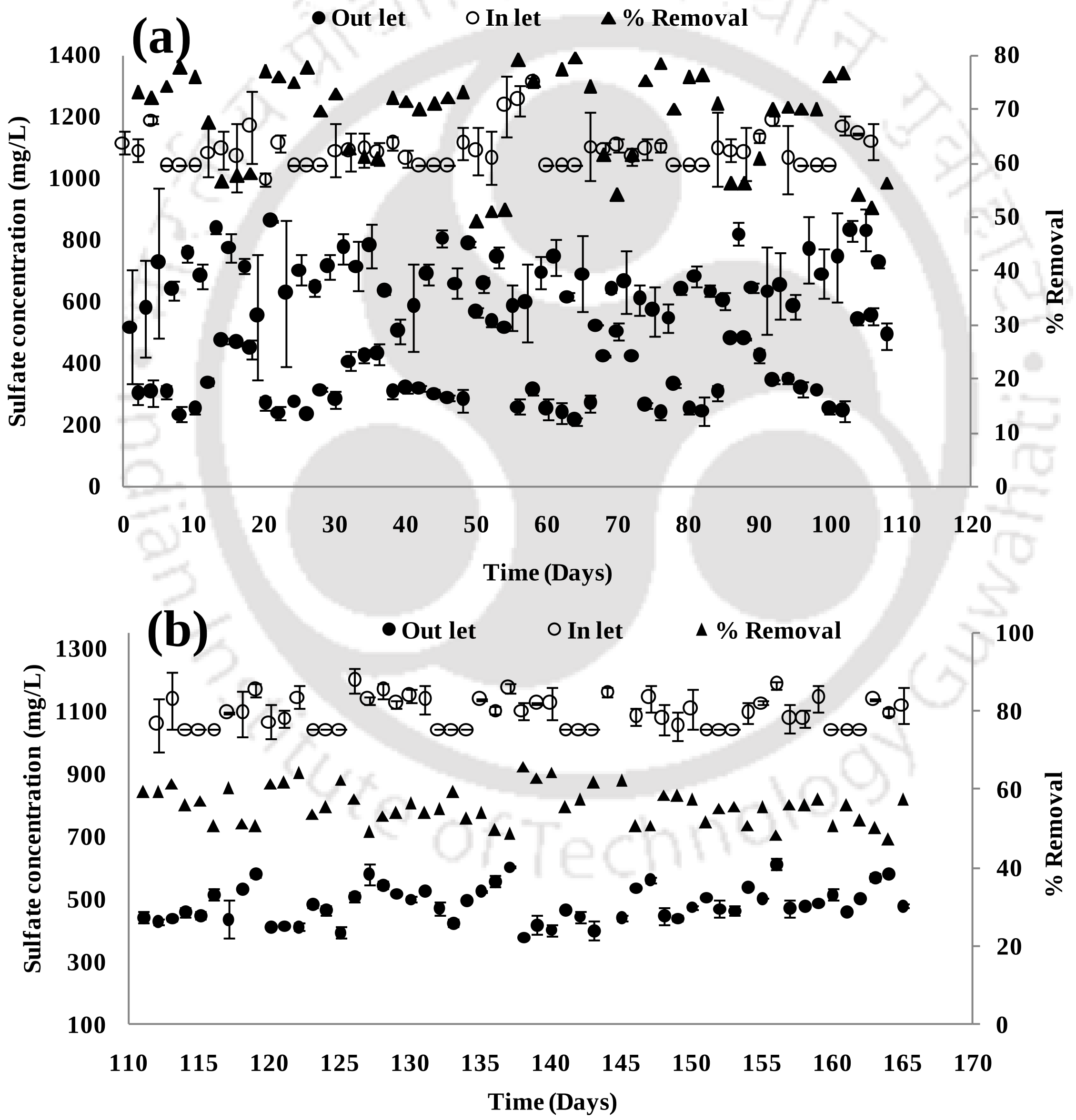


Figure 4. 31 Sulfate reduction in the presence of different heavy metals using the An-RBC reactor: (a) 48 h RT and (b) 24 h HRT (●: Sulfate outlet, ○: Sulfate inlet, ▲: % Sulfate removal).

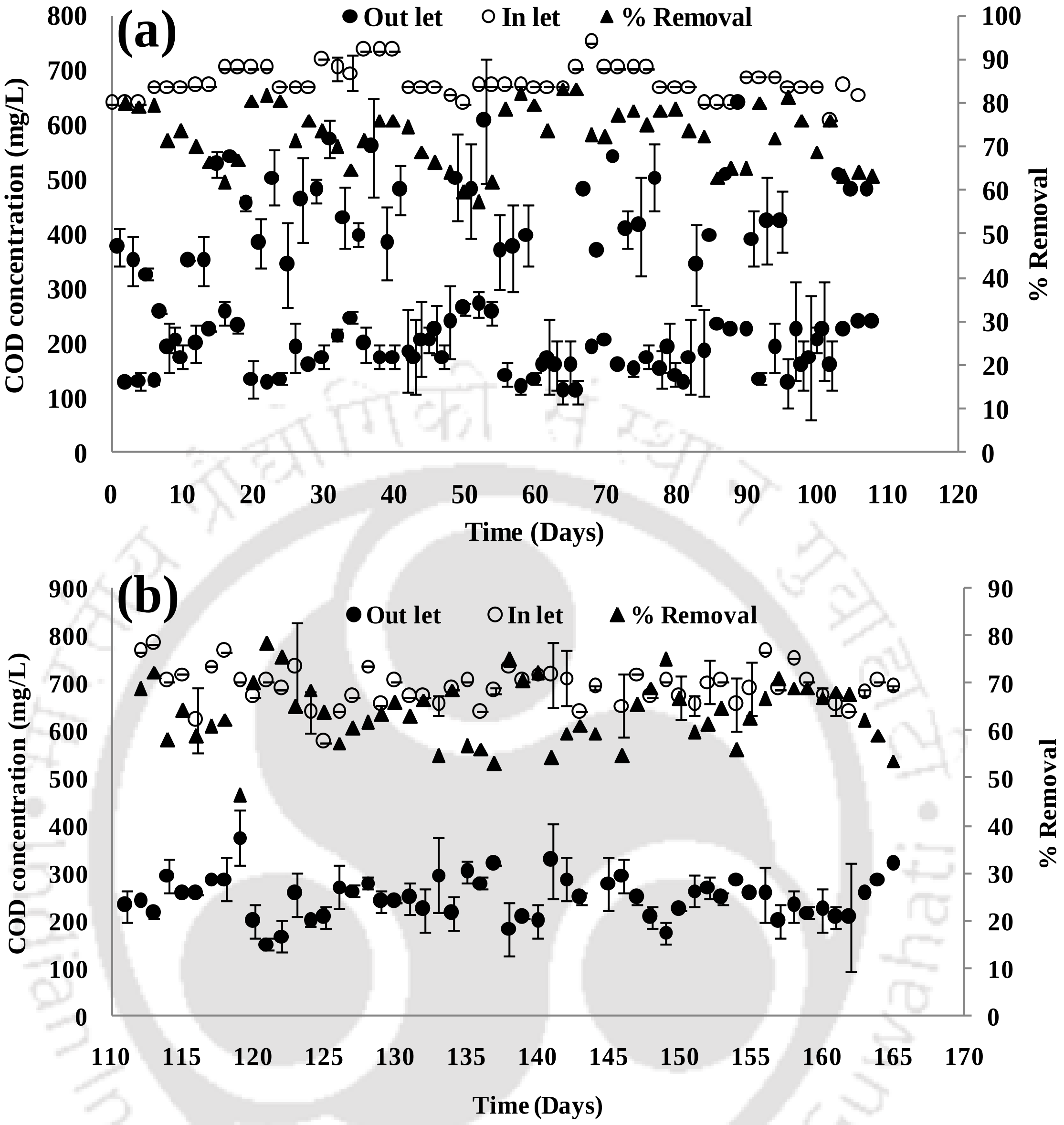


Figure 4. 32 COD removal in the presence of different heavy metals using the An-RBC reactor: (a) 48 h RT and (b) 24 h HRT (●: COD outlet, ○: COD inlet, ▲: % COD removal).

The results of metal removal (Fig. 4.30) obtained in this study revealed that at both 48 h and 24 h HRT values, metal removal was maximum at their respective low inlet metal concentrations, similar to the results obtained previously using the DFCR (Fig. 4.22). Maximum removal of these metals achieved at a low inlet metal concentration level indicates very good SRB activity in the An-RBC reactor suggesting that the reactor was well capable of handling low strength metallic wastewater (Fig. 4.30). This can be attributed to the tolerance of SRB to these metals at low concentrations (Martins et al., 2009); very high removal efficiency of the metals at their low inlet loading is also attributed to the earlier acclimatization of SRB to sulfate reduction during the initial startup phase of the An-RBC reactor. Similar results were reported by Martins et al. (2009) in their study using high metal resistant SRB consortium for the removal of Fe(III), Zn(II) and Cu(II) without any effect on the SRB activity at inlet concentrations of 400 mg/L, 150 mg/L and 80 mg/L, respectively. Sulfide generated in the continuous experiments using the An-RBC reactor is shown in Fig. 4.33 for the two HRT conditions, which matched with the afore-mentioned sulfate reduction values. These results were slightly more than that obtained previously using the DFCR (Fig. 4.25).

