Investigation of algal-microbial biofilms for acid mine drainage treatment

Sanaz Orandi

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PANEL OF SUPERVISORS

Principal Supervisor

A/Prof. David M. Lewis

Ph.D. (University of Adelaide)
School of Chemical Engineering
The University of Adelaide
Email: david.lewis@adelaide.edu.au
Phone: +61 8 83135503
Fax: +61 8 83134373

Cooperative Supervisors

Dr. Navid R. Moheimani

Ph.D. (Murdoch University)
Algae R&D Center
School of Biological Sciences & Biotechnology
Murdoch University
Email: n.moheimani@murdoch.edu.au
Phone: +61 8 93602682
Fax: +61 8 9360 6651

A/Prof. Peter J. Ashman

Ph.D. (University of Sydney)
School of Chemical Engineering
The University of Adelaide
Email: peter.ashman@adelaide.edu.au
Phone: +61 8 83135072
Fax: +61 8 83134373
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The thesis is composed of the following papers.


The following outcomes were resulted from this thesis included:

1- Orandi, S., Lewis, D. M., Eslami, A, Mohebbi, A (2012) A novel approach to exploit indigenous mining algal-microbes in a rotating biological contactor for the removal of heavy metals from acid mine drainage, Annual Conference International Mine Water Association (IMWA) 2012, Bunbury, Western Australia. (Oral presentation, Received best student presentation award, see full paper in Appendix A)


4- Orandi, S., Lewis, D. M., Moheimani, N. (2011) A novel approach to develop and maintain an algal biofilm derived from an indigenous mining microbial consortium in a
rotating biological contactor, 4th Congress of the International Society for Applied Phycology, Halifax, Canada. (Poster)

Additionally, this work was presented in the 3 min thesis (3MT) completion held at the University of Adelaide and won the runner up prize.
If I want to summarise my thesis in one sentence,

I would say:

The God-given environment not only serves us with treasures to sell, but also offer balms to put on remaining scars

Sanaz Orandi
March 2013
توانا و هرک دانابود

کدوال را دانش نشاده دام
فرح زده ی بهار نابی در
پنداش کرمس دیو شاد
برخ وگر چند سختی آیدبه
زنادان بنال دل سنگ کوه
توانا و هرک دانابود

جمال ایوان اسم فردوسی
Acknowledgement

A few years ago, when I was asked about my most ambitious wish, I would respond “getting my PhD, building upon my Master’s findings”. However, to achieve this goal I had to go through the toughest time in my life.

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Sanaz Orandi

March 2013
ABSTRACT

Mining wastewaters, typically acid mine drainage (AMD) have been listed as one of the most severe types of contaminated surface waters containing heavy metals (e.g. Cu, Zn, Cr, Pb) and toxic metalloids (e.g. As, Cd, Sb). AMD convey these elements into water bodies and threaten aquatic life and human health, consequently. AMD is required to be treated before discharge to the environment, particularly in arid areas with scarce water resources. To date, neutralisation and evaporation have been commonly used at mine sites to decrease the elemental contents of contaminated surface waters. However, these techniques are expensive or ineffective for removing recalcitrant elements e.g. Mn; and produce large volumes of contaminated sludge. In recent decades, the exploitation of microorganisms for treating wastewaters, particularly municipal wastewaters, has significantly improved water treatment technologies, and are referred to as biotreatment/bioremediation. High efficiency, cost effectiveness and sustainability are associated with biotreatment. The application of biotreatment has been investigated for AMD treatment and documented extensively. However, the results are limited and not comprehensive enough for an applicable system to be deployed in mine sites. The main objective of my PhD research was to establish and develop an effective and sustainable AMD biotreatment system for removing metals/metalloids, applicable for mine sites. The indigenous mine microorganisms were used as biosorbents in a biotreatment system, obtained from AMD resources at Sarcheshmeh copper mine, Iran. The microbial sample contained mainly filamentous and unicellular green micro-algae, *Klebsomidiun* sp. and *Chlamydomonsae* sp.; bacteria, *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Pseudomonas* sp.; and fungi, *Aspergillus* sp. and *Penicillium* sp. The AMD, from which the indigenous microbial consortium was collected, was analysed to quantify its cation and anion (including nutrients PO$_4^{3-}$ and NO$_3^-$) contents. The analysis data was used to synthesise a multi-ion AMD composed of 25 components (cations and anions at concentrations 0.005-100 mg/L), high sulphate (>1000 mg/L) and low pH (~3). The indigenous microbial assembly was maintained in synthetic AMD (Syn-AMD) *in vitro*. For the biotreatment investigations, a laboratory-scale photo-rotating biological contactor (PRBC) was designed and used to immobilise the microbial consortium as an algal-microbial biofilm. The PRBC was initially operated in batch mode, using Syn-AMD and indigenous microbes as PRBC solution and inoculum, respectively.
An algal-microbial biofilm (60g dry weight) was successfully grown on the discs’ surfaces in the PRBC after 12 weeks. The PRBC was then operated at both batch and continuous modes to investigate the efficiency of the system for removing different elements from the Syn-AMD. Batch systems were conducted in 7-day periods under pH 3 and 5. The batch results showed that the algal-microbial biofilm system was able to reduce the concentration of major elements from 10 to 60% at pH 3 in the order of Na > Cu > Ca > Mg > Mn > Ni > Zn, whereas higher results (40-70%) were recorded for these elements at pH 5 in the order of Cu > Mn > Mg > Ca > Ni > Zn > Na. The removal trend for each element contained maximum and minimum removal values that occurred during the experiment. The removal efficiency of the system for trace elements varied extensively between 3 and 80% under both pH conditions.

The efficiency of the system was also evaluated in continuous condition, by introducing Syn-AMD (pH~3) into the PRBC at the flow rate of 10 ml/min and hydraulic retention time of 24 h. The operation of PRBC within a 28-day period showed similar removal efficiency (10-60%) compared with the batch operation, for most of elements. The chemical composition of treated water was examined daily within 28 days and the results revealed absorption (7 days) and desorption periods occurring alternatively. The increase and decrease of pH by 0.5 and 0.2 were recorded at the same time of absorption and desorption periods, which was attributed to mobilisation and immobilisation mechanisms occurring in the algal-microbial biofilm.

The system was operated for a further 10 weeks continuously and the results demonstrated the average weekly removal for major elements from 20 to 50% in the order of Cu> Mg> Ni> Na> Mn> Ca> Zn whereas for trace elements varied broadly between 10 and 80%. Scanning electron microscopy (SEM) analysis illustrated the accumulation of heavy metals in/on the biofilm. Biofilm analysis also revealed the presence of different elements up to 10% of the dried biomass.

The results demonstrated the effectiveness and sustainability of indigenous environmental friendly algal-microbial biofilm to be exploited for removing most of elements from AMD. The results offer a potentially sustainable approach for the primary treatment of AMD at mine sites.
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CHAPTER ONE
CHAPTER 1: INTRODUCTION

1.1 Overview

Industrialisation has been presented as the hallmark of civilisation. Although, industrial activities have created important environmental issues such as water contamination. Industrial wastewaters, mainly resulting from mining, milling and surface finishing industries, are contaminated with toxic elements and heavy metals. The polluted waters drain or discharge into the water bodies which are often the source of irrigation or drinking water for the towns downstream. In many countries, municipal wastewater treatment facilities are not equipped for removing traces of heavy metals; therefore every consumer is exposed to a quantity of pollutants in the consumed water (Gadd, 2010; Gilmour and Riedel, 2009).

Mining industry is one of the major units that release toxic elements and heavy metals such as As, Cd, Cu, Zn, Pb and Ni to water bodies and soil. Mining activities increase the concentration of these elements in water compared to their normal concentrations in each area (Lottermoser, 2010). Mining wastewaters, typically known as acid mine drainage (AMD), are acidic and contain the elements Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, Zn which are considered the major pollutants (Sparks, 2005). These elements are not biodegradable and their presence in water affects the ecological balance (Baker and Banfield, 2003). The treatment of AMD, particularly from the aspect of removing toxic heavy metals and metalloids, is the focus of this thesis.

AMD treatment has been limited to chemical precipitation in most mine sites, which is not effective for removing pollutant elements present in low concentrations. To remedy this problem advanced wastewater treatment is required. Biotreatment and/or biosorption has emerged as an alternative technique and has been successfully used in municipal wastewater treatment system. This technique depends on the efficiency of microorganisms and designed system. The reported research evaluates the capability of biotreatment for AMD treatment. The primary contribution of this work has been to provide insight into the capabilities of indigenous mining microorganisms that are resistant species and adapted to survive in acidic and contaminated mining wastewaters.
Additionally, in order to participate in the development of advanced AMD treatment methods, this study established an algal-microbial biofilm from indigenous mining microorganisms in a laboratory-scale photo-rotating biological contactor (PRBC) for removing variety of elements included heavy metals at different concentrations.

1.2 Background - Impact of mining activities on water quality

Water is an essential resource for mining activities such as mineral processing, hydrometallurgical extraction, coal washing and dust suppression. Additionally, water is an unwanted by-product from mine dewatering processes in open pits and underground mining operations. Mines located in wet climates may have to pump more than 100,000 litres per minute. The water quality in used and unwanted water both are influenced by mining activities (Lottermoser, 2010).

Mining excavation and extraction of ore minerals from a reductive environment exposes them to the oxidative environment of surface and accelerates their oxidation (Nganje et al., 2010). The common minor constituents of the Earth’s crust are sulphide minerals. Sulphides comprise the major proportion of the rocks in some mines, in particular metallic ore deposits (Cu, Fe, Zn, Pb, Au, Ni, U), phosphate ores, coal seams, oil shales and mineral sands (Lottermoser, 2010; Brake et al., 2001 a,b). Sulphide minerals are not stable in an oxidative environment. These minerals react with oxygen and water leading to the release of hydrogen and sulphate ions which influences the water quality by decreasing the pH. In some cases the pH of water decreases to 2 - 4 (Bhattacharya et al., 2006; Das et al., 2009a). Surface waters that are affected by these influences are referred to as AMD (Lottermoser, 2010; Kalin et al., 2004). The excavation of mines, particularly open-cut mines, produces a huge amount of overburden and low-grade waste rocks that create massive piles around mine sites. These wastes typically contain sulphide minerals and are one of the major the resources that effectively contribute to AMD formation around mine sites (Lottermoser, 2010). However, the formation of acidic waters can also occur through the natural weathering of the sulphide-bearing rocks, along the outcrops or the scree slopes of ore deposit. In general, sulphide-rich and carbonate-poor materials are expected to produce acidic drainages (Lottermoser, 2010).
Sulphide minerals, typically pyrite and chalcopyrite, contain heavy metals such as Cd, Sn, Pb, Cu, Fe, Hg, Ni, Zn and Cr in their atomic lattice. The oxidation of the sulphide minerals causes the release of embedded heavy metals (Fe, Cu, Pb, Zn, Cd, Co, Cr, Ni, Hg), metalloids (As, Sb), and other elements (Al, Mn, Si, Ca, Na, K, Mg, Ba) (Lottermoser, 2010; Das et al., 2009 a,b; Bhattacharya et al., 2006). Most of these metals and metalloids are dissolved under acidic conditions. Therefore, AMD is an extreme and common example of poor mine water quality and can be a source of severe contamination in surface and ground waters (Niyogi et al., 2002; Costley and Wallis, 2000).