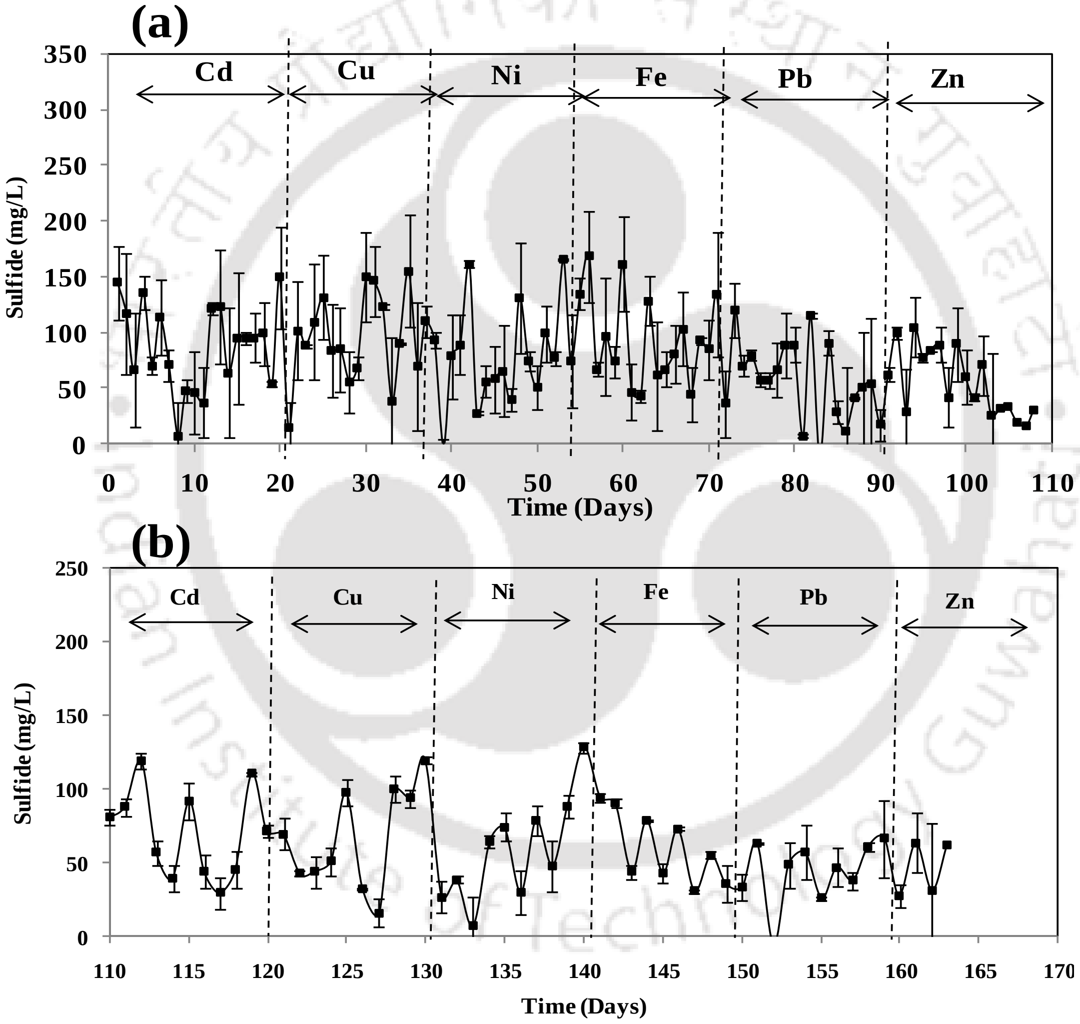


Figure 4. 33 Sulfide generated in presence of the different heavy metals using the An-RBC reactor: (a) 48 h RT and (b) 24 h HRT (■ Sulfide generated (mg/L)).

A slight reduction in the metal removal at a high inlet metal concentration (Fig. 4.30), particularly at 90 mg/L and above indicated inhibition of SRB activity (Kieu et al., 2011), as also observed previously in case of the DFCR (Fig. 4.22). This is also consistent with that reported in the literature, however, using fluidized bed reactor system (Nevatalo et al., 2010; Sahinkaya and Gungor, 2010). The inhibition of SRB activity at a high inlet metal concentration is also evident from the sulfate and COD reduction values presented in Figs. 4.31 and 4.32. Furthermore, a high residual COD in the effluent which is still well-below the permissible discharge standards for wastewater is attributed to the formation of acetate from lactate (Widdel, 1988; Omil et al., 1996; Lens et al., 1998; Nagpal et al., 2000).

At low inlet metal concentration, a high sulfate reduction efficiency and, therefore, a high sulfide content was observed (Figs. 4.31a and 4.33a). Minimum inhibitory concentration level of different heavy metals on SRB as reported in the literature is presented in Table 4.1 (Azabou et al., 2007). In the present study, the metals at their respective low inlet metal concentrations did not inhibit the sulfate reduction by SRB (Fig. 4.30), which also matched well with the results obtained using the DFCR.

However, an increase in the influent metal concentration decreased the sulfate reduction efficiency and the sulfide content. All the results of sulfide generation (Fig. 4.33) matched well with the results obtained using the DFCR (Fig. 4.25) and are consistent with those reported by Christensen et al. (1996) and Jong and Parry (2003). A decrease in the sulfide content at a high influent metal concentration can be attributed to (a) its utilization of metal precipitation and (b) volatile hydrogen sulfide formation (Jong and Parry, 2003).

Metal removal observed in this study is maximum at 48 h HRT than at 24 h HRT at their respective high inlet metal concentrations (Fig. 4.30a), which is consistent with the results obtained using the DFCR (Fig. 4.22a). The sulfate reduction and COD removal were also better at 48 h HRT than at 24 h HRT (Figs. 4.31a and 4.32a), and the values are slightly higher than the results obtained using the DFCR (Figs. 4.23a and 4.24a).

The metal removal mechanism in the continuously operated An-RBC reactor is thus attributed to the precipitation of metals as metal sulfide salts *via* sulfate reduction by SRB in the reactor. Compared to other reactor systems, RBC reactor system used in this study offers a high interfacial area on its rotating discs for biomass growth and attachment, which also provide sufficient contact between the microbes and contaminants present in the wastewater (Pakshirajan and Kheria, 2012). Together with this attached biomass, the suspended biomass present in the reactor vessel further improves the wastewater treatment efficiency in terms of sulfate reduction and removal of COD and heavy metals.

Figure 4.34 shows the performance of the An-RBC reactor in terms of metal removal rate as a function of ILR. It can be seen that the metal removal efficiency is maximum at a low ILR suggesting that SRB activity is unaffected due to the metal at a concentration below 75 or 150 mg/L (Fig. 4.34). However, with an increase in the ILR, the metal removal rate and metal removal efficiency decreased, indicating an inhibitory effect of the metals above 90 or 175 mg/L of inlet concentration on SRB activity (Fig. 4.34).

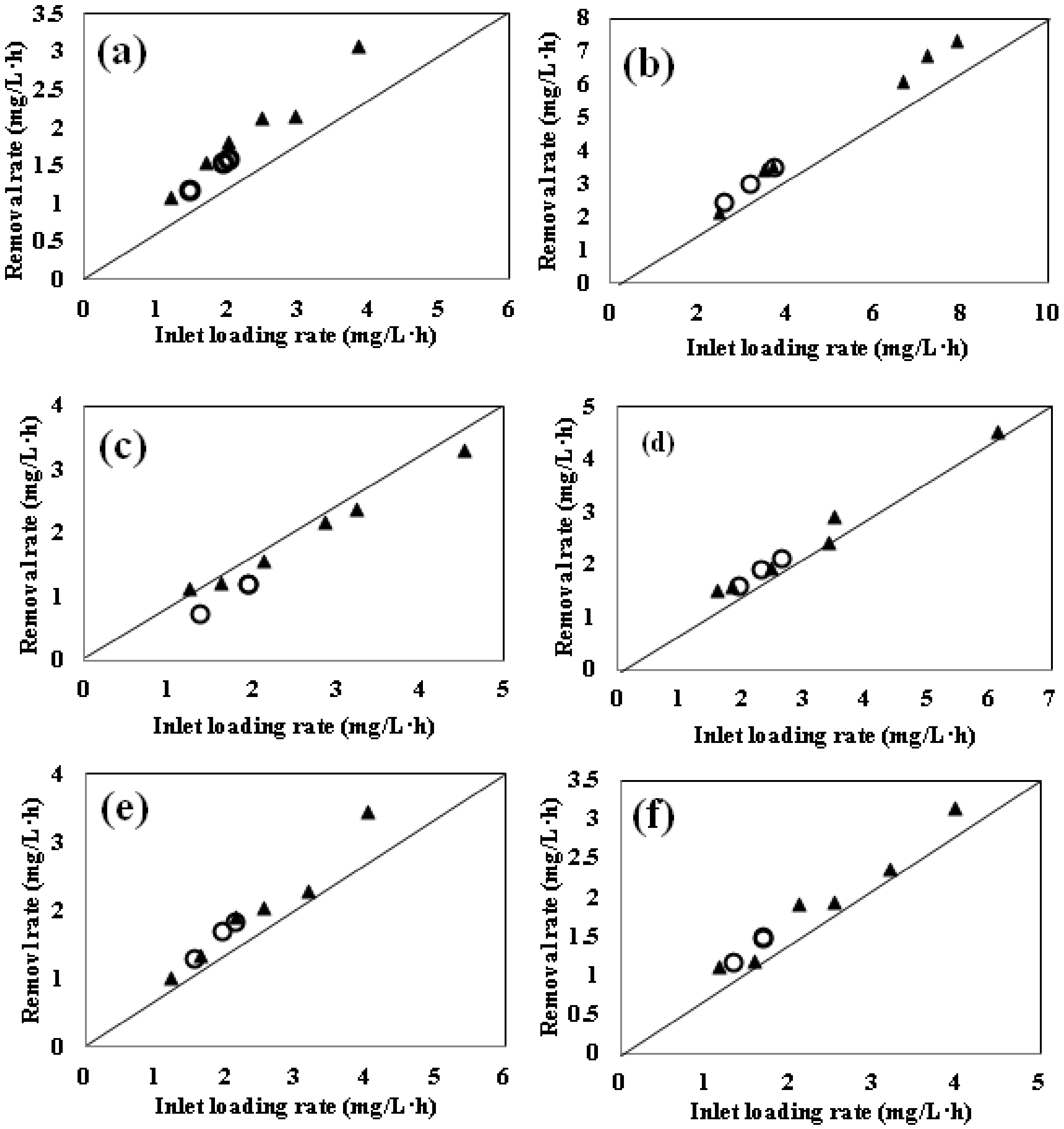


Figure 4. 34 Removal rate of different heavy metals as a function of inlet loading rate using the An-RBC reactor: (a) Cd(II), (b) Cu(II), (c) Ni(II), (d) Fe(III), (e) Pb(II) and (f) Zn(II) (▲: Metal removal rate from single component system; ○: Metal removal rate from multi-metal solution).

These observations were similar to those obtained using the DFCR (Fig. 4.26). Hence, it could be surmised that metal loading rates greater than 3.64 mg/L·h in case of Cu(II) and 1.87 mg/L·h, in case of Fe(III), Pb(II), Ni(II), Zn(II) and Cd(II) are toxic and inhibitory to SRB activity and are therefore, detrimental to the performance of the An-RBC reactor. Among the different metals tested in this study, Cu(II) removal was maximum at all inlet concentration levels as explained earlier. It has been reported that copper sulfide is least soluble compared with the other metal sulfides owing to its very low solubility product value of 6×10^{-37} (Hill et al., 2005).

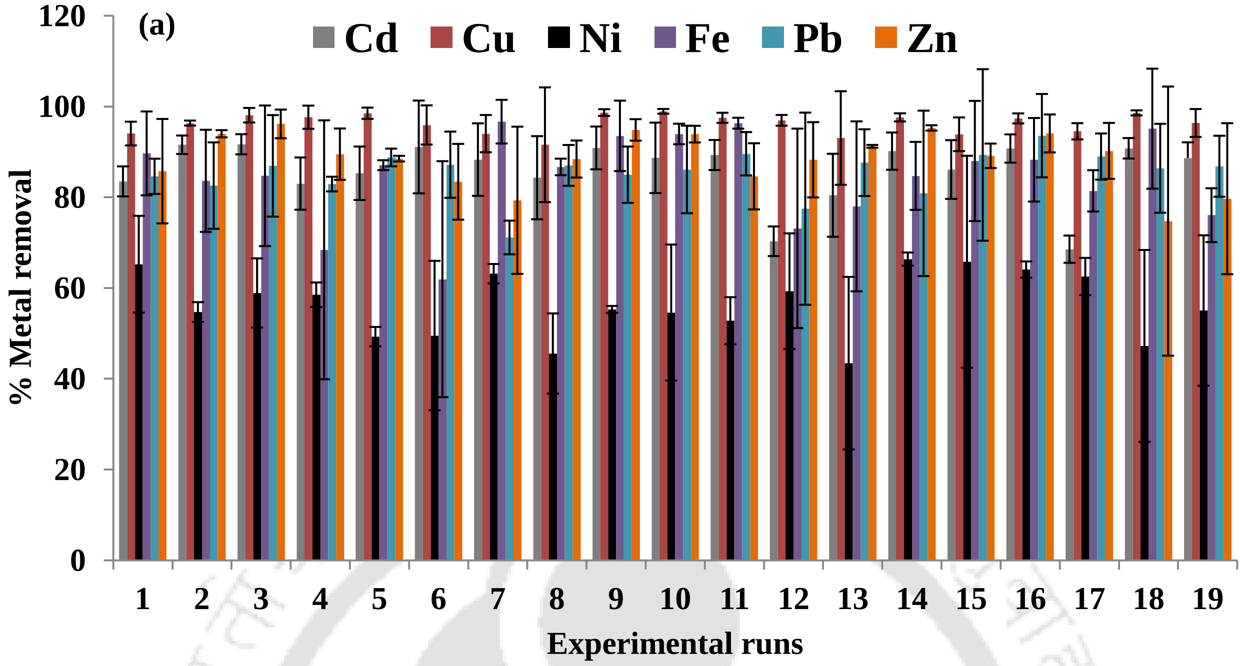
4.3.2 Heavy metal removal from multi-component system

This mixture study was aimed at investigating the performance of the An-RBC reactor for continuous metal removal from multi-metal solution.

4.3.2.1 Heavy metal and COD removal and sulfate reduction

Figure 4.35a presents simultaneous removal of Ni(II), Cd(II), Fe(III), Zn(II), Pb(II) and Cu(II) from multi-metal solution using the An-RBC reactor. A maximum removal of Cu(II) (98.4%), followed by Zn (II) (96%), Fe(III) (95%), Pb(II) (93.5%), Cd(II) (91%) and Ni(II) (65.7%) was obtained at their respective low level combination (experimental run 5; Table 3.8). These results were slightly lower than the metal removal from multi-component system obtained previously using the DFCR (Fig. 4.27). Maximum metal removal was achieved at a low concentration combination of these metals, i.e. at 50 mg/L each of Zn(II), Cd(II), Pb(II) and Ni(II) and at 100 mg/L Cu(II) (Fig. 4.35a). Metal removal was minimum at their respective high level combination (experimental run 15; Table 3.8) (Fig. 4.35a), which were slightly better than the metal removal from multi-component system obtained using the DFCR (Figs. 4.27a). All these results clearly reveal the dependence of metal removal efficiency by SRB on the metal and their initial concentration level in the mixture.

Figure 4.35b presents COD and sulfate reduction efficiencies and sulfide generated consequent to the experimental runs using the An-RBC reactor. Maximum sulfate reduction efficiency was obtained with metals added at their respective low initial level combination (experimental run 5) and in experimental run 1 (> 71%) (Fig. 4.35b).



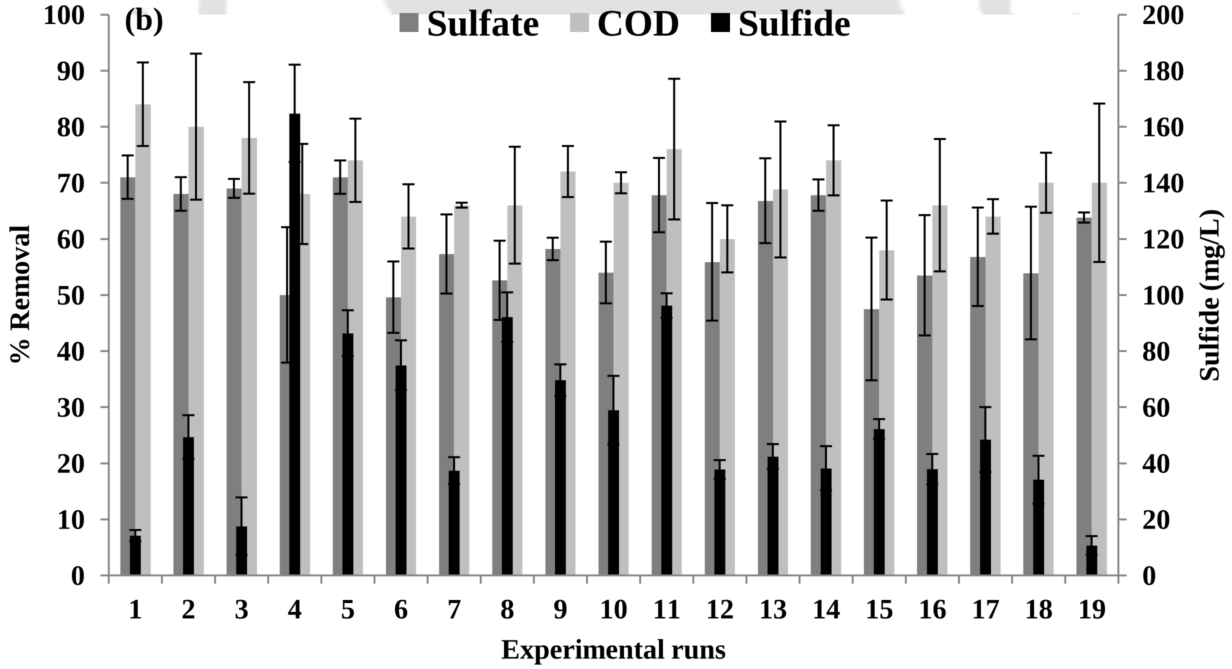


Figure 4. 35 (a) Continuous heavy metal removal from mixture, (b) Continuous COD removal, sulfate reduction and sulfide generated in the mixture experiments using the An-RBC reactor.

Maximum COD removal was obtained in experimental runs 5 and 2 (> 78%) (Fig. 4.35b). Sulfide generated was maximum in experimental runs 4, 11, 8 and 5, respectively. Both COD removal and sulfate reduction were minimum in experimental run 15 (metals added at their respective high initial level combination).

These results of COD removal, sulfate reduction and sulfide generated were slightly higher than that obtained using the DFCR (Fig. 4.27b). It could be observed from Fig. 4.35 that removal of different heavy metals correlated well with sulfate removal, confirming that the metal removal by SRB is due to sulfide precipitation. Copper removal was maximum amongst the other heavy metals, which is similar to the observation made in all earlier studies (Fig. 4.27a).

Figure 4.34 also compares the removal rate of metals from single and multi-metal solution obtained using the An-RBC reactor. It can be noted that the removal rate of Ni(II) from mixture was affected compared with its removal from single metal solution (Fig. 4.34c), whereas removal rate of all other metals was stable and unaffected in both single and mixture systems (Fig. 4.34). Therefore, from Fig 4.34, it can be observed that metal loading rates greater than 3.7 mg/L·h in case of Cu(II) and more than 1.69 mg/L·h in case of Ni(II), Pb(II), Cd(II), Pb(II) and Fe(III) are toxic and inhibitory to SRB activity and, thus, unfavorable to the performance of the An-RBC reactor for metal removal from mixture.

Thus, both DFCR and An-RBC reactors showed maximum removal of the metals from mixture at low inlet concentration combination; but, the An-RBC reactor showed better performance over DFCR in terms of maximum metal removal efficiency, sulfate reduction and COD removal at a high inlet concentration combination as evident from Figs. 4.27 and 4.35 which is attributed to the explanation provided in the Section 4.3.1.2.