1.3 Acid formation processes

Acid formation in mine waters occurs through various oxidation reactions. Pyrite, as the most abundant sulphide mineral, occurs in most geological environments. It is commonly associated with coal and metal ore deposits. The oxidation of pyrite occurs either in the presence or absence of microorganisms which are known as biotic and abiotic chemical oxidation processes, respectively. These processes, biotic and abiotic degradation, can be caused by oxygen (direct oxidation) or by oxygen and iron (indirect oxidation). The divalent and trivalent states of iron both play a central role in the indirect oxidation of pyrite. The different pyrite oxidation mechanisms can be summarised as follows (Lottermoser, 2010):

- Oxidation by oxygen (abiotic direct oxidation)
- Oxidation by oxygen in the presence of microorganisms (biotic direct oxidation)
- Oxidation by oxygen and iron (abiotic indirect oxidation)
- Oxidation by oxygen and iron in presence of microorganisms (biotic indirect oxidation)

Oxygen directly oxidises pyrite in the abiotic and biotic direct oxidation processes through the following stoichiometric chemical reaction (Eq. 1.1):

$$\text{FeS}_2(s) + 7/2 \text{O}_2(g) + \text{H}_2\text{O}(l) \rightarrow \text{Fe}^{2+}(\text{aq}) + 2\text{SO}_4^{2-}(\text{aq}) + 2 \text{H}^+(\text{aq})$$

(Eq. 1.1)

The pyrite oxidation is primarily accomplished by indirect oxidation which involves the chemical oxidation of pyrite by oxygen and ferric iron (Fe$^{3+}$), which occurs in three interconnected steps, presented in the following reactions (Eq. 1.2-1.4):
FeS\textsubscript{2(s)} + 7/2 O\textsubscript{2(g)} + H\textsubscript{2}O\textsubscript{2(l)} → Fe\textsuperscript{2+}\textsubscript{(aq)} + 2SO\textsubscript{4}^{2-}\textsubscript{(aq)} + 2 H\textsuperscript{+}\textsubscript{(aq)} + energy \quad \text{(Eq. 1.2)}

Fe\textsuperscript{2+}\textsubscript{(aq)} + 1/4O\textsubscript{2(g)} + H\textsuperscript{+}\textsubscript{(aq)} → Fe\textsuperscript{3+}\textsubscript{(aq)} + 1/2H\textsubscript{2}O\textsubscript{(l)} + energy \quad \text{(Eq. 1.3)}

FeS\textsubscript{2(s)} + 14 Fe\textsuperscript{3+}\textsubscript{(aq)} + 8H\textsubscript{2}O\textsubscript{(l)} → Fe\textsuperscript{2+}\textsubscript{(aq)} + 2SO\textsubscript{4}^{2-}\textsubscript{(aq)} + 16 H\textsuperscript{+}\textsubscript{(aq)} + energy \quad \text{(Eq. 1.4)}

In these reactions aq, l and s stand for aqueous, liquid and solid phases of different components.

The indirect pyrite oxidation is exothermic and releases energy (Eq. 1.2-1.4). In the initial step (Eq. 1.2), pyrite is oxidised by oxygen and dissolved ferrous iron (Fe\textsuperscript{2+}), sulphate and hydrogen ions are produced. The second step (Eq. 1.3) represents the oxidation of ferrous iron to ferric iron by oxygen which occurs under low pH. In the third reaction (Eq. 1.4), the generated Fe\textsuperscript{3+} via the second reaction acts as the oxidising agent in additional pyrite oxidation and generates more Fe\textsuperscript{2+} which can be oxidised to Fe\textsuperscript{3+}. As the above reactions show the indirect pyrite oxidation form a continuing cycle of Fe\textsuperscript{2+} conversion to Fe\textsuperscript{3+} and subsequent oxidation of pyrite by Fe\textsuperscript{3+} to produce Fe\textsuperscript{2+} (Lottermoser, 2010). The equations 1.2-1.4 depict the main reactions in mine sites responsible for the production of AMD. The presence of oxidising bacteria accelerates the processes of sulphide oxidation, 500,000 times (Das et al., 2009 a, b).

The acidic nature of AMD results from the release of hydrogen ions and sulphate anions through the aforementioned reactions. Additionally, as most metals and metalloids are soluble under acidic conditions, concentrations of heavy metals, metalloids and other elements rise from 0.01 to 1-9 x 10\textsuperscript{3} mg/L in AMD. High sulphate (>1000 mg/L), iron precipitates (100 to 1-9 x 10\textsuperscript{3} mg/L) and increased total dissolved solids (TDS) (100 to more than 1-9 x 10\textsuperscript{4} mg/L) are the typical problems of AMD (Lottermoser, 2010). The large volume of generated AMD with the mentioned environmental problems is a big issue for mine sites. An average of 15.5 ML/d was reported as the discharged volume of AMD (pH~3) from the West Rand Mining Basin in Gauteng Province, South Africa, which highlights the huge volume of released AMD (Hobbs and Cobbing, 2007).

### 1.4 Importance of AMD treatment

Discharging AMD to water bodies, rivers and lakes reduces their water quality. The AMD affected waters are a persistent and potentially severe source of surface and ground water
pollution around mine sites that can continue for a long time, even after mining closure. Dissolved heavy metals in AMD are introduced into the food chain and accumulate in the microorganisms (Baker and Banfield, 2003; Lottermoser et al., 1999). The accumulation of heavy metals through the trophic chain creates toxic effects and teratogenic changes which threat the health of humans, plants and animals (Ahluwalia and Goyal, 2007; Malik, 2004). Dangerous concentrations of toxic metals in vegetables and grains growing in the contaminated soils are of growing concern due to their accumulation in terrestrial and aquatic habitats being associated with adverse effects on the biota and human health. The extreme toxicity of heavy metals is due to their damaging effects on nerves, liver and bones. These elements block functional groups of vital enzymes. As an example, Ni is listed as a possible human carcinogen (group 2B), and causes reproductive problems and birth defects. A range of detrimental effects of heavy metals on fauna and flora are well documented (Gadd, 2010; Malik, 2004).

The contamination of surface and ground waters by mining effluents, especially in arid areas with scarce water resources, is a serious environmental issue. Additionally, the increased solubility of metals in the acidic waters leads to the loss of considerable amount of precocious metals through mining wastewaters. These outcomes and stricter environmental regulations for mine sites add the necessity of treating AMD and removing heavy metals before discharging to the environment.

1.5 Conventional treatment techniques for metal ion removal from aqueous solutions

The conventional established methods for removing metal ions from aqueous solutions mainly include reverse osmosis, electro-dialysis, ultra-filtration, ion exchange, chemical precipitation and phyto-remediation. These methods are listed in Table 1.1 with their general description and disadvantages of application. The disadvantages included incomplete metal ion removal, high reagent requirement, generation of toxic sludge which require further treatment and add costs, limit the application of most methods for AMD treatment (Fu and Wang, 2011; Silverira et al., 2009; Das et al., 2008; Ahalya et al., 2003). Chemical precipitation and phyto-remediation have been the most commonly used methods for AMD treatment which are described in the next sections.
Table 1.1 Established techniques for removing metal ions from aqueous solutions (Ahalya et al., 2003)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Process</th>
<th>Disadvantage</th>
</tr>
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<tbody>
<tr>
<td>Reverse Osmosis</td>
<td>Metal ions are separated by a semi-permeable membrane at a pressure greater than osmotic pressure caused by dissolved solids in wastewater</td>
<td>Expensive</td>
</tr>
<tr>
<td>Electrodialysis</td>
<td>Metal ions are separated through semi-permeable ion selective membrane using an electrical potential between two electrodes which causes a migration of cations and anions towards respective electrodes</td>
<td>Clogging membrane due to metal hydroxides formation</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Pressure driven membrane operations that use porous membranes for metal ions removal</td>
<td>Sludge generation</td>
</tr>
<tr>
<td>Ion-exchange</td>
<td>Metal ions from dilute solutions are exchanged with ions held by electrostatic forces on the exchange resin or polymers</td>
<td>High cost, Partial removal of certain ions</td>
</tr>
<tr>
<td>Chemical Precipitation</td>
<td>Precipitation of metals by the addition of coagulants such as alum, lime, iron salts and other organic polymers</td>
<td>Produce large amount of sludge containing toxic compounds</td>
</tr>
<tr>
<td>Phyto-remediation</td>
<td>Using certain plants to clean up soil, sediment, and contaminated water with metals</td>
<td>Requires long time for metal ion removal, Plant regeneration for further biosorption is difficult</td>
</tr>
</tbody>
</table>

1.6 Established methods for metal ion removal from AMD

AMD treatment technologies are site-specific, and multiple remediation strategies are commonly required to achieve successful removal of metal ions from AMD (Brown et al., 2002). Currently, the most commonly used methods in mine sites include evaporation, neutralisation, controlled release and dilution by natural waters; and wetlands contribute to the multiple remediation strategies for improving AMD quality before discharging to the environment. These techniques can be basically categorised as active or passive methods:

- Active treatment methods, such as neutralisation and chemical precipitation, require continuous addition of chemical reagents, mechanical devices to mix the reagent with water and active maintenance and monitoring of the system.
- Passive treatment methods such as wetlands which benefit the biological processes to neutralise acidity and reduce dissolved metal concentrations. Such methods require little or no reagents, active maintenance and monitoring, or mechanical devices.

The most commonly applied methods, neutralisation and precipitation; and wetlands in mining area are explained individually as follows.

### 1.6.1 Neutralisation and precipitation

Currently, neutralisation and precipitation are the most commonly applied treatment methods to remove metals from AMD and raise the pH to an alkaline level. The procedure involves collecting AMD and selecting an appropriate neutralising reagent that is mixed with the AMD.

A large variety of natural, by-product or manufactured chemical reagents are used for neutralisation included limestone (CaCO$_3$), caustic lime (CaO), hydrated lime (Ca(OH)$_2$), dolomite (CaMg(CO$_3$)$_2$), caustic magnesia (Mg(OH)$_2$), magnesite (MgCO$_3$), soda ash (Na$_2$CO$_3$), caustic soda (NaOH) and ammonia (NH$_3$) (Lottermoser, 2010). Equations 1.5 to 1.7 demonstrate the treatment process by using limestone, where AMD contains Pb and Zn ions. Hydrogen ions are consumed; bicarbonate ions are generated and dissolved metals (Pb and Zn) are converted into the less soluble minerals such as sulphates, carbonates, and hydroxides.

$$\text{CaCO}_3(s) + \text{H}^+(\text{aq}) + \text{SO}_4^{2-}(\text{aq}) + \text{Pb}^{2+}(\text{aq}) \rightarrow \text{PbSO}_4(s) + \text{HCO}_3^-(\text{aq}) \quad \text{(Eq. 1.5)}$$

$$\text{CaCO}_3(s) + \text{Pb}^{2+}(\text{aq}) \rightarrow \text{PbCO}_3(s) + \text{Ca}^{2+}(\text{aq}) \quad \text{(Eq. 1.6)}$$

$$\text{CaCO}_3(s) + \text{Zn}^{2+}(\text{aq}) + 2\text{H}_2\text{O}(l) \rightarrow \text{Zn} (\text{OH})_2(s) + \text{Ca}^{2+}(\text{aq}) + \text{H}_2\text{CO}_3(\text{aq}) \quad \text{(Eq. 1.7)}$$

Beneficial chemical reagents, including limestone, are low cost materials with ease of use; and form a dense and easily handled sludge. Neutralisation has the potential to remove many heavy metals such as Cd, Cu, Fe, Pb, Ni and Zn from AMD. This process leads to the precipitation of metals as hydroxides, which are then typically landfilled. However, the precipitation of all elements does not always occur and many metals are dissolved in water within a broad range of pH (Brookins, 1988). Additionally, neutralisation is ineffective in treating aqueous solutions where concentrations of the contaminants are low (1-100mg/L). Slow reaction rates of chemical
agents where ions bond with limestone particles forming iron precipitates are also a disadvantage of this method. A rapid mixing unit is required to prevent coating of the chemicals with reaction products (Perry and Green, 2008). Furthermore, the precipitates inhibit the neutralisation reactions and cause excessive reagent consumption (Lottermoser, 2010). Active mixing requires high energy and large sedimentation tanks and infrastructure requirements. In addition any valuable metals present in AMD are lost in the solid phase sludge (Perry and Green, 2008).

After the neutralisation process, the treated AMD is diverted to an open pond systems for evaporation, which is not a sustainable remedy and results in huge volumes of laden heavy metal sediments (Silverira et al., 2009; Zinck, 2006; Aube and Zinck, 2003).

1.6.2 Wetlands

Wetland treatment has been successfully used for some wastewaters including agricultural, storm water runoff and AMD. Treatment takes place in an organically-rich and water-saturated shallow ponds, exploiting chemical, physical and biological processes. The wetland processes aim to increase pH and decrease metal and sulphate concentrations via oxidation and reduction reactions, cation exchange and adsorption of metals onto the organic substrate, adsorption of metals by precipitating Fe$^{3+}$ hydroxides, and metal uptake by plants (Brown et al., 2002). Some aquatic plants, growing in wetlands, are able to uptake large amounts of heavy metals and metalloids. Additionally, filtering of suspended solids and colloidal matter from the mine water as well as sedimentation and retention of these precipitates by physical entrapment are additional benefits of using wetlands (Brown et al., 2002).

Wetlands are aesthetically attractive, passive, low-cost with minimal maintenance, and are sustainable. However, this method is a preferred option for the completion or partial treatment of small volumes of AMD, which has low total dissolved solids (TDS). High metal concentrations in AMD can adversely affect the aquatic plants in wetlands. Furthermore, this method cannot be used for the treatment of AMDs with high iron concentration and low pH, as these parameters causes stress to the growing plants in wetlands (Lottermoser, 2010).

To maintain the wetlands in a permanently saturated condition, sufficient year-round supply of water is required (Lottermoser, 2010). It is commonly stated that favourable responses to metals
load were observed during the wet seasons whereas the efficiency for metal removal was significantly reduced at dry seasons. A wetland without sufficient water supply acts as a chemical time bomb as it is a source of metals, metalloids, and sulphate (Zhuang, 2009).

1.7 Biotreatment/bioremediation

As explained previously, limited methods are currently applied for AMD treatment. However, the disadvantages of these techniques strongly advise the development of the more efficient and applicable methods for AMD treatment and heavy metal removal.

Biotreatment or bioremediation has been considered as an alternative efficient and cost effective technique to treat different types of wastewaters, included AMD (Gadd, 2010; Das et al., 2009a,b; Natarajan, 2008; Prasad, 2007; Ahluwalia and Goyal, 2007). These biotechnological methods can be defined as any process that uses microorganisms, green plants or their enzymes to return the original or uncontaminated nature of the polluted environment (Gadd, 2004; Malik, 2004). Biotreatment is based on metal binding capacities of various biological materials and depend on the considerable efficiency of microorganisms. Algae, bacteria and fungi have been proved to be potential metal biosorbents (Gadd, 2010). Biological methods have been developed during the last decades as a polishing stage in wastewater treatment schemes (Gadd, 2010; Das et al., 2009a,b; Natarajan, 2008; Prasad, 2007; Ahluwalia and Goyal, 2007; Gadd, 2004; Kalin et al., 2004; Ahalya et al., 2003).

Biotreatment can be undertaken by dead or pre-treated cells; and live cells. When biosorbents are exposed to metal ions, ions can be entrapped in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is independent of the biological metabolic cycle and is known as “biosorption” or “passive uptake” which is the basis of biotreatment using dead/pre-treated cells (Volesky, 2001).

Using live cells is the basis of the other technique in biotreatment. In this technique, the metal ions can be absorbed onto the cell walls and also pass across the cell membrane through the metabolic cycle. This method which is referred as “bioaccumulation” or “active uptake” benefits from the metal uptake by both active and passive modes. This biphasic uptake of metals
is carried out by an initial rapid phase of biosorption followed by the slower, metabolism-dependent active uptake of metals (Malik, 2004).

Main advantages of biotreatment/bioremediation by the metabolically mediated or physico-chemical removal potential of microbial biomass over conventional treatment methods include (Ahalya et al., 2003):

- Availability and low cost of biologic materials
- Environmentally friendly concerning energy and material consumption
- Reduced amount of sludge production for disposal compared with chemical precipitation
- Efficient for very low residual metal concentration (less than mg/L level) compared to other common physico-chemical processes
- Can be metal specific
- Possibility of metal recycling and recovery

1.7.1 Biosorption- Passive Systems

During 1980s and 1990s, a few pilot installations and commercial scale units were constructed in the USA and Canada based on biosorption process by inactive cells (Tsezos et al., 2007). In these biotreatment system, biological sorbent particles (Biosorbents) were used which were made by immobilisation of biomass in a matrix such as polyethylene (Bennett et al., 1991) silica or polyacrylamide gels (Darnal et al., 1986). The results confirmed the applicability of biosorption as the basis for metals sequestering/recovery, in particular from high volume of dilute complex wastewaters. However, these pilot plants helped the researchers to realise the limitations associated with the application of inactive microbial biomass in an industrial biotreatment system (Tsezos et al., 2007). The limitations were mainly including the cost of formulating the biomass into biosorbent material, the negative effect of solution matrix co-ions on the targeted metals, and the reduced flexibility of the biological material which made recycling and reuse of the biosorbent more difficult (Tsezos et al., 2007). Therefore, developing a continuous biotreatment system based only on biosorptive removal of metals using inactive microbial biomass is not sustainable, efficient and effective (Tsezos et al., 2007; Malik, 2004; Ahalya et al., 2003). The application and development of a biotreatment system by active microbial cells offer a better solution for removing metal/metalloids which include the biosorption process, because it
contributes as a parallel mechanism to metabolically mediated uptake mechanisms (Tsezos et al., 2007; Malik, 2004; Ahalya et al., 2003).

1.7.2 Biotreatment- Active Systems

The application of live microbial cells for biotreatment has received a lot of attention in recent decades (Gadd, 2010). Application of active and growing microbial cells benefit from (Tsezos, 2007; Malik, 2004; Ahalya et al., 2003):

- Active and passive biosorption and bioaccumulation mechanisms in cells
- Ability of self-replenishment
- Continuous metabolic uptake of metals after physical adsorption
- Potential for optimisation through development of resistant species
- The ability of cells to detoxification of the diffused metals into the cells and bond to intracellular proteins or chelating them before being incorporated into vacuoles and other intracellular sites
- Avoiding separate biomass production process, e.g. cultivation, harvesting, drying, processing and storage prior to the usage
- The ability of microbes for removing most pollutants via a single stage process
- Unlimited capacities of live cells for removing dissolved and fine-dispersed metallic elements via immobilisation

1.8 Chemical equilibrium models

The effect of water chemistry on microbes can be predicted with chemical equilibrium models. These models such as Free Ion Activity Model (FIAM) and Biotic Ligand Model (BLM) have been developed to quantify the manner in which water chemistry affects the speciation and biological availability of metals in aquatic systems. The bioavailability and bioreactivity of metals control their potential to cause adverse effects. The toxicity of metals such as Cu is affected by various species of each element and chemical environmental factors, in particular organic matter, pH, Ca, Mg, and Na concentrations. The BLM includes the modifying effect of these factors on the interaction of metals with a biological receptor which is called the biotic ligand and predicts the toxicity of these factors to aquatic organisms. The BLM has gained extensive interest amongst the scientific, regulated and regulatory communities because of its
potential to be used for assessing water quality criteria (WQC) and its application in performing aquatic risk assessments for metals. The BLM is used as a tool to outline Ambient Water Quality Criteria (AWQC) for surface water by the Environmental Protection Agency (EPA) (Alen and Janssen, 2006).

The FIAM is the most commonly used model to explain bio-uptake fluxes outside of the cell. This model is based on the following assumptions (Hassler et al., 2009):

1- The primary site for metal interactions with living organisms is the cell’s plasma membrane. The interactions can be described as a surface complexation reaction.

2- A pseudo-equilibrium is established between metal species in the bulk solution and at the biological surface because the metal transport towards the membrane in solution and surface complexation reaction occurs rapidly.

3- The biological response depends on the concentration of metal surface complex.

4- In the range of metal concentrations of toxicological interest, the concentration of free sites remains virtually constant and variations in metal surface complex follow variations in free metal ion complexes.

5- The nature of the biological surface remains constant during exposure to the metal of interest

The aforementioned assumptions limit the applicability of this model to defined systems. However, for systems which involve divalent trace metals, inorganic media or filtered seawater, fixed pH, and a known ligand concentration, the results of the model are consistent with experimental results.

Use of these models has been successful in predicting the results of metal uptake by microorganisms (Lamekas and Slaveykova, 2007). However, the failure of these models to elucidate metal bioaccumulation has also been recorded (Hassler and Wilkinson, 2003).