Utgikar et al. (2004) in their study demonstrated that the toxic effect of Zn and Cu in mixture was notably greater than their individual toxic effect. In another study using an anaerobic semi continuous stirred tank reactor containing SRB for metal removal from mixture, Kieu et al. (2011) reported about 94-100% metal removal each of Cu, Ni, Zn and Cr without any effect on the performance of the reactor. It is evident from the results obtained from different studies, including the present study, that the toxic and inhibitory effects of different metals in mixture on SRB are controlled by parameters, such as pH, temperature, HRT, type of metal and its concentration, microbial community and the bioreactor configuration which is evident from this present study (Kieu et al., 2011).

4.3.2.2 Statistical analysis

Tables 4.9 and 4.10 (Appendix F.1 and F.2) present the results of ANOVA and student *t* test of metal removal using the An-RBC reactor. Furthermore, from the results of ANOVA (Table 4.9) and student *t* test (Table 4.10) of metal removal from mixture using the An-RBC reactor, it can be seen that Ni(II) concentration positively favored its own removal with a P value of 0.08 (Table 4.10), whereas individual effect of increase in Ni(II), Cu(II) and Zn(II) inhibited Pb(II) removal with a P value < 0.07 owing to their competitive effect for sulfide precipitation.

An increase in Cu(II) and Cd(II) concentration inhibited Zn(II) removal (P < 0.1; Table 4.10), whereas Pb(II) concentration negatively affected Cu(II) removal (P < 0.25; Table 4.10) both due to their similar solubility product constant values. Individual effect of an increase in Cd(II) concentration favored Fe(III) removal, whereas an increase in Cu(II) concentration inhibited Fe(III) removal.

4.3.2.3 Characterization of metal bio-precipitates formed in the reactor

To understand the morphology and elemental composition of metal bio-precipitates formed in the reactor, FESEM-EDX analysis was performed. Figure 4.36a shows EDX spectrum with elemental composition of the biomass collected from experimental run 5 (An-RBC reactor); insert to Fig. 4.36a represent FESEM image of the precipitate. X-ray dot mapping of precipitates obtained from experimental runs 5 collected from the reactor is presented in Fig. 4.36b.

Figure 4.36 confirms metal sulfide precipitation by SRB with the presence of other elements which constituted the liquid media. Different metals precipitated in the experimental run 5 are highlighted with circle in Fig. 4.36a. FESEM-EDX results shown in Figs. 4.28 and 4.36 match well with each other and further support that sulfidogenesis is the governing mechanism for metal removal by SRB in both the An-RBC and DFCR reactors (Jin et al., 2007). Metal removal order followed using the two reactors, i.e, Cu > Zn > Cd > Pb > Fe > Ni (Figs. 4.27 and 4.35) also closely correlated with the solubility product values of corresponding metal sulfide salts (Hill et al., 2005).

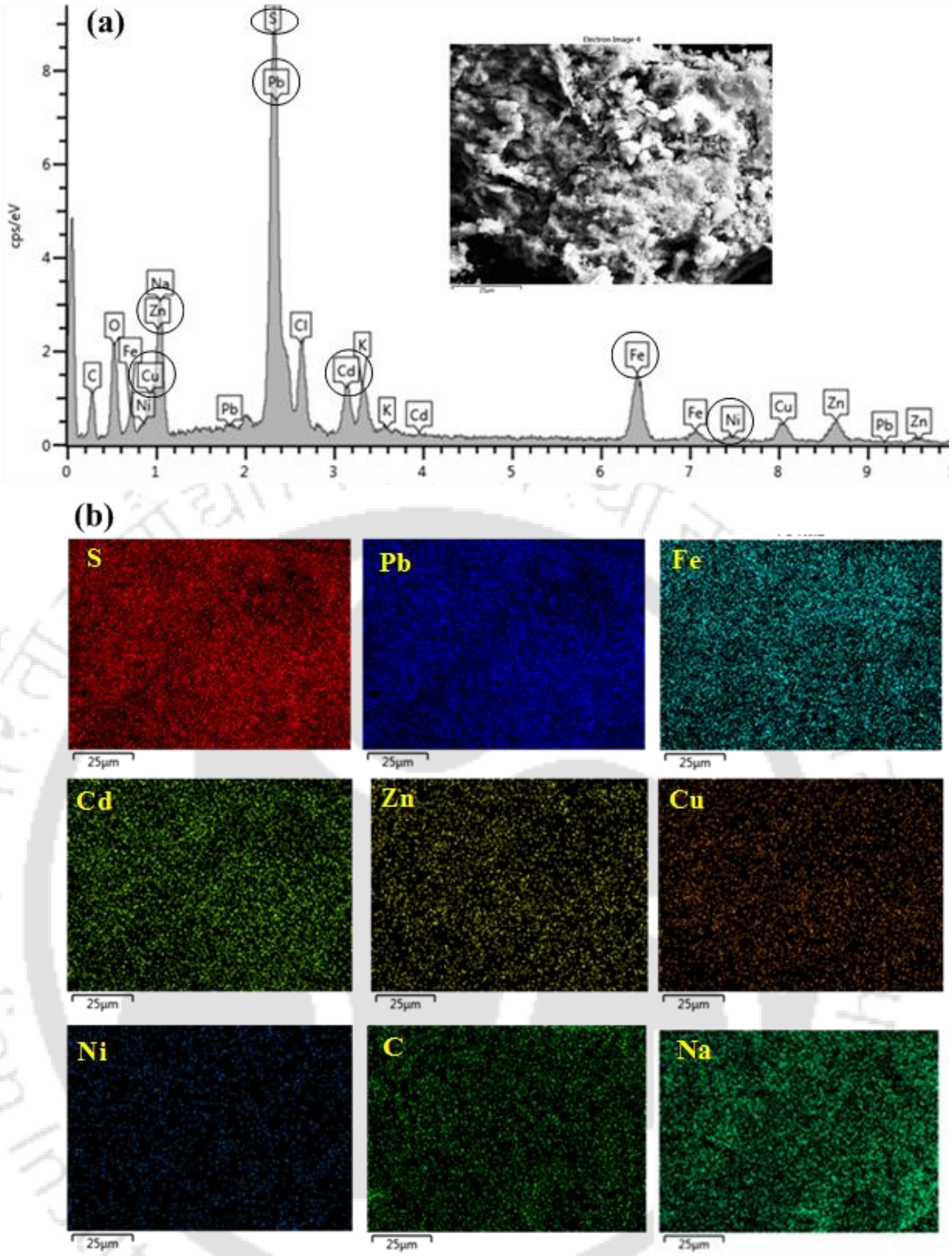


Figure 4. 36 (a) EDX spectrum with the image of the precipitate (experimental run # 5) collected from the An-RBC reactor and (b) X-ray dot mapping of the same precipitate.

Overall, it could be surmised that the An-RBC reactor (the passive biofilm bioreactor) is more suited than the DFCR with immobilized SRB beads for metal removal from wastewater which showed better performance in terms of maximum metal removal efficiency, sulfate reduction and COD removal at a high inlet metal concentration without any biomass washout problems.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5. Summary and Conclusions

The present study on heavy metal removal by biogenic sulfide precipitation using sulfate reducing bacteria (SRB) was performed under batch and continuous operation conditions. The key findings of the study are discussed in this section.

- Anaerobic biomass used in this study was collected from different sources that included wastewater treatment plant (WWTP) located at Indian Institute of Technology Guwahati, Guwahati, Assam, India, a lab-scale upflow anaerobic packed bed reactor (UFAR) and another lab-scale anaerobic packed bed reactor (APR) treating sulfate rich water containing arsenic. Among the three biomass types investigated in this study, the anaerobic biomass collected from UFAR showed very high removal of different metals at both low and high initial concentrations. The metal removal by this biomass followed the order: Cu > Fe > Ni > Pb > Cd > Zn. At a high initial concentration of 50 mg/L of the metals, biomass activity, including its growth, sulfate reduction, chemical oxygen demand (COD) reduction, were inhibited, thus resulting in a reduced removal of these metals by SRB from different sources. The heavy metal removal mechanism was attributed to the capability of the biomass to reduce sulfate to sulfide, thereby resulting in precipitation of the metals as their sulfide salts.
- Fourier transform infrared (FTIR) spectrometer characterization of the biomass grown in the absence and in presence of the metals further confirmed the involvement of sulfate, hydroxyl, amine and amide groups on the biomass for its heavy metal binding. Transmission electron microscope equipped with energy dispersive spectroscopy (TEM-EDS) and field emission scanning electron microscope equipped with energy dispersive X-ray spectroscopy (FESEM-EDX) analysis of the bacteria following metal removal further confirmed that the metal precipitates formed were associated with the cell surface. Overall, this study proved very good potential of the anaerobic biomass from UFAR for heavy metal removal from sulfate rich wastewater, and was therefore, chosen for further investigations.
- Simultaneous precipitation of different heavy metals from multi-component system by SRB from UFAR was evaluated. A very high removal of the different metals was achieved at low initial concentration combination of the metals and the results matched well with the results obtained in the case of single-metal solution. However,

at a high metal concentration combination, sulfate and COD reduction were inhibited, thus resulting in a slightly reduced removal of these metals by SRB which also matched with the results of single-metal study.