These models were not considered in the reported study as it was based on fundamental research focused on the application of algal-microbial biofilms for metal/metalloid adsorption.
1.9 Objectives and achievements

The overall objective of my PhD research was to develop a reliable, sustainable and effective system for AMD biotreatment. The PhD study aimed to exploit an indigenous algal-microbial consortium, naturally found in AMD’s periphytons at the Sarcheshmeh copper mine in Iran, as an efficient biosorbert. This aim was targeted and resulted from the previous observations and findings (Orandi et al., 2009, Orandi et al., 2007) that specific strains of micro-algae, bacteria and fungi were shown to form an efficient bio-assembly for sorption of heavy metals. The experiments were designed to mimic their natural habitat in vitro and utilise their absorptive nature for AMD treatment. The main objectives of the reported research are as follows:

- Quantify AMD composition in the field (in-vivo), including metal ions and nutrients content
- Synthesising a complex AMD for in-vitro investigations
- Investigate the applicability of the synthetic AMD to maintain indigenous microbial growth during in-vitro investigations
- Isolation, numeration and identification of indigenous AMD microbes
- Immobilisation and development of an algal-microbial biofilm in a laboratory scale photo-rotating biological contactor (PRBC)
- Quantify removal potential of the indigenous AMD biofilm in PRBC for 18 elements included heavy metals and toxic metalloids
- Quantify accumulated metals/metalloids in the algal-microbial biofilm

This study provides a realistic understanding of an AMD biofilm’s potential for removing various metal ions from AMD at different initial concentrations under low pH conditions (3-5). This project participated in the development of a novel system to mitigate AMD-related contamination in mining sites. The main objectives which were driven from in vivo and in vitro investigations are depicted in a flow diagram shown in Fig.1.1.
Fig. 1.1 Flow diagram indicating the main aspects reported in this thesis.
1.10 References


Brake, S., Connors, K., Romberger, S. (2001a) A river runs through it: impact of acid mine drainage on the geochemistry of West Little Sugar Creek pre- and post-reclamation at the Green Valley coal mine, Indiana, USA. Environmental Geology. 40: 1471-1481.


CHAPTER TWO
CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Many studies have reported that specific species of microorganisms have considerable potential for removing heavy metals from different types of contaminated wastewaters, typically at neutral or alkaline pH (Gadd, 2010; Romera et al., 2006; Munoz and Guieysse, 2006; Mehta and Gaur, 2005). Selection of resistant and effective species of microorganisms is a pre-requisite for the biotreatment of acidic and contaminated AMD waters (Boshoff et al., 2004; Russell et al., 2003). Indigenous AMD microorganisms were selected as a target biosorbent for biotreatment investigations in this study. Scientific literature relevant to the exploitation of microbes for AMD treatment and a practical biotreatment system for the mining industry are presented and reviewed in this chapter.

2.2 Extremophilic indigenous microorganisms in AMD

AMD often exerts environmental pressure to aquatic life by chemical stress, from low pH and high concentration of heavy metals; and physical stress, from metal oxide deposits (Das et al., 2009a; De la pena and Barreiro, 2009). Additionally, low macro-nutrient levels such as organic carbon, nitrate and phosphate in AMD restrict microbial growth (Lottermoser, 2010; Brake et al., 2001). Many studies investigated the impact of AMD on aquatic life (Smucker and Vis, 2011; Bray et al., 2008). These studies show that there is limited biodiversities in AMD which are included extremophilic micro-algae, bacteria, fungi and yeasts (Malik, 2004; Johnson and Hallberg, 2003; Brake et al., 2001; Nordstrom, 2000).
### 2.2.1 Green micro-algae in AMD

The acidophilic species of micro-algae, *Klebsormidium*, *Euglena*, *Microspora*, *Mougeotia*, *Ulothrix*, *Stigeoclonium* and *Chlamydomonas*, are often found in AMD and are able to grow at low pH ~ 0.05 (*Das et al.*, 2009, a, b; *Prasad*, 2007; *Orandi et al.*, 2007). Abundance and distribution of green micro-algae e.g. *Klebsormidium* sp. and *Chlamydomonas* sp. were reported as the indicators of AMD and high iron concentration (*Novis* and *Harding*, 2007; *Valente* and *Gomes*, 2007).

*Klebsormidium* sp. are an unbranched filamentous micro-algae classified in the phylum of Chlorophyta (Green algae). This alga is widespread and often abundant in dense mats in streams impacted by AMD. *Klebsormidium* was once considered as a relative of *Ulothrix*. However, it is now well known that it is superficially like *Ulothrix* and in more recent classifications it has been placed in the different group, *Charophyceae*, based on cell features in the division process (*Leliaert et al.*, 2012; *Rindi et al.*, 2011, 2008; *Novis*, 2006). The narrow filaments in *Klebsormidium* mostly contain cylindrical or slightly barrel-shaped cells, 5–15 µm broad and 1–3 times long as broad. The diameter of filaments in *Klebsormidium* is much less than in *Ulothrix.* Each cell body contains plate-like or ribbon-like chloroplasts. These curved chloroplasts cover a relatively small proportion of the cell wall. The parietal plate chloroplasts superficially resemble members of the *Ulotrichales*. However, the chloroplasts in *Ulothrix* which are similarly curved, occupy a greater proportion of the cell wall (*Bellinger and Sigee*, 2010). The filamentous green micro-algae, composed of elongated squared cells of around 5-6 µm in diameter, were reported in the Tinto River in Spain. The reported algae were forming long filaments up to 200-300 mm in length (*Espana et al.*, 2007).

In addition to filamentous micro-algae, uni-cellular micro-algae such as *Chlamydomonas* are also abundant in AMD (*Das et al.*, 2009 a,b; *Kalin et al.*, 2006). The haploid cells of *Chlamydomonas* have two anterior flagella. The cells contain chloroplast and “eye spot” that perceives light. This alga is classified in the division of *Chlorophyta*. Micro-algae are the only phototrophic form of life in AMD that provide organic carbon for heterotrophic microorganisms such as fungi and bacteria (*Amaral Zettler et al.*, 2002).
2.2.2 Fungi and yeasts in AMD

Fungi growth occurs over a wide pH range (1-11) and fungi were detected in AMD or acidic industrial wastewater (Brake and Hasiotis, 2010). The resistant strains of fungi which are able to thrive in AMD are reviewed by Das et al. (2009 a,b). Fungi such as Aspergillus, Fusarium, and Penicillium were reported mainly from mine sites (Das et al., 2009a). Representatives of yeasts e.g. Candida, Rhodotorula and Trichosporon are also usually isolated from streams carrying AMD (Baker et al., 2004). Fungi and yeasts are considered heterotrophic organisms as their metabolism relies solely on carbon fixed by other organisms (Gadd, 2007).

The ecological study of fungal populations in the acidic Tinto River in Southern Spain revealed unexpected levels of microbial richness, as 154 strains of filamentous fungi and 90 strains of yeasts were isolated from this River. The isolated Fungal strains belonged to the genus Penicillium and Scytalidium, Bahusakala, Phoma, and Heteroconium. The isolated strains of yeasts belonged to 6 genera Rhodotorula, Cryptococcus, Tremella, Holtermannia, Leucosporidium, and Mrakia (Lopez-Archilla et al., 2004). Another investigation on the microbial diversity of a highly acidic runoff (pH ~ 0.9) from the Richmond Mine at Iron Mountain (Northern California) revealed that the majority of isolated microorganisms (68%) belonged to fungi, Dothideomycetes and Eurotiomycetes sp (Baker et al., 2004). Presence of Geotrichum sp. and Aspergillus sp. in Sarcheshmeh copper mine run off was also reported (Orandi et al., 2007).

2.2.3 Bacteria in AMD

Numerous types of bacteria including Thiobacillus thiooxidans, T. ferrooxidans, Ferrobacillus sulfooxidans, Leptospirillum ferrooxidans, T. concretivorus, T. thioparus, Sulfobacillus thermosulfidooxidans and Metallogenium have been isolated from AMD waters (Lottermoser, 2010). Aguilera et al., (2010) reported Acidithiobacillus ferrooxidanse and Leptospirillum ferrooxidans as the most abundant bacterial species in the AMD in the Rio Tinto, Spain. Orandi et al. (2007) reported the bacteria, Pseudomonas spp. and Tiobacillus spp. isolated from acidic (pH ~ 3) drainages at Sarcheshmeh copper mine, Iran.
Most of these acidophilic/extremophilic bacteria are chemo-litho-trophic e.g. *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and are sustained by the energy derived from pyrite oxidation in mine sites (Baker *et al.*, 2004). They need small amount of nitrogen and phosphorous for their metabolism, and their energy requirements are provided through the oxidation of Fe$^{2+}$, hydrogen sulphide, thiosulphate, sulphur, and metal sulphides. They also have the ability to transform inorganic carbon into cell building material, which may originate from the atmosphere or from the dissolution of carbonates (Escobar *et al.*, 2008; Yang *et al.*, 2008, Cabrera et al., 2005; Baker and Banfield, 2003).

*Acidithiobacillus ferrooxidans* is a rod-shaped Gram-negative bacterium. The cells are non-sporing, singly or occasionally in pairs or chains, depending on the growth conditions. Highly motile with a single flagellum and non motile strains have been reported. The optimal pH level for growth is 1.5 – 2.5 (Valdes *et al.*, 2008). *Leptospirillum ferrooxidans* is also Gram-negative and has spiral-shaped cells, 0.3-0.5 microns wide and 0.9-3.0 microns in length. *Pseudomonas* are the other Gram-negative bacteria with rod-shaped cells and heterotrophic. Some of the species were reported commonly from mine sites (Palleroni, 2010; Novis and Harding, 2007; Das *et al.*, 2009 a,b).

Acidophilic strains of *Bacillus* were also reported from mine sites (Raja and Omine, 2012). These heterotrophic bacteria are Gram-positive and rod-shaped. Some of species are able to form tough, protective endospores, allowing the organism to tolerate the extreme conditions of AMD. They are mostly reported as an obligate aerobe (Madigan and Martinko, 2005).

Prior to the design of any biotreatment system for AMD waters, selecting resistant microbes is a necessary primary step. However, many reported studies used un-adapted strains of microbes for biotreatment investigations. For example, Costley and Wallis (2001, 2000, 1999) used activated sludge as the initial inoculum for their biotreatment system using a rotating biological contactor for removing Cu, Zn and Cd from synthetic wastewater. The enrichment processes were conducted to obtain the microbial population acclimatised to 100 mg/L of Cu, Zn and Cd (Costley and Wallis, 1999).
2.3 Role of indigenous microorganisms in AMD

Many investigations have been carried out to understand the role of indigenous microorganisms in AMD resources or AMD contaminated waters (Das et al., 2009b; Malkoc and Nuhoglu, 2003; Niyogi et al., 2002; Brake et al., 2001). The investigations showed, the extremophilic microbial community are not only adapted to survive under the harsh environment of AMD, but also participate significantly in improving the degraded quality of AMD waters (Gadd, 2010; Lottermoser, 2010; Prasad, 2007).