- FTIR spectroscopy analysis of the biomass confirmed the presence of functional groups in the SRB that were similar to that of an earlier reported SRB *Desulfovibrio*. species. TEM-EDS and FESEM-EDX analyses of the bacteria following metal removal further confirmed that the metal precipitates formed were associated with the cell surface and the heavy metal removal mechanism was attributed to the capability of the SRB to reduce sulfate to sulfide, thereby resulting in precipitation of the metals as sulfide salts. This mixture study proved very good potential of SRB in the anaerobic biomass for metal precipitation with a high tolerance towards different metals in a multi-component system.
- Actively immobilized SRB biomass was tested for its efficiency to remove heavy metals from aqueous solution under both batch and continuous conditions. Immobilized SRB biomass in the form of beads showed high metal removal even at high initial metal concentrations; metal removal results were better than that obtained using free/suspended biomass. Surface characterization of the immobilized SRB beads using FESEM-EDX confirmed metal sulfide formation and precipitation exterior to the bead surface.
- A very high metal removal efficiency was obtained at a low metal loading rate using the continuous downflow column reactor (DFCR) packed with the immobilized SRB beads and the metal removal followed an order that represented their solubility product values. Metal loading rates up to 4.29 mg/L·h for Cu(II) and up to 2.20 mg/L·h, for Fe(III), Zn(II), Ni(II), Pb(II) and Cd(II) were found to be favorable for their removal using the DFCR. Continuous metal removal from a mixture of the heavy metals showed that metal removal was maximum at their respective low inlet concentrations. However, the removal values were reduced at a high inlet concentration of the heavy metals, which matched well with low sulfate reduction and COD removal efficiencies. Metal precipitate analysis using FESEM-EDX revealed the potential of SRB in precipitating metals as metal sulfides *via* sulfate reduction. Continuous heavy metal removal from both single and multi-metal solution system using the DFCR demonstrated a very good potential of the immobilized SRB biomass for achieving a stable wastewater treatment performance. However, biomass washout from the reactor could not be overcome.

- Continuous heavy metal removal study was performed using anaerobic rotating biological contactor (An-RBC) reactor in which, the SRB exist mainly as passively attached biofilm onto its rotating discs. A very high metal removal efficiency was obtained at a low metal loading rate, which matched with the results obtained using the DFCR. Sulfate and COD reduction values confirmed that a high metal loading is inhibitory to SRB activity. These results are relatively better than the results obtained using the DFCR. Metal loading rates greater than 3.64 mg/L·h in case of Cu(II) and 1.87 mg/L·h, in case of Fe(III), Pb(II), Ni(II), Zn(II) and Cd(II) are detrimental to the performance of the An-RBC reactor. V3 -V4 metagenomics sequencing and analysis revealed that SRB immobilized in the An-RBC reactor is predominant with *Desulfovibrio vulgaris*. sp.
- Continuous metal removal from mixture of the heavy metals showed that Cu(II) removal was maximum (> 98%), followed by Zn (II) (96%) and other heavy metals at their respective low inlet concentrations and metal removal order followed in the mixture study was Cu > Zn > Cd > Pb > Fe > Ni, which matched well with the results obtained using the DFCR. Metal loading rates less than 3.7 mg/L·h in case of Cu(II); less than 1.69 mg/L·h for Ni(II), Pb(II), Zn(II), Fe(III) and Cd(II) were favorable for the performance of the An-RBC reactor toward metal removal from mixture which is better compared to the results obtained using the DFCR. Metal precipitate analysis using FESEM-EDX revealed the potential of SRB in precipitating metals as metal sulfides *via* sulfate reduction; similar findings were observed in the results observed using the DFCR. Overall, the passive biofilm bioreactor (An-RBC reactor) could be chosen for large-scale treatment of sulfate and metal rich wastewater owing to a better removal efficiency of metal, sulfate and COD.

Scope for Future Work

Following are some suggestions for future work based on this thesis:

- Recovery of metals from the precipitates formed in the reactors
- Long-term reactor performance prediction using real wastewater containing heavy metals and sulfate based on machine learning techniques (e.g. artificial neural networks)
- Cost-benefit analysis

- Geochemical modeling to predict the impact of treated water on the environment
- Microbiology and biochemistry of SRB treating complex metallic wastewater with cheap and abundantly available organic compounds
- Kinetic modeling of the reactor for metal removval
- Metal speciation and precipitate analysis to decipher the metal precipitation mechanism by SRB in sulfidogenic bioreactors treating metallic wastewater

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APPENDIX

Appendix

A. Statistical design of experiments

Experiments are always considered as major tool for practically validating a theoretical hypothesis about scientific knowhow of a process or phenomenon, where the observation is expected to be correlated with some known associated process parameter. In order to identify important factors and their contribution towards an observed response, normal approach of using one variable at a time involves a large number of experiments to be carried out which often cannot elucidate any significant interaction among the process variables (Ryan et al., 2007).

Statistical experimental designs are potent tools for improving the efficiency of experimentation and they facilitate understanding about the system being investigated with a minimum number of experiments. Inclusion of replicate test conditions allows the estimation of random, experimental variation. Statistical analysis of data generated from the experiment clearly establishes the relationship between the measured parameter of interest (response) and the process parameters (input factors or factors) being studied.

The factors may have individual, simple effects on the response (referred to as main effects) or may have effects that are interdependent (referred to as interaction effects). Since the designed experiments are generated on the basis of statistical theory, confidence in the results obtained and conclusions drawn are clearly defined (Montgomery, 1997; Altekar et al., 2006). Several statistical design types are available and their choice is determined by the aims of the experiment and information available about the experimental background. They can be categorized as (i) Plackett Burman design (ii) Taguchi design (iii) full and factorial design (iv) response surface analysis and (v) Box-Behnken design.

Application of statistical design of experiments in process development can improve yield of a process, reduce process variability, reduce time and overall costs. One of the striking features of the statistical design of experiments is a scientific approach of planning the experiments which aids in analyzing the experimental data trough suitable statistical methods that facilitates in drawing valid, meaningful and objective conclusions. Statistical methodology plays significant role in analyzing the data where there is a chance of experimental errors (Montgomery, 1991).

Full factorial design

Many experiments involve the study of the effects of two or more factors / parameters against a response(s). Owing to the inefficient understanding and requirement of extensive experimental work with respect to use of the classical method, factorial designs are widely used in such experiments involving several factors where it is necessary to study the interaction effects of the factors on the final response(s). Since the test variables are referred as 'factors', hence the term 'factorial' has been used. The 2-level full factorial design which is most popular, includes all possible factor combinations at two levels, low and high denoted by "–" and "+", respectively, for each of the factors.

A suitable number of runs carried out to replicate the levels of variables at their center point (0) provide an estimate of the residual error associated with the experiments and also the curvature of the response. It is a powerful tool for understanding complex processes whose detailed mechanisms are unknown and for describing factor interactions in multiple factor systems. For a process involving k factors at only 2 levels, a complete replicate of such a design requires 2 × 2 ×…. × 2 = 2k observations, which is called a 2^k full factorial design.

The effect of a factor is described as the change in response produced by a change in the level of the factor. This is frequently called a main effect because it refers to the primary factors of interest in the experiment (Montgomery, 1991). In some experiments, it can be found that the difference in response between the levels of one factor is not the same at all levels of the other factors and this occurs when there is an interaction between the factors. In such a case, when an interaction is large, the corresponding main effects entail little practical meaning. Moreover, in the presence of significant interaction, it is necessary to usually examine the levels of one factor; say A, with levels of the other factor fixed to draw conclusion about the main effect of the factor A.

Thus, when compared to one-factor-at-a-time experiments, full factorial designs have several advantages. Furthermore, when interactions are likely to be present, a factorial design is necessary to avoid misleading conclusions. The 2 k design, in specific is particularly used in the screening experiments, when there are likely to be many factors to be investigated whose interactions may be significant. It provides the smallest number of runs with 'k' factors that can be studied in a complete design (Montgomery, 1991). Finally, full factorial design allows the effects of a factor to be estimated at several levels of the other factors, yielding conclusions that are valid over a range of experimental conditions. On the other hand Plackett-Burman design is a very efficient screening design with minimal number of experimental runs, and is useful for detecting large main effects, assuming all interactions are negligible.

Fractional factorial experiments

Factorial design aids in obtaining the data at every combination of the levels. Usually, factorial and fractional factorial designs (FFD) are considered as cornerstone of process development and product formation and their improvement on industrial scale (Montgomery, 1991). A FFD is useful when there are large number of factors and use of full factorial design leads to large number of experiments. Generally, in a fractional factorial design, total number of runs is $N = 2^{k-p}$, which is a fraction of the total number of experiments. Therefore, FFD is useful when not all the factors in a design are important and this will result in a small set of experiments starting with screening of large number of factors. Properly chosen FFDs for 2-level experiments possess desirable properties of being both balanced and orthogonal.

Plackett-Burman design

Different statistical methods, for instance, Plackett Burman design has been developed to avoid the application of full factorial design of experiments in industrial experimentation when the number of factors is so large that these methods are impractical. By assuming a simplified linear hypothesis, the problem of determining main effects with maximum precision is reduced which involves different combination of factors. In practical, all solutions of this have been found useful when each factor appears at two levels, whereas the solutions for more than two levels are reasonably limited. This type of design uses two levels for each factor, the extreme denoted by "+" and the nominal by "-" (Plackett and Burman, 1946). Plackett Burman designs have been used in numerous varieties of chemical and biochemical studies, analytical as well as synthetic; in practice, designs with 12 and 20 runs seem to have been well known. Plackett Burman designs are best applicable for screening purposes in systems where it is necessary to identify a few main factors affecting the response, and where interactions are not significant, but it is worth noting that if some of the factors seem to show high effect values, it signifies that interactions are indeed present (Analytical Methods Committee, AMCTB No 55).

By assuming a simplified linear hypothesis, the problem of determining main effects with maximum precision is reduced which involves different combination of factors. In practical, all solutions of this have been found useful when each factor appears at two levels, whereas the solutions for more than two levels are reasonably limited. This type of design uses two levels for each factor, the extreme denoted by "+" and the nominal by "-" (Plackett and Burman, 1946). The Plackett–Burman design is based on a first-order polynomial equation (Tasharrofi et al., 2011) of the form as expressed in equation 5:

$$\mathrm{Y} = \beta_0 + \sum \beta_i X_i \qquad (1)$$

Where, Y is the response (% Metal removal), β_0 is the model coefficient, β_i is the linear coefficient, and X_i is the level of the independent variable.