Microorganism cell walls generally contain functional groups including hydroxyl (-OH), phosphoryl (-PO$_3$O$_5$), amino (-NH$_2$), carboxyl (-COOH), sulphydryl (-SH), which result in an overall negative charge on the cell surface. Metal ions with positive charges are adsorbed onto the cell surface (Mehta and gaur, 2005). The cell walls of green micro-algae are mainly composed of cellulose which has a fiber-like structure and an amorphous matrix of various polysaccharides (Bayramoglu et al., 2006). The Polysaccharides, typically carbohydrates carry carboxyl groups and are found in the microorganism cell walls. Fungal cell walls contain up to 80–90% polysaccharides, with proteins, lipids and polyphosphates. Bacterial cell walls also contain peptidoglycans, which is made of polysaccharides. The negative charge of carboxyl group in these microorganisms contribute in adsorbing metals onto their cell walls (Wang and Chen, 2009; Romera et al., 2006). The potential of bacteria, algae and fungi for removing heavy metals and accumulating them on the cell surface or into the cytoplasm have been documented extensively (Gadd, 2010; Das et al., 2009b; Mehta and Gaur, 2005).

Recent studies highlight the potential of indigenous AMD microorganisms, especially microalgae, for removing metal/metalloids from AMD (Trzcinska and Skowronska, 2012; Souza-Egipsy, 2011; Bayramoglu et al., 2006; Aguilera et al., 2006, 2007, 2010; Munoz and Guieysse, 2006). A previous study by Skowronska (2003) investigated resistance and accumulation of elevated Zn concentrations for two strains of green algae. One species, referred as Zn-tolerant, was isolated from ditches containing mining water and another species referred as Zn-sensitive was isolated from unpolluted lake water. The Zn-tolerant species was able to accumulate significantly more Zn and Pb than the Zn-sensitive species. Additionally, the Zn-tolerant species was capable to detoxify the excess of accumulated zinc more efficiently than the Zn-sensitive species. The indigenous microorganisms isolated from AMD can offer efficient biosorbents for AMD treatment. However, there is no adequate information that quantifies the metal removal
trends of these microbes over a prolonged treatment period. Understanding the biosorption trend of these microorganisms is required before the exploitation in a biotreatment system can be achieved.

2.4 Metal-microbial cell interactions

Most metals/metalloids are in soluble and mobile forms under the acidic conditions of AMD. The immobilisation of these elements to the less mobile species is the main target of treatment processes, in order to minimise bioavailability, transport and propagation of pollution through water streams.

Despite a dramatic increase in the published literature relating to the biosorption of metals/metalloids from AMD, this technology is not yet applicable to mine sites (Tsezos, 2007; Ahalya et al., 2003). Many research processes are still at the laboratory scale and cannot be exploited in an industrial context (Gadd, 2010). The majority of searches have been conducted to prove the potential of biosorption by employing different microbial strains in batch systems. In these biosorption studies, the removal/immobilisation of elements by the microbial cells has been mainly emphasised (Gadd, 2009; Das et al., 2009). Immobilisation takes place through a number of processes which reduce the free metal/metalloid ion species. However, immobilisation may promote solubilisation of metals in some circumstances by shifting the equilibrium to release more metal into solution, which is referred as mobilisation or desorption in biotreatment studies (Gadd, 2010; Gadd, 2004). The efficiency of biotreatment systems are controlled by immobilisation and mobilisation processes. To date, biosorption studies which demonstrated immobilisation have been extensively documented whereas mobilisation/desorption studies have not been addressed adequately (Das et al., 2009). Characteristics of metal removal by microorganisms are not truly determined unless the sorption studies are complemented by desorption studies (Das et al., 2009).

Costley and Wallis (1999) used a laboratory scale RBC to investigate the efficiency of an immobilised activated sludge for removing Cu, Zn and Cd from a contaminated synthetic wastewater. They investigated the effect of rotational speed, namely 3, 15 and 25 rpm, on heavy metal removal within a 7-day continuous period. The result was very promising for Cu removal, >90%. However, the results for Zn and Cd showed more desorption was achieved than absorption
(Costley and Wallis, 1999). The results and discussion of this research were expanded mainly on the metal removal capacity of the system whereas desorption trends were not discussed adequately. However, the authors stated that the microbial cells may exhibit resistance mechanisms to tolerate high concentrations of heavy metals as a prevention mechanism for the initial metal uptake, or alternatively providing a means of expelling ions from the cells (Costley and Wallis, 2001). The re-solubilisation of Zn and Cd which sorbed during the adaptation period of inoculum was also attributed to their desorption results (Costley and Wallis, 1999). To design an efficient and applicable system for AMD biotreatment at mine sites, adequate understanding of the microbial performances, in particular by indigenous mine microbes, for immobilisation and mobilisation of metals/metalloids, is required.

2.5 Metal immobilisation processes

Microbial cells are capable to immobilise metal/metalloid ions through physico-chemical or metabolically dependent mechanisms. The immobilisation process is commonly referred as biosorption (Tezos, 2007; Gadd, 2004).

The complex structure of microbial cells contributes to the various biosorption mechanisms. According to the dependence on the cell's metabolism these mechanisms can be divided into two groups: 1) non-metabolism dependent/passive, and 2) metabolism dependent/active (Ahalya et al., 2003). Additionally, according to the place where biosorption occurs in microbial cells, they can be further classified as (Gadd, 2010; Ahalya et al., 2003):

1. Extracellular accumulation/ precipitation
2. Cell surface sorption and precipitation
3. Intracellular accumulation

The aforementioned mechanisms are explained as follows:

2.5.1 Intracellular accumulation

Intracellular accumulation results from the transport of the metals/metalloids across a microbial cell membrane. This kind of biosorption is dependent on the cell’s metabolism and only occurs
with viable cells. The accumulation process may occur due to the transportation of essential ions such as K\(^+\), Mg\(^{2+}\) and Na\(^+\) during the cell’s metabolism. This occurs because of other ions including heavy metals with the same charge and ionic radius are absorbed through the transport system in the cell (Tsezos, 2007).

2.5.2 Physical adsorption

Physical adsorption or biosorption is conducted with the help of van der Waals' forces. Previous studies showed that the biosorption of U, Cd, Zn, Cu and Co in algae, fungi and yeasts took place through electrostatic interactions between the metal ions in solutions and the cell walls. Examples are biosorption of copper by the bacterium _Zoogloea ramigera_ and the alga _Chiarella vulgaris_ (Aksu _et al._ 1992) and biosorption of chromium by the fungi _Ganoderma lucidum_ and _Aspergillus niger_ (Ahalya _et al._, 2003).

Biosorption is a common process among live or dead cells for removing/immobilising elements. However, the efficiency of this process is affected by environmental conditions. For example, biosorption efficiency is adversely affected by the presence of cations with similar charges and radiances in a multi-ion solution. Additionally, biosorption can be adversely affected by decreasing pH, temperature and microbial mass density (Ahalya _et al._, 2003).

2.5.3 Ion Exchange

Polysaccharides enclosed in microbial cell walls contain bivalent ions such as Ca\(^{2+}\), and Mg\(^{2+}\) that can be exchanged with the counter bivalent metal ions such as Co\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\) and Zn\(^{2+}\) resulting in the biosorptive uptake of heavy metals (Brake and Hasiotis, 2010). Muraleedharan and Venkobachr (1990) showed that Cu was up taken by fungi _Ganoderma lucidum_ and _Aspergillus niger_ due to the ion exchange mechanism.

2.5.4 Complexation

Complex formation on the microbial cell surface after the interaction between the metal and active groups is another biosorption process that removes metals from solution. Carboxyl groups
in carbohydrates of the microbial polysaccharides are responsible for metal complexation. Aksu et al. (1992) stated that the biosorption of copper by *Calluna vulgaris* and *Zoogloea ramigera* occurred through both adsorption and formation of coordination bonds between metals and active groups of cell wall polysaccharides, amino and carboxyl groups. Complexation was found to be the only mechanism attributed for the accumulation of Ca, Mg, Cd, Zn, Cu and Hg by *Pseudomonas syringae* (Ahalya *et al.*, 2003). However, complexation does not always result in metal removal and accumulation in microbial cells. Microorganisms may also produce organic acids such as citric, oxalic, gluonic, fumaric, lactic and malic acids that can chelate toxic metals, resulting in the formation of metallo-organic molecules (Gadd, 1999; Gadd and Sayer, 2000). These organic acids help in the solubilisation of metal compounds and their leaching from their surfaces which is known as the refurbishing trait of live cells to survive in metal contaminated waters (Ahalya *et al.*, 2003).

### 2.5.5 Precipitation

Precipitation takes place by either metabolism-dependant or independent processes. The metal removal from solution in the former case is often associated with active defence system of microorganisms. Microbial cells produce some compounds, such as phosphate and sulphides, in response to the presence of toxic metals which favour the precipitation of metals. Consequently, soluble metals (Me$^{2+}$) are transformed to insoluble hydroxides, carbonates, phosphates and sulphides (reactions 1- 4 shown below). These reactions may take place simultaneously or sequentially in a biological process. The relative importance of the reactions depend on the microbial culture composition and environmental conditions such as dissolved oxygen, and the presence of alternative electron acceptors such as sulphates.

1) Me$^{2+}$ + 2OH$^-$ → Me (OH)$_2$
2) Me$^{2+}$ + HCO$_3^-$ → Me CO$_3$ + H$^+$
3) 3Me$^{2+}$ + 2HPO$_4^{2-}$ → Me$_3$(PO$_4$)$_2$ + 2H$^+$
4) Me$^{2+}$ + HS$^-$ → MeS + H$^+$

Bio-precipitation can also result from the ability of microorganisms to alter the pH or alkalinity of their micro-environment. The cells alter their microenvironment due to their normal or induced metabolic activity, resulting in micro-precipitation of metal ions (Tsezos, 2007; Gad 2004).
Immobilisation of metal ions resulting from the various biosorption mechanisms can occur simultaneously (Tsezos, 2007; Gadd, 2004). However, the metal-cell interactions also involve processes that lead to mobilisation of metals/metalloids. Mobilisation mechanisms play an important role in biotreatment/bioremediation processes. The dominant mechanisms are explained below.

2.5.6 Chelation
The other metal mobilisation mechanism of microbes is chelation which is conducted by microbial metabolites and siderophores. Fe is an essential element for the growth of microorganisms. To obtain the required Fe, some microbes excrete siderophores, which are low molecular weight ligands to aid iron assimilation from precipitates (Gadd, 2001). In fact, this ability of microbes can be useful in biotreatment as the siderophores are also able to bind other metals such as Mg, Mn, Cr (III), Ga (III) and radionuclides such as Pt (IV) and decrease their bioavailability. The chelated metals are adsorbed to the biomass and/or precipitated (Gadd, 2010, Gadd, 2004).