Analysis of Variance

Analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures in which the observed variance is partitioned into components due to different explanatory variables. In general terms, ANOVA explains any variation in the statistically derived model and significance of the model parameters. The model parameters, usually indicated in ANOVA, are the main effects, interaction effects and error terms, and their significance in the model is represented by Fischer ‘F’ and associated P values. The other terms of ANOVA table are degrees of freedom (df), sum of squares (SS) and mean squares (MS). The MS value of a model term in an ANOVA table is obtained by dividing SS over df and its F value is obtained by dividing MS due to the model term by MS due to error. Normally, larger F and lower P values of a model term in ANOVA indicate good significance of the term over others.

Student ‘*t*’ test

In general, a *t*-test is any statistical hypothesis test in which the test statistic has a Student's *t* distribution if the null hypothesis is true. It is generally applied when sample size is small enough and using an assumption of normality and the associated z-test leads to incorrect inference. The Student *t*-distribution in probability and statistics is a probability distribution that arises in the problem of estimating the mean of a normally distributed population when the sample size is small. It is the basis of the popular Student *t*-tests for the statistical significance of difference between two sample means and for confidence intervals for difference between two population means.

The Student *t*-distribution is a special case of the generalized hyperbolic distribution. A test of the null hypothesis is that the means of two normally distributed populations are equal. Given two data sets, each characterized by its mean, standard deviation and number of data points, one can use some kind of '*t*' test to determine whether the means are different, provided that the underlying distributions can be assumed to be normal. All such tests are usually called Student *t* tests, though strictly speaking that name should only be used if the variances of the two populations are also assumed to be equal.

Statistical experimental designs (factorial design and response surface analysis) have been applied for (i) bio-precipitation of toxic metals using mixed sulfate reducing bacterial culture (White and Gadd, 1996a) (ii) to study the effect of process variables on sulfate reduction process and (iii) to study the effect of pH and different heavy metals on the growth of SRB (Kikot et al., 2010). Other than these reports, available literature on the use of these statistical experimental designs for continuous metal removal is very limited. From literature, it is clear that factorial design of experiments have been utilized and reported that the statistical approach yielded a better and meaningful interpretation of the results.

B. Chemical precipitation of heavy metal by sodium sulfide

Industrially on large-scale, heavy metal removal from wastewater is carried out through hydroxide precipitation, however, advantages associated with the use of sulfide precipitation for heavy metal removal over traditional methods has attained a wide interest. On the other hand, use of chemical sulfide precipitation for metal removal is limited due to the difficulty in controlling the dosage of sulfide (due to the very low solubility of the metal sulfide and thus the sensitivity of the process to the dose) and problems associated with the toxic and corrosive nature of excess sulfide (Veeken et al., 2003)

The results in the present study reveal that metal removal was almost very low with different concentration levels of sulfide (5, 10, 15 and 25 mg/L) added. A large difference was found between the theoretical and the experimental data, and this was attributed to the formation of colloidal particles of metal sulfides (MeS) (Ennaassia et al., 2002). With the formation of colloidal particles, the separation of the solid from the aqueous phase would not be complete and thus, the analysis of the aqueous phase would include the contribution of the colloidal MeS particles, thereby underestimating the metal removal compared to the equilibrium conditions (Ennaassia et al., 2002). It was also reported that metal removal was lower when a higher sulfide dose was used since the higher sulfide concentration resulted in much smaller particles. This is in agreement with the present study results for metal removal as well using chemically added sulfide.

C. Identification of SRB culture

Significance of Use of V3-V4 region of 16S rRNA gene

The 16S small ribosomal subunit gene (16S rRNA), in particular, has been widely used to study and characterize bacterial community compositions in a variety of ecological niches including host associated communities, such as the endogenous human microbiome, and host-free communities, such as soil and ocean environments. Several aspects of the 16S rRNA gene make it optimal as a marker for these types of studies. First, it is ubiquitous among prokaryotic life. Second, its size and high degree of functional conservation result in clock-like mutation rates throughout prokaryotic evolution. Third, and most importantly, the 16S rRNA gene includes both conserved regions, which can be used for designing amplification primers across taxa, as well as nine hyper-variable regions (V1-V9), which can be effectively used to distinguish between taxa.

With the advent of massively parallel sequencing technologies, which generally yield short reads, focus has shifted from sequencing the full 16S rRNA gene to sequencing shorter sub-regions of the gene at great depth. By calculating the geodesic distance between different regions, the phylogenetic relationships based on the V4 sequences were closest to those based on the full-length sequences. This analysis suggests that V4 ranks first in sensitivity as a marker for bacterial and phylogenetic analysis, which is same as taxonomic results obtained using the RDP (Ribosomal Database Project) classifier. In addition, V1-V3 was also highly recommended. That is why a combination of V3-V4 region of 16S rRNA gene was selected for analysis.

Figure C shows the relative percentage presence of *Desulfovibrio*. sp. excluding unknown or uncultured bacteria. V3 -V4 region of 16S rRNA gene analysis revealed that SRB immobilized in the An-RBC reactor is predominant with *Desulfovibrio*. sp.

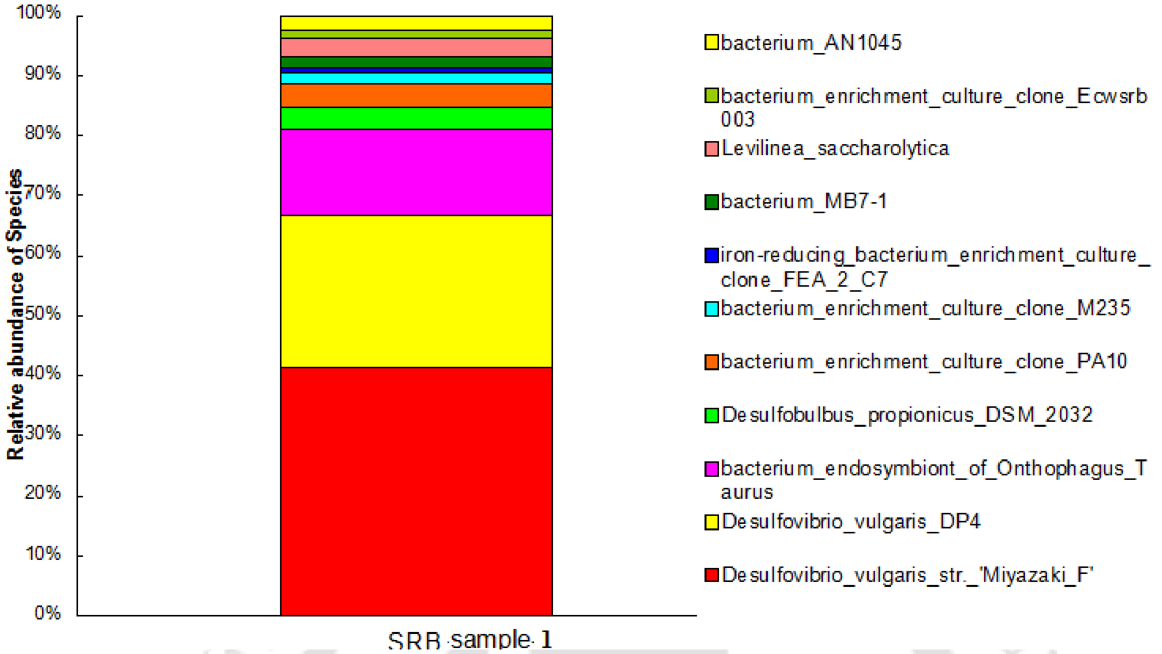

Figure. C The relative percentage presence of *Desulfovibrio*. sp. excluding unknown or uncultured bacteria.

D.1 Analysis of variance of heavy metal removal from a multi-component system by SRB

Table 4.5 Analysis of variance of heavy metal removal from a multi-component system by SRB

Source	**DF**[1]	**Seq SS**[2]	**Adj MS**[3]	**F**[4]	**P**[5]
Cd(II)[a]					
Main effects	6	188.434	31.406	1.02	0.5
Cd	1	161.26	161.26	5.26	0.07
Cu	1	7.038	7.038	0.23	0.652
Ni	1	0.042	0.042	0	0.972
Fe	1	8.687	8.687	0.28	0.617
Pb	1	1.849	1.849	0.06	0.816
Zn	1	9.559	9.559	0.31	0.601
Residual Error	5	153.387	30.677		
Total	11	341.821			
Cu(II)[b]					
Main	6	47.3236	7.8873	2.5	0.167
Cd	1	1.7787	1.7787	0.56	0.487
Cu	1	22.6875	22.6875	7.18	0.044
Ni	1	0.1045	0.1045	0.03	0.863
Fe	1	6.3365	6.3365	2.01	0.216
Pb	1	4.296	4.296	1.36	0.296
Zn	1	12.1203	12.1203	3.84	0.107
Residual Error	5	15.7949	3.159		
Total	11	63.1185			
Ni(II)[c]					
Main	6	460.785	76.797	3.19	0.112
Cd	1	11.408	11.408	0.47	0.522
Cu	1	99.187	99.187	4.12	0.098
Ni	1	303.007	303.007	12.59	0.016
Fe	1	13.868	13.868	0.58	0.482
Pb	1	7.207	7.207	0.3	0.608
Zn	1	26.107	26.107	1.08	0.345
Residual Error	5	120.337	24.067		
Total	11	581.122			
Fe(III)[d]					
Main	6	481.629	80.271	6.27	0.031
Cd	1	4.248	4.248	0.33	0.589
Cu	1	77.521	77.521	6.06	0.057
Ni	1	104.312	104.312	8.15	0.036
Fe	1	239.771	239.771	18.74	0.008
Pb	1	54.955	54.955	4.29	0.093
Zn	1	0.822	0.822	0.06	0.81
Residual Error	5	63.976	12.795		
Total	11	545.605			