2.6 Metal mobilisation processes
The main mechanisms responsible for the mobilisation of elements by microbial cells in particular indigenous mining microorganisms include: autotrophic and heterotrophic leaching; chelation by microbial metabolites and siderophores; redox transformations; and methylation, which are defined as follows (Gadd, 2010; Gadd, 2004):

2.6.1 Leaching process
Microorganisms are able to acidify their environment which results from proton (H+) efflux through the plasma membrane H+-ATPases, charge balance maintenance, and accumulation of respiratory carbon dioxide. Acidification occurs when there is competition between hydrogen and metal ions in a metal-anion complex or in a sorbed form, resulting in the release of free metal cations (Gadd, 1999; Gadd and Sayer, 2000). For example, a strain of *Penicillium simplicissimum*
was used to leach Zn from insoluble ZnO contained in industrial filter dust (Gadd, 2010; Gadd, 2004).

2.6.2 Oxidation and reduction
The immobilisation and mobilisation of elements is also carried out by some bacteria through reduction and oxidation processes which is named redox transformations. Anaerobic bacteria increase the solubility of elements by reduction such as Fe (III) to Fe (II) and Mn (IV) to Mn (II) (Gadd, 2004; McLean et al., 2002). Although this function depends on the elemental property and the result is opposite for some elements such as U (VI) to U (IV) and Cr (VI) to Cr (III) whose reduction leads to their immobilisation (Smith and Gadd, 2000).

2.6.3 Methylation
Methylation of some elements include Hg, As, Se, Sn, Te and Pb is known as the other mechanism affecting elemental solubility which is mediated by a range of bacteria and fungi under aerobic and anaerobic conditions. A number of different metal/metalloids may transform enzymatically by methyl groups of microbial cells. The formation of methylated metal compounds by these processes differs in their solubility, volatility and toxicity. For example, methylated species of Se, (CH$_3$)$_2$Se and (CH$_3$)$_2$Se$_2$, are volatile and are often lost from the substrates (Gadd, 2010; Gadd, 1993). The bioremediation of contaminated land and water was successfully achieved using microbial methylation of Se at Kesterson Reservoir, California, reducing the selenium concentrations to acceptable levels via volatilisation (Gadd, 2010; Gadd 2004).

Microorganisms in AMD affect water chemistry through the aforementioned metal-microbial interactions that lead to reduce or increase the concentration of metal/metalloids. However, the effect on water quality, over a prolonged period has not been adequately investigated. To design a biotreatment system for reducing the metal/metalloid content of AMD, it is required to understand the change in elemental concentrations over time.
2.7 Immobilised microbial biofilm for AMD treatment

To select metal-resistant species for AMD treatment, instead of depending on the isolation of a single species, a better approach could be achieved by designing a consortium of metal-resistant and biosorptive potential of microorganisms (Gadd, 2010). A multi-species consortium can withstand a wider range of extreme conditions encountered with AMD, such as low pH and toxic elements. A good example of a multi-species consortium can be found in a biofilm community. A biofilm is an assemblage of single or multiple species of bacteria, fungi and algae that are attached to an abiotic or biotic surface by their secreted extra-cellular polymeric substances (EPS) (Ahluwalia and Goyal, 2007; Mehta and Gaur, 2005). The rich exopolymer content of the biofilms is beneficial for both biosorption of dissolved metals and entrapping dispersed solids (Malik, 2004). Additionally, biofilm formation facilitates the microbial immobilisation which is one of the objectives of this research. Biofilm formation processes, indigenous AMD biofilms and the importance of biofilm communities for AMD treatment are described in the next sections.

2.8 Biofilm formation and its significance in water treatment

More than 99% of microorganisms existing on Earth are biofilms in almost every environment that is moist with adequate nutrient flow (Singh et al., 2006). Biofilm formation is a natural complex process where clusters of microbial cells attach to a rough surface and stick together by secreted EPS.

A biofilm formation process is depicted in Fig. 2.1. The life of a biofilm initiates from planktonic or free floating cells. In order to form a biofilm, a planktonic cell must first interact with a surface for attachment. The process starts with the attachment of microbial cells (Fig. 2.1. step 1). After initial association with the surface, a planktonic cell can dissociate from the surface and return to the planktonic state or irreversibly be reattached to the surface. Irreversible attachment involves the production of EPS and a monolayer of cells is produced in this step (Fig. 2.1. step 2). The process continues with cell agglomeration forming a multi-layer cell structure (Fig. 2.1. step 3). A mature biofilm is developed (Fig. 2.1. step 4) and finally the cell detachment step releases some cells that proceed to a new formation cycle (Fig. 2.1. step 5). The thick layer of multi-cellular aggregates are known as biofilm (Singh et al., 2006; Mehta and Gaur, 2005; Qureshi et al., 2005).
EPS plays an important role in binding cells to a surface and protecting them from the surrounding environment. EPS is mainly composed of polysaccharides, proteins, nucleic acids and phospholipids. Carbohydrates and proteins are the pre-requisite of biofilm establishment. EPS protect the cells in the biofilm by providing a diffusive barrier to any toxic compounds that could harm the cells. The other role of EPS is to provide a barrier to contain nutrients for cell growth (Qureshi et al., 2005).

The biofilm structure is composed of microbial cells immobilised in a heterogeneous and very porous matrix with a high amount of water as it comprises 95-97% of the matrix. Water is bound to the capsules of the microbial cells or solvent. The matrix is a complex of secreted cells products (EPS), absorbed nutrients, cell lysis products and even particulate material and detritus from the nearby surrounding environment distributed within the interstitial voids (Bae et al., 2000). The diffusion function within the matrix is related to the water-binding capacity and mobility of the biofilm (Van Hullebusch et al., 2003).
Water and nutrient diffusion into the interior of a biofilm is highly limited. Once a mature biofilm is developed, water channels facilitate the deeper conveyance of water and nutrients into the biofilm. The architecture of the biofilm develops in response to shear forces. In low shear environments, the biofilm forms thick layers as mushroom-like masses. Under high shear stress environment, the biofilm is flatter or form long strands (Singh et al., 2006). However, high shear forces lead to biofilm detachment. Additionally, nutrient starvation is another reason for biofilm detachment (Qureshi et al., 2005).

Microorganisms that secrete polymers and form biofilms gain high microbial biomass. The high density of this microbial biomass provides and maintains optimal conditions for growth. The optimal conditions include pH, localised solute concentrations and reductive oxidative potential (REDOX) potential in the vicinity of the cells. This is achieved by the unique architecture of the biofilm and controlled circulation of fluids within it. Additionally, high microbial biomass facilitates immobilisation of minerals, nutrient and metals from the surrounding liquid phase (Singh et al., 2006; Van Hullebusch et al., 2003).

Spath et al., (1998) investigated the role of EPS in binding aqueous metal species from wastewater. He evaluated the sorption of Cd and Zn to the EPS and cell components of a biofilm collected from a sequenced batch biofilm reactor. Although EPS has a high density of charged functional groups, 80% of the total Cd content was sorbed to the cellular component of the biofilm. A laboratory study also investigated EPS production and metal adsorption by Spirulina sp. A steady increasing amount of Cu removal was observed along with an increased level of EPS production (Das et al., 2009).

2.9 Indigenous algal-microbial biofilm in AMD

Indigenous AMD microorganisms thrive as extensive biofilms along the acidic drainages, attached to a substrate (Aguilera et al., 2010; Aguilera et al., 2007; Aguilera et al., 2006 a, b; Levings et al., 2005). Biofilm development was studied by Aguilera et al. (2010, 2007, 2006 b) in an extremely acidic river, Rio Tinto in Spain. The biofilm was mainly composed of the autotrophic species of flagellated or filamentous green micro-algae (60%), chemo-litho-trophic bacteria and heterotrophic microbes such as bacteria, fungi, amoebae, small flagellates and ciliates. Coexistence and synergistic relationships among the multi-species of the indigenous
AMD biofilm protected them from environmental stresses and enabled them to survive under the extreme conditions (Aguilera et al., 2010; Das et al., 2009 a,b; Levings et al., 2005; Amaral-Zettler et al., 2002, Niyogi et al., 2002).

Green micro-algae play a significant role among the extremophilic microbes which participate in AMD biofilm. The bright green colour of the algal mat denotes the presence of chlorophyll. The evidence of their photosynthesis activity is the presence of oxygen bubbles at the water-air interface (España et al., 2007). The photo-synthetically produced oxygen enhances the functions of iron oxidising bacteria. Micro-algae provide organic carbon for heterotrophic microorganisms through photosynthesis and they are the basis of food chains in AMD (Amaral Zettler et al., 2002). These extremophilic micro-algae have adapted to grow under low nutrients levels in AMD. Mining wastewaters including AMD are considered to be limiting in NO$_3$ and PO$_4$ which are the major nutrient requirements for algae (Dunbabin and Bowmer 1992). NO$_3$ is mainly derived from explosive materials which used for blasting operations and phosphorous is resulted from slow weathering and erosion of the surrounding rocks (Lottermoser, 2010; Thomson and Tracey 2005). The autotrophic property of algae influences AMD waters as they assimilate nitrate and inorganic carbon from the AMD environment. This occurrence raises the alkalinity nature of water, therefore directly decreasing the acidity of water (Das et al., 2009b).

The algal-biofilms colonise acidic drainages released from waste piles and tailings at mine sites. The biofilms cover almost 100% of the stream substrate from the discharge point up to several meters along the stream. The development of the concentrated biofilm within this distance is due to availability of Fe ion. At the release point of AMD, the oxygen content is depleted due to the respiration of heterotrophic bacteria which oxidise pyrite (FeS$_2$), indicating that Fe is available in AMD in its ionic form. Fe is an essential element for growth of micro-algae (España et al., 2007). Additionally under the low pH (~3) conditions found in AMD, higher organisms (e.g. grazers) are not present and micro-algae can dominate the biofilm.

Micro-algae have also the ability to produce EPS, especially under nutrient stress conditions. EPS participates in biofilm formation and metal removal (Sutherland, 2005). Aguilera et al. (2008) analysed the composition of EPS extracted from 12 biofilms, isolated from the Rio Tinto River, Spain. The results showed the heavy metal content of the biofilm closely resembled the water composition (Das et al., 2009).
Indigenous AMD biofilms dominated with micro-algae are efficient biosorbents for the primary biotreatment of AMDs with low pH and high Fe$^{2+}$ ion composition.

2.10 AMD treatment using biofilm reactors

Novel biotreatment technologies for industrial wastewater treatment including AMD are based on the establishment and maintenance of specific active biofilms on an appropriate solid support medium. The key advantages of using biofilm treatment systems include (Tsezos et al., 2007):

1) The natural attachment of biofilms creates high densities of microorganisms per unit volume of reactor which directly improve the treatment performance in small reactor volumes, which is economically advantageous.

2) The immobilised microorganisms in biofilm structures minimise the requirement for sedimentation after treatment process.

3) The immobilised biofilms survive and withstand concentrated AMD discharges of toxic wastes due to the protective nature of EPS in biofilms.

4) Once a biofilm is established, the microbes are reinforced and protected by the matrix, so the elimination of biofilm is very difficult. Therefore, a sustainable system can be designed which requires minimal maintenance and dosing of nutrients.

The significant advantage of immobilised microorganisms in a biofilm structure has led to the development of biofilm reactors in bio treatment industry for metal bearing wastewaters such as AMD. Biofilm reactors facilitate an effective contact between biofilms and wastewaters (Tsezos et al., 2007; Ahalya et al., 2003).

During the late 1980s and 1990s novel biofilm reactor systems were developed and applied (Tsezos et al., 2007). Systems such as small-granule fixed-beds, fluidised beds, rotating biological contactors, trickling filters, and hybrid suspended biofilm which support development and maintenance of biofilms were developed. The relatively recent pilot and industrial biofilm
reactors used in mining and metallurgical wastewater polishing treatment for sequestering various targeted pollutants including toxic metal/metalloids are moving bed sand filter reactor (Diels et al., 2003), anaerobic packed bed reactors (Jong and Parry, 2003) and rotating biological contactors (Costley and Wallis, 2001). The configuration and operation process method of these reactors are described in the following sections.

2.10.1 Moving bed sand filter reactors

In moving bed sand filters, sand granules act as supporting carrier material for biomass immobilisation. The microbial biofilm that forms on sand grains interacts with the soluble targeted metal species in wastewater. The key characteristic of this reactor is that the sand is continuously circulated and washed. The excess of produced biomass with the sequestered metal ions is also continuously removed. Biofilm growth is supported by the addition of a selected carbon sources such as acetate. Acetate is metabolised by microbial biomass and increases alkalinity which alters the chemical microenvironment of the biofilm by increasing the pH (Tsezos et al., 2007).

The application of moving bed sand filters for development of metal reducing biofilms was tested by Diels et al. (2003). A moving bed sand filter with a diameter of 3 m, a filter bed height of 4 m and a filter bed volume of 12 m$^3$ was used which was filled with quartz sand with a grain-size diameter between 1.2 and 2.0 mm. The applied filtration velocity and feed flow was between 6 and 7 m/h, and 20 and 45 m$^3$/h, respectively. The sand circulation speed of 0.5 - 1.0 cm/min and wash water flow of 3-5 m$^3$/h was used. The contact time to empty the reactor was about 36 min. The inoculum was a consortium composed of resistant, metal biosorbing/bioprecipitating bacteria including Pseudomonas mendocina AS302, Arthrobacter spp. BP7/26 and Ralstonia eutropha CH34. The used wastewater for treatment contained mainly Co (1.5 mg/L), Ni (0.7 mg/L), Zn (0.2 mg /L) and Cu (0.1 mg /L). Nitrate (3.4 mg/L) and carbon substrate (8 mg /L) was supplied to the system as nutrients to maintain biofilm growth. The reactor was operated for 18 months continuously without loss of activity. Ni, Co, Zn and Cu were removed between 80% and 100%. Fe removal was 60-80% and for other metals Al, Ag, Cr, As and Se, the removal was up to 80%. The bio-sludge analysis revealed the concentration of heavy metals up to 10% of the dry weight. The results showed the reliable technology of sand filter reactor for metals removal from a low concentration solution by specific resistant microbial strains (Diels et al., 2003). However in the
reported study, few metals were included in the wastewater composition at low concentrations, whereas AMD contains many of these elements at more than 100 mg/L. Additionally, the acidic nature of AMD with high amounts of sulphate that limit bio-removal efficiency were not considered.

2.10.2 Anaerobic biofilm reactors using sulphate reducing bacteria

Anaerobic biofilm reactors by the exploitation of sulphate reducing bacteria (SRB) are the most common biofilm reactors in mining. These reactors are used to produce biogenic hydrogen sulphide under anaerobic conditions. Reactor configurations such as packed or fluidised beds have been tested for pilot or commercial applications (Kaksonen et al., 2007). The wastewater is fed into the bioreactor where the SRB biomass is grown and metal precipitation occurs simultaneously in the cells microenvironment. It is also possible to collect the produced H$_2$S to use subsequently for the precipitation of the metal ions in the wastewater as metal sulphides. This technology can be applied in mining and metallurgical industry for treatment of AMD and metal/sulphate bearing wastewaters (Tsezos et al., 2007).

Jong and Parry (2003) used a bench-scale up flow anaerobic packed bed reactor for removing sulphate and heavy metals by SRB in short-term runs. Contaminated water with Cu, Zn, Ni, Fe, Al, As (5-50 mg/L), Mg (>500 mg/L) and sulphate (~2500mg/L) was treated, over a 14 day period at 25°C. The reactor was filled with silica sand and SRB was employed as inoculum. Organic substrate and sulphate were added to the reactor at loading rates of 7.43 and 3.71 kg d$^{-1}$ m$^{-3}$, respectively. The results showed that more than 97.5% of the initial concentrations of Cu, Zn and Ni was removed, whilst only > 77.5% and > 82% of As and Fe were removed, respectively. Additionally, >82% reduction in sulphate concentration was recorded. In contrast, the concentration of Mg and Al remained unchanged during the treatment period. The pH of water was increased from ~ 4.5 to 7.0 due to the SRB activity. Additionally, the removal of sulphate and metals was enhanced in comparison to controls not inoculated with SRB (Jong and Parry, 2003). The results of the aforementioned study highlighted the potential of SRB bacteria for removing various elements. However, some elements such as Mg and Al were not removed. Additionally, SRB bacteria are heterotrophic and nutrient provision is required for their growth (Ahalya et al., 2003).
2.10.3 Rotating Biological Contactor (RBC)

The RBC facilitates the immobilisation of microorganisms as a fixed biofilm, attached to the moving support media (Ibrahim et al., 2012; Rodgers and Zhan, 2003). An RBC unit typically consists of a series of closely spaced flat or corrugated discs that are mounted on a horizontal shaft. Generally, the discs are partly submerged (40%) in the solution. The shaft continually rotates by a mechanical motor or a compressed air drive. The media rotation promotes oxygen transfer and maintains the biomass in aerobic conditions. The rotation of the discs creates shear forces that enhance the stripping off of the excess biomass (Cortez et al., 2008; Patwardhan, 2003; Rodgers and Zhan, 2003). In the case of using mesh material for discs, the rotation and shear stress maintains a constant population of microorganism and conserves the media from clogging. Several units can be constructed as series or parallel arrangements to improve the treatment results (Ibrahim et al., 2012; Cortez et al., 2008; Patwardhan, 2003).

From the most commonly used type of biofilm reactors for AMD treatment, a RBC was selected for the biotreatment investigations reported in this research. The significant advantages of RBCs and the design parameters are described in the next section. Additionally, a few case studies indicating removal of metal/metalloids by microbial biofilms in RBCs are presented in this chapter.

2.11 significant advantages of RBC and design parameters

Rotating biological contactors (RBCs) are one of the most efficient bioreactors that are commonly used to treat municipal and industrial wastewater (Ibrahim et al., 2012; Mathure and Patwardhan, 2005). Easy construction and expansion, simple process control and monitoring, simple and feasible design and operation, high interfacial areas are generated by the discs that is independent of the rotation speed, resistance to shock and toxic loads, low land occupancy, low energy consumption and low cost of operation and maintenance are the principal advantages of using RBCs. The efficiency of RBCs depend on several design parameters that are listed in Table 2.1 (Ibrahim et al., 2012; Cortez et al., 2008, Mathure and Patwardhan, 2005, Kargi and Eker, 2001; Costely and Wallis 2000).
Table 2.1 RBC design and operation parameters

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effect</th>
<th>Optimised parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotational speed</td>
<td>Important in biofilm growth, metal removal efficiency and nutrient and oxygen mass transfer</td>
<td>1-10 rpm for 1–4 m diameter mounted on shafts around 5–10 m long</td>
</tr>
<tr>
<td>Hydraulic flow</td>
<td>Increasing flow rates decrease removal efficiency</td>
<td>The range in full-scale size: 1.292–6.833 dm³/m²h</td>
</tr>
<tr>
<td>Hydraulic retention time (HRT)</td>
<td>Longer contact times improve removal efficiency</td>
<td>24 hour</td>
</tr>
<tr>
<td>RBC media</td>
<td>More surface area is favourable (Corrugated and cellular mesh), considering costs</td>
<td>PVC, polycarbonate sheets, High Density Polyethylene (HDPE)</td>
</tr>
<tr>
<td>Staging</td>
<td>Multi staging increase efficiency</td>
<td>Using baffles in a tank or using a series of tanks</td>
</tr>
<tr>
<td>Temperature</td>
<td>Directly affects the microbial activity</td>
<td>Optimised temperature is between 20 to30 °C</td>
</tr>
<tr>
<td>RBC medium submergence</td>
<td>Increased submersion increases the volume capacity and reduces RBC staging requirement, Depends on operation type, microorganisms and characteristics of wastewater</td>
<td>40-60%</td>
</tr>
</tbody>
</table>

From the point of configuration, RBCs provide flat support media for the growth of indigenous AMD algal-microbial biofilms which are used as the biosorbent in this study. The discs’ surfaces can resemble AMD substrate surface for the attachment and growth of algal-microbial biofilms, and the rotational speed is adjustable to protect the biofilm from detachment. Additionally, the rotation of discs allows the biofilm to be exposed to a light source, which can be installed on top of the reactor or side area, or exposed to sunlight. The rotational speed is easily adjustable to an optimised speed for maximum metal uptake and biofilm growth. The partial submersion and rotation of discs of the RBC provide enough air around the layer of biofilm which enhance the aerobic microbial performance in biofilm structure. The biofilm in RBCs aid the accumulation of metal/metalloids in a low volume of biosorbent which can be recovered in low volumes of water.
In the case of biofilm detachment, it can regrow due to presence of an initial layer and the resistant nature of the biofilm that provides a viable biosorbent in RBCs.

2.12 RBC application for removing metal/metalloids from wastewaters

A series of studies was carried out by Costley and Wallis (1999, 2000, 2001) using a RBC for removing Cu, Zn and Cd from a synthetic wastewater. They used a laboratory scale RBC, inoculated with the enriched culture of sewage-activated sludge for biofilm development. A nutrient broth was formulated and used to supply carbon whilst minimising the possibility for metal complexation. The RBC contained 10 L wastewater and the discs were submersed to a level of 40%. The rotation speed of disc was adjusted at 10 rpm (revolution/min) and the flow rate was set to 6.9 ml/min. Both parameters were already optimised for achieving maximum biofilm growth and metal removal. The RBC was operated continuously for multiple sorption (84 days) and desorption (48 h) cycles with a HRT of 24 h. After the operation period (84 days) the average metal removal for Cu, Zn and Cd achieved up to 81.8%, 49.7% and 30.1%, respectively. The results were relatively constant during the operation (Costley and Wallis, 2001).