Pb(II)[e]					
Main	6	121.968	20.328	13.37	0.006
Cd	1	5.936	5.9361	3.9	0.105
Cu	1	14.301	14.3008	9.4	0.028
Ni	1	31.818	31.8176	20.92	0.006
Fe	1	3.63	3.63	2.39	0.183
Pb	1	66.27	66.27	43.57	0.001
Zn	1	0.013	0.0133	0.01	0.929
Residual Error	5	7.604	1.5209		
Total	11	129.572			
Zn(II)[f]					
Main	6	206.329	34.388	4.12	0.071
Cd	1	0.124	0.124	0.01	0.908
Cu	1	2.651	2.651	0.32	0.598
Ni	1	20.489	20.489	2.45	0.178
Fe	1	28.213	28.213	3.38	0.126
Pb	1	6.871	6.871	0.82	0.406
Zn	1	147.982	147.982	17.71	0.008
Residual Error	5	41.77	8.354		
Total	11	248.099			

[a] (S= 5.5 PRESS= 885.0 R^2= 55.12%)
[b](S= 1.7 PRESS= 90.80 R^2= 75.13%)
[c](S= 4.9 PRESS= 693.14 R^2= 79.2%)
[d] (S= 3.3 PRESS= 331.5 R^2= 89.09%)
[e] (S= 1.2 PRESS= 44.19 R^2= 94.1%)
[f] (S= 2.8 PRESS= 240.59 R^2= 83.1%)

[1] Degree of freedom; [2] Sum of squares; [3] Mean sum of squares; [4] Fisher's value; [5] Probability

[a] Cadmium; [b] Copper; [c] Nickel; [d] Iron; [e] Lead; [f] Zinc;

S: standard error; PRESS: predicted residual error sum of squares; R^2: coefficient of determination

D.2 Analysis of variance of sulfate and COD removal in the presence different heavy metals in the study

Table 4.6 Analysis of variance of sulfate and COD removal in the presence different heavy metals in the study

Source	DF[1]	Seq SS[2]	Adj MS[3]	F[4]	P[5]
% Sulfate Removal[a]					
Main	6	340.702	56.784	3.47	0.097
Cd	1	14.747	14.747	0.9	0.386
Cu	1	268.03	268.03	16.36	0.01
Ni	1	14.077	14.077	0.86	0.397
Fe	1	1.266	1.266	0.08	0.792
Pb	1	14.286	14.286	0.87	0.393
Zn	1	28.296	28.296	1.73	0.246
Residual	Error	5	81.937	16.387	
Total	11	422.639			
% COD Removal[b]					
Main	6	836.46	139.41	2.68	0.15
Cd	1	10.08	10.08	0.19	0.678
Cu	1	385.33	385.33	7.4	0.042
Ni	1	85.33	85.33	1.64	0.257
Fe	1	235.85	235.85	4.53	0.087
Pb	1	44.85	44.85	0.86	0.396
Zn	1	75	75	1.44	0.284
Residual	Error	5	260.5	52.1	
Total	11	1096.96			

[a] (S= 4.04 PRESS= 471.95 R^2= 80.61%)

[b] (S= 7.21 PRESS= 1500.48 R^2= 76.25%)

[1] Degree of freedom; [2] Sum of squares; [3] Mean sum of squares; [4] Fisher's value; [5] Probability

S: standard error; PRESS: predicted residual error sum of squares; R^2: coefficient of determination

E.1 ANOVA of heavy metal removal using the DFCR

Table 4.7 ANOVA of heavy metal removal using the DFCR

Variable	**% Cd**		**% Fe**		**% Cu**		**% Ni**		**% Pb**		**% Zn**	
Source	F	P	F	P	F	P	F	P	F	P	F	P
Effects	4.2	0.2	9.4	0.1	0.4	0.9	0.5	0.8	3.8	0.2	1.1	0.5
Cd	**14.3**	**0.063**	0.3	0.6	0.0	0.9	0.3	0.7	**7.8**	**0.1**	**4.4**	**0.2**
Cu	6	0.1	2.3	0.3	0.8	0.5	0.1	0.8	**4.2**	**0.2**	0.6	0.5
Ni	0.7	0.5	**9.1**	**0.09**	0.1	0.8	1.6	0.3	**2.8**	**0.2**	0.1	0.7
Fe	2	0.3	0.1	0.8	0.5	0.6	0.8	0.5	**3.8**	**0.2**	0.0	0.9
Pb	2	0.3	7	0.1	0.8	0.5	0.1	0.8	**2.7**	**0.2**	1.2	0.4
Zn	0	0.9	**37.7**	**0.02**	0.0	0.9	0.2	0.7	1.7	0.3	0.4	0.6
2-Way	4	0.2	2.5	0.3	0.2	1.0	0.4	0.8	1.5	0.5	0.5	0.8
Cd*Cu	8.3	0.1	3	0.2	0.2	0.7	0.1	0.8	0.1	0.8	0.3	0.6
Cd*Ni	0.2	0.7	**10.5**	**0.08**	0.0	1.0	0.4	0.6	0.9	0.4	0.1	0.9
Cd*Fe	0.6	0.5	2.7	0.2	0.2	0.7	0.8	0.5	0.3	0.7	0.1	0.8
Cd*Pb	**16**	**0.057**	0	1	0.2	0.7	0.6	0.5	0.1	0.8	0.0	1.0
Cd*Zn	1.3	0.4	0	1	0.0	0.9	0.8	0.5	**3.2**	**0.2**	2.1	0.3
Cu*Fe	1.4	0.4	0.2	0.7	0.1	0.8	0.1	0.8	0.8	0.5	0.9	0.5
Cu*Zn	0.1	0.8	0.8	0.5	0.3	0.6	0.4	0.6	**5.0**	**0.2**	0.0	0.9
3-Way	4.1	0.2	4.4	0.2	0.3	0.7	1.0	0.5	1.6	0.4	1.6	0.4
Cd*Cu*Fe	5.9	0.1	2	0.3	0.2	0.7	1.9	0.3	0.7	0.5	**3.2**	**0.2**
Cd*Cu*Zn	2.2	0.3	6.9	0.1	0.5	0.5	0.0	0.9	2.6	0.3	0.0	1.0
Curvature	1.2	0.4	0	1	0.1	0.8	0.4	0.6	0.0	0.9	1.1	0.4

^{a}(S= 2.6 R^2= 97%)
^{b}(S= 2.1 R^2= 98%)
^{c}(S= 2.5 R^2= 66%)
^{d}(S= 13 R^2= 81%)
^{e}(S= 5 R^2= 94%)
^{f}(S= 5.6 R^2= 88%); F: Fisher's value; P: Probability; ^{a}Cadmium; ^{b}Iron; ^{c}Copper; ^{d}Nickel; ^{e}Lead; ^{f}Zinc; S: standard error; R^2: coefficient of determination

E.2 Student's *t* test for estimating the coefficients of individual and interaction effects of different heavy metals and their significance on metal removal using the DFCR

Table 4.8 Student's *t* test for estimating the coefficients of individual and interaction effects of different heavy metals and their significance on metal removal using the DFCR

Variable	**% Cd[a]**			**% Fe[b]**			**% Cu[c]**			**% Ni[d]**			**% Pb[e]**			**% Zn[f]**		
Term	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P
Constant		130	0		145	0		154.8	0.0		16.3	0.0		65.8	0.0		62.0	0.0
Cd	**5.1**	**3.8**	**0.06**	0.6	0.6	0.6	-0.3	-0.2	0.9	-3.5	-0.5	0.7	**-7.1**	**-2.8**	**0.1**	**-6.0**	**-2.1**	**0.2**
Cu	-3.3	-2.5	0.1	-1.7	-1.5	0.3	1.1	0.9	0.5	-1.8	-0.3	0.8	**-5.2**	**-2.0**	**0.2**	-2.2	-0.8	0.5
Ni	1.1	0.8	0.5	**-3.3**	**-3**	**0.09**	-0.4	-0.3	0.8	-8.4	-1.3	0.3	**-4.3**	**-1.7**	**0.2**	-1.1	-0.4	0.7
Fe	-1.9	-1.4	0.3	0.3	0.3	0.8	-0.9	-0.7	0.6	5.9	0.9	0.5	**-5.0**	**-2.0**	**0.2**	-0.4	-0.2	0.9
Pb	-1.9	-1.4	0.3	-2.9	-2	0.1	-1.1	-0.9	0.5	-2.1	-0.3	0.8	**-4.2**	**-1.6**	**0.2**	-3.1	-1.1	0.4
Zn	-0.3	-0.2	0.9	**-6.7**	**-6**	**0.02**	0.2	0.2	0.9	-2.8	-0.4	0.7	-3.3	-1.3	0.3	1.8	0.6	0.6
Cd*Cu	3.8	2.9	0.1	-1.9	-1	0.2	0.6	0.5	0.7	-1.6	-0.3	0.8	-0.8	-0.3	0.8	-1.6	-0.6	0.6
Cd*Ni	0.6	0.4	0.7	**3.5**	**3.2**	**0.08**	0.0	0.0	1.0	4.1	0.6	0.6	-2.4	-1.0	0.4	-0.6	-0.2	0.9
Cd*Fe	-1	-0.8	0.5	-1.8	-1	0.2	-0.6	-0.5	0.7	-5.8	-0.9	0.5	-1.3	-0.5	0.7	0.9	0.3	0.8
Cd*Pb	**5.4**	**4**	**0.05**	-0.1	-0.1	1	0.5	0.4	0.7	5.0	0.8	0.5	-0.6	-0.2	0.8	-0.1	-0.1	1.0
Cd*Zn	1.5	1.1	0.4	0	0	1	-0.1	-0.1	0.9	6.0	0.9	0.5	**-4.5**	**-1.8**	**0.2**	4.1	1.5	0.3
Cu*Fe	1.6	1.2	0.4	-0.5	-0.5	0.7	0.4	0.3	0.8	-1.5	-0.2	0.8	2.3	0.9	0.5	-2.6	-0.9	0.5
Cu*Zn	0.4	0.3	0.8	-1	-0.9	0.5	-0.7	-0.6	0.6	4.2	0.6	0.6	**-5.7**	**-2.2**	**0.2**	-0.3	-0.1	0.9
Cd*Cu*Fe	-3.3	-2	0.1	1.5	1.4	0.3	-0.5	-0.4	0.7	-9.0	-1.4	0.3	2.0	0.8	0.5	**-5.1**	**-1.8**	**0.2**
Cd*Cu*Zn	2	1.5	0.3	-2.9	-2.6	0.1	-0.9	-0.7	0.5	-1.3	-0.2	0.9	-4.1	-1.6	0.3	0.0	0.0	1.0
Ct		-1.1	0.4		0	1		-0.3	0.8		0.6	0.6		-0.2	0.9		-1.1	0.4

[a]Cd(II) removal; [b]Fe(III) removal; [c]Cu(II) removal; [d]Ni(II) removal; [e]Pb(II) removal; [f]Zn(II) removal; Effect; T: T value; P: probability

F.1 ANOVA of heavy metal removal using the An-RBC reactor

Table 4.9 ANOVA of heavy metal removal using the An-RBC reactor

Variable	**% Ni** [a]		**% Pb** [b]		**% Zn** [c]		**% Cu** [d]		**% Fe** [e]		**% Cd** [f]	
Source	F	P	F	P	F	P	F	P	F	P	F	P
Effects	4.4	0.2	10.6	0.08	8.5	0.1	1.2	0.5	3.3	0.2	0.4	0.9
Cd	0	1	0.9	0.4	**23.5**	**0.04**	2.0	0.3	**7.4**	**0.1**	0.0	0.9
Cu	5	0.2	**13**	**0.06**	**8.9**	**0.096**	1.1	0.4	**8.1**	**0.1**	0.9	0.5
Ni	**10.8**	**0.082**	**29**	**0.03**	3.2	0.2	0.1	0.8	0.1	0.8	0.0	0.9
Fe	3.5	0.2	7.7	0.1	8.3	0.1	0.2	0.7	1.8	0.3	0.3	0.7
Pb	5.2	0.1	0.4	0.5	0	0.9	**3.4**	**0.2**	2.3	0.3	0.6	0.5
Zn	1.7	0.3	**12**	**0.07**	7.4	0.1	0.5	0.5	0.3	0.6	0.4	0.6
2-Way	0.9	0.6	1.4	0.4	4.6	0.2	1.2	0.5	3.0	0.3	0.2	0.9
Cd*Cu	1.1	0.4	0.1	0.7	0.5	0.5	2.4	0.3	0.8	0.5	0.5	0.6
Cd*Ni	1.8	0.3	0.01	0.9	7.2	0.1	0.7	0.5	1.7	0.3	0.2	0.7
Cd*Fe	3.2	0.2	0.9	0.4	0	0.9	1.7	0.3	**5.2**	**0.1**	0.1	0.8
Cd*Pb	0.1	0.8	3	0.2	**15.2**	**0.06**	0.2	0.7	**8.0**	**0.1**	0.1	0.8
Cd*Zn	0	1	3.1	0.2	**8.7**	**0.098**	0.2	0.7	**5.1**	**0.2**	0.2	0.7
Cu*Fe	0.2	0.7	2.3	0.2	0	0.9	**3.2**	**0.2**	0.0	0.9	0.2	0.7
Cu*Zn	0.1	0.8	0.1	0.7	0.4	0.6	0.1	0.7	0.0	1.0	0.2	0.7
3-Way	2.1	0.3	4	0.1	4.1	0.2	0.5	0.7	0.5	0.7	0.4	0.7
Cd*Cu*Fe	3.6	0.2	8.1	0.1	5.3	0.1	0.9	0.4	0.4	0.6	0.2	0.7
Cd*Cu*Zn	0.5	0.5	0.01	0.9	2.9	0.2	0.1	0.7	0.6	0.5	0.5	0.5
Curvature	2.9	0.2	3.2	0.2	12.1	0.1	0.3	0.7	1.0	0.4	0.0	0.9

[a](S= 4.6 R^{2}= 95%)
[b](S= 2.2 R^{2}= 98%)
[c](S= 2.4 R^{2}= 98%)
[d](S= 2.1 R2= 89%)
[e](S= 6 R2= 95%); F: Fisher's value; P: Probability
[f](S= 6.8 R2= 69%); [a]Nickel; [b]Lead; [c]Zinc; [d]Copper; [e] Iron; [f]Cadmium; S: standard error; R^{2}: coefficient of determination

F.2 Student's *t* test for estimating the coefficients of individual and interaction effects of different heavy metals and their significance on metal removal using the An-RBC reactor

Table 4.10 Student's *t* test for estimating the coefficients of individual and interaction effects of different heavy metals and their significance on metal removal using the An-RBC reactor

Variable	**% Ni** [a]			**% Pb**[b]			**% Zn**[c]			**%Cu**[d]			**% Fe**[e]			**% Cd**[f]		
Term	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P	Effect	T	P
Constant		47	0		151	0		141	0		181.9	0		55.3	0		51.4	0
Cd	0	0	1	-1.1	-1	0.4	**-6**	**-4.9**	**0.04**	-1.5	-1.4	0.3	**8.3**	**2.7**	**0.1**	0.4	0.1	0.9
Cu	-5.2	-2.2	0.2	**-4.1**	**-3.6**	**0.07**	**-3.7**	**-3**	**0.1**	-1.1	-1.0	0.4	**-8.6**	**-2.8**	**0.1**	-3.1	-0.9	0.5
Ni	**7.7**	**3.3**	**0.08**	**-6.1**	**-5.4**	**0.03**	2.2	1.8	0.2	-0.2	-0.2	0.8	-0.8	-0.3	0.8	0.3	0.1	0.9
Fe	-4.4	-1.9	0.2	-3.1	-2.8	0.1	-3.6	-2.9	0.1	-0.5	-0.5	0.7	-4.0	-1.3	0.3	-1.7	-0.5	0.7
Pb	-5.4	-2.3	0.1	-0.8	-0.7	0.6	-0.1	-0.1	0.9	**-2.0**	**-1.9**	**0.2**	-4.6	-1.5	0.3	-2.6	-0.8	0.5
Zn	-3.1	-1.3	0.3	**-4**	**-3.6**	**0.07**	3.4	2.7	0.1	-0.8	-0.7	0.5	-1.7	-0.6	0.6	-2.2	-0.7	0.6
Cd*Cu	2.5	1.1	0.4	-0.4	-0.4	0.8	-0.9	-0.7	0.5	1.6	1.5	0.3	2.7	0.9	0.5	2.4	0.7	0.6
Cd*Ni	3.2	1.4	0.3	0.1	0.1	0.9	3.3	2.7	0.1	-0.9	-0.8	0.5	4.0	1.3	0.3	-1.5	-0.4	0.7
Cd*Fe	-4.2	-1.8	0.2	1.1	1	0.4	0.1	0.1	0.9	-1.4	-1.3	0.3	**7.0**	**2.3**	**0.1**	1.2	0.3	0.8
Cd*Pb	0.6	0.3	0.8	-2	-1.8	0.2	**4.8**	**3.9**	**0.06**	-0.4	-0.4	0.7	**8.6**	**2.8**	**0.1**	-1.1	-0.3	0.8
Cd*Zn	0	0	1	-2	-1.8	0.2	**3.7**	**3**	**0.09**	-0.4	-0.4	0.7	**-6.9**	**-2.3**	**0.2**	1.6	0.5	0.7
Cu*Fe	1.1	0.5	0.7	-1.7	-1.5	0.3	-0.2	-0.2	0.9	1.9	1.8	0.2	-0.3	-0.1	0.9	1.4	0.4	0.7
Cu*Zn	0.6	0.3	0.8	-0.4	-0.4	0.8	-0.8	-0.6	0.6	-0.4	-0.4	0.7	-0.1	0.0	1.0	-1.6	-0.5	0.7
Cd*Cu*Fe	-4.5	-1.9	0.2	-3.2	-2.9	0.1	2.9	2.3	0.1	1.0	1.0	0.4	2.0	0.7	0.6	1.5	0.5	0.7
Cd*Cu*Zn	-1.7	-0.7	0.5	-0.1	-0.1	0.9	-2.1	-1.7	0.2	0.4	0.4	0.7	2.3	0.8	0.5	2.5	0.7	0.5
Ct		1.7	0.2		1.8	0.2		3.5	0.1		0.5	0.7		1.0	0.4		-0.1	0.9

[a]Ni(II) removal; [b]Pb(II) removal; [c]Zn(II) removal; [d]Cu(II) removal; [e]Fe(III) removal; [f]Cd(II) removal; Effect; T: T value; P: probability

G. Simple cost estimation of the An-RBC reactor set up

Table. Simple cost estimation of the An-RBC reactor set up

Single time investment (instruments cost) (a)	**Cost**
1 hp DC Electric Motor 1500 rpm capacity with regulator	~$229.9
Peristaltic pump	~$1072.88
N_2 Cylinder	~$229.9
Workman cost	~$153.27
Raw material cost (b)	
Nylon rod	~$11.5
Perspex sheet 10 mm size (8×6)	~$68.97
PMMA Cylinder and other materials	~$114.95
Silicon tubings	~$76.63
Operation and maintenance costs (c)	
Power consumption: 7.5/- per Unit for connected load more than 5 KW	~$7.66
During the operation of the reactor	per month
Consumables (Media preparation; water; silicon tubings; grease etc.)	~76.63
Total (a+b+c)	**$2042.29**

LIST OF PUBLICATIONS

List of Publications Based on This Research Work

Referred International Journals

- Gopi Kiran, M., Pakshirajan, K., Gopal Das., 2016. Heavy metal removal using sulfate reducing biomass obtained from a lab-scale upflow anaerobic packed bed reactor, J Environ Eng. 142 (9), C4015010. (DOI: 10.1061/(ASCE)EE.1943-7870.0001005.).
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