In another example, Travieso et al., (2002) used a rotary drum which was covered by 0.5 mm wide polyurethane bands for immobilisation of a micro-algal biofilm. The pure culture of micro-alga *Scenedesmus obliquus* was used as an inoculum which was obtained through serials of dilution and antibiotic treatment. The reactor was operated with a synthetic wastewater containing 140 ml of municipal sewage and 0.1 g/l of cobalt salt (CoSO$_4$. 7H$_2$O) which gave a final concentration of 3000µg/l and pH ranging between 8.6 and 8.9. The reactor was operated in a 20-day batch mode at a constant rotational speed of 2 rpm. The authors reported 94.5% cobalt removal after 10 days. In the aforementioned research, algae were successfully immobilised on the rotary system and the result was promising for Co removal which is a typical toxic element in wastewaters such as AMD.

Kapoor et al. (2004) studied the biological oxidation of Fe$^{2+}$ under acidic conditions by using oxidising bacteria in a bench-scale RBC. The RBC feed wastewater was composed of FeSO$_4$. 7H$_2$O at 0.08 g/L and MgSO$_4$.7H$_2$O at 0.04 g/L concentration. (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ were used at concentrations of 0.08 g/L and 0.1g/L to supply nutrients. Potable water was used for making up the solution and concentrated sulphuric acid was added to bring the pH to between 1.9-2.0. The RBC was able to achieve 50% Fe$^{2+}$ oxidation efficiency after 24 h operation.
The examples described above used RBCs for removing metals/metalloids from metal bearing synthetic wastewaters, not particularly AMD. The applications of RBC for AMD treatment has been mostly focused on Fe\textsuperscript{2+} removal which is one of the major environmental issues related to AMD. For example, Olem and Unz (1977, 1980), who possibly were the pioneers for AMD treatment by RBCs used a pilot-scale RBC to evaluate ferrous iron oxidation. The actual AMD (pH ~ 2.7), which was released from a coal mine, were introduced to the RBC. The indigenous iron oxidising bacteria developed a biofilm in the RBC that mediated the transformation of Fe\textsuperscript{2+} to the less soluble ferric state, Fe\textsuperscript{3+}. The RBC was operated for 11 months at the optimum operating speed of 10 rpm. The hydraulic loadings of 0.11 and 0.22 m\textsuperscript{3}/ day m\textsuperscript{2} resulted in the oxidation of 240 mg/L influent Fe\textsuperscript{2+} to produce effluent Fe\textsuperscript{2+} of 2 and 5 mg/L, respectively. The results indicated the applicability and efficiency of the system to be used for primary treatment of AMDs with low pH (~3) and high Fe\textsuperscript{2+} ions. However, the applicability of RBC for removing other metals/metalloids was not assessed in the reported study.

The aforementioned studies (see sections 2-10-1 to 2-12), reported on the application of sand bed filters and anaerobic reactors; and RBCs, indicated the significant potential of microbial biofilms, mainly from bacteria, developed in the biofilm reactors for removing metal/metalloids from contaminated wastewaters. However, there are significant gaps which limit the exploitation of their results as an applicable biotreatment system on mine sites which include:

- Exploitation of bacterial biofilms requires regular and appropriate nutrient dosing for their growth which add to costs and maintenance requirements and limit the feasibility of their usage. Costley and Wallis (2001) stated that biotechnological approaches for metal removal from low metal contaminated voluminous wastewaters can be economically viable only if cheap carbon and nutrient sources can be provided. In the reported study, only the case studies conducted by Olem and Unz (1977, 1980) did not require nutrient supply as the iron oxidising bacteria used the released energy from the oxidation process of iron and captured carbon dioxide from air.

- The majority of biotreatment investigations have reported metal removal efficiency using synthetic AMD or synthetic metal bearing solutions, composed of only a few specific heavy metals. The multi-ion composition of AMD composed of various metal/metalloids at high or low concentrations, high sulphate concentration and low pH, which can adversely affect the
biotreatment results, were not considered adequately. The application of the biotreatment systems with real AMD resulted in low removal efficiency (Gadd, 2012; Gadd, 2009; Ahalya et al., 2003).

- Many biotreatment studies were conducted in batch systems and do not show the metal removal trends in continuous process. Furthermore, the reported results emphasise removal efficiency and do not describe the negative results or desorption stages adequately.

- The cleansing role of indigenous AMD microorganisms in particular micro-algae and fungi has not been taken into account for the biotreatment systems.

2.13 Conclusion

Water contamination is one of the most important environmental issues resulting from mine sites and mining activates. AMD stands for the acidic and metal contaminated mine water which must be treated before discharging to the environment. An efficient treatment system must be designed to remove the contaminating elements, reduce iron precipitations and increase pH. Conventional treatment methods (as presented in Chapter 1) are not efficient for removing some elements particularly at low concentrations and can be expensive. Biotreatment, using live cells, can be a solution to AMD pollution. However, biotreatment investigations in the mining industry have remained limited to applications of SRB bacteria. Prior to design any alternative and novel biotreatment system for AMD waters, selecting multi-species of resistant microbes is a primary pre-requisite to withstand the harsh AMD conditions. The extremophilic indigenous AMD microorganisms, particularly phototrophic eukaryotes, were found to be efficient biosorbents for AMD treatment and their metal removal potential has revived interests as demonstrated by recent studies. These microbes thrive in AMD resources as attached biofilms to drainages substrates. The presence of autotrophic (green microalgae), lithotrophic (bacteria) and heterotrophic (bacteria and fungi) microbial species in AMD biofilms, has modified a low nutrient dependence system, which relies on the low concentrations of available nitrate and phosphate in AMD waters. The indigenous AMD microbes are adapted to the acidic and contaminated waters, whilst they are able to remove and accumulate many metals actively and passively, through different biochemical and biological mechanisms. To immobilise the algal-microbial consortium as biofilm and achieve efficient treatment, a suitable configuration of photo-bioreactors is required.
From the most commonly used types of biofilm reactors for AMD treatment, the RBC was selected for the development of indigenous AMD algal-microbial biofilms in this research. The subsequent chapters are described:

- Chapter 3 describes the general materials and methods which were undertaken to conduct the investigations. This chapter illustrates the field (in-vivo) observations and studies.

- Chapter 4 contains the results from the in-vivo measurements and presents the materials and procedures for synthesising a complex AMD and the investigation on the applicability of the synthetic AMD for the maintenance of indigenous microbe’s during in vitro investigations. This chapter was published in the Journal of Environmental Science and Pollution Research.

- Chapter 5 presents the design and application of a photo-rotating biological contactor (PRBC) for treatment investigation. Additionally, biofilm establishment and development in the PRBC are presented associated with some of the treatment results obtained during continuous PRBC operation. This chapter was published in the Journal of Industrial Microbiology and Biotechnology.

- Chapter 6 presents the microbial diversity in the collected indigenous AMD algal-microbial consortium samples from field (in vivo) and algal-microbial biofilm in the PRBC (in vitro). The work has been submitted to the Journal of Applied and Environmental Microbiology.

- Chapter 7 represents the biotreatment investigation in a batch process. This chapter was published in the Journal of Applied Microbiology and Biotechnology.

- Chapter 8 discusses the biotreatment investigations for the continuous process. This chapter has been submitted to the Journal of Environment International.

- Chapter 9 presents a general discussion and concludes the research reported in the thesis.
2.14 References


Costley, S.C. and Wallis, F.M. (2000) Effect of flow rate on heavy metal accumulation by rotating biological contactor (RBC) biofilms. Journal of Industrial Microbiology and


CHAPTER THREE
CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

The material and methods used in this research is divided into two sections: 1) in vivo studies and 2) in-vitro studies as follows:

- In-vivo studies: Sample collection from AMD and indigenous AMD microorganisms at Sarcheshmeh copper mine.

- In-vitro studies: elemental and nutrient analysis of AMD, assessment of biofilm formation from the indigenous microbes, preparation of synthetic AMD, algal-microbial culture and maintenance in-vitro, PRBC design and set-up, biofilm development in a PRBC, microbial identification studies on the indigenous microorganisms and biofilm samples, batch treatment study, and continuous treatment study. The in-vitro studies were conducted in the micro-algae laboratory of the University of Adelaide.

Field information from Sarcheshmeh copper mine, AMD resources and indigenous AMD biofilm locations are presented in this chapter to show the connections between the in-vivo and in-vitro studies. General introductions for the in-vitro studies are also presented in this chapter.

3.1.1 Site study- Sarcheshmeh copper mine

Sarcheshmeh copper mine is the largest porphyry Cu-Mo deposit in Iran and it is also included in the list of giant porphyry copper deposits in the world (Khorasanipour et al., 2011; Sillitoe, 2010; Cook et al., 2005). The mine is located in 160 km west of Kerman province, 55 km southwest of Rafsanjan city, south-eastern Iran (29°56′40″N and 55°52′20″E). Sarcheshmeh is placed in the Central Iran Volcanic Belt, which is principally composed of a folded volcano-sedimentary complex (Fig. 3.1) (Atapour and Aftabi, 2007). The ore deposit contains approximately 1,200 million tonnes of ore with an average of 1.2 % Cu, 0.03 % Mo, 3.9 g/t (3.9x10^{-4} %) Ag and 0.11
g/t Au (0.11x10^{-4} \%) (Aftabi and Atapour 2011; Khorasanipour et al., 2011, Shahabpour and Doorandish, 2008).

Sarcheshmeh is located in a semi-arid area with annual temperature between -20 and 32°C, mean rainfall of 440 mm, and annual evaporation of about 1,170 mm (Khorasanipour et al., 2011). The catchment area of Sarcheshmeh is about 21 square kilometres and it is known as one of the rainiest regions in Kerman (Sahraei Parizi et al., 2005). However, the mine is located only 50 km from the Rafsanjan basin that is bordered by the Lut Desert in southeast Iran where the average annual rainfall is only 95mm (Razavi, 1991). The scarce resources of water in this area signify the importance of the Sarcheshmeh water resource. Mining activities in Sarcheshmeh has adversely affected the quality of surface waters by oxidation of sulphide minerals and AMD production. Regardless of the mining activity, the natural weathering and of oxidation of sulphide minerals in this area has affected the water quality (Shahabpour and Doorandish, 2008; Orandi, 2006).
Fig. 3.1 a) World distribution of giant porphyry copper deposits, included Sarcheshmeh copper mine (adapted from Sillitoe, 2010), b) Location of Sarcheshmeh in Iran (adapted from Khorasanipour et al. (2011))
3.1.2 AMD resources at Sarcheshmeh copper mine

Currently, Sarcheshmeh is it the largest open-pit mine in Iran. Majority of porphyry Cu deposits are mined by open-pit methods and, less commonly by underground methods (Berger et al., 2008). The excavation of open mines produces a huge amount of overburden and low grade waste rocks that has been piled around mine sites. The volume of waste rocks will depend on the depth and geometry of the deposit. On average, about 1.5 tons of waste rocks and overburden must be removed for every ton of ore grade mined in porphyry Cu deposits (Khorasanipour et al., 2011).

Open-pit mining in Sarcheshmeh has created a deep oval cavity with considerable height difference. This topography directs surface water runoff toward the mine site. To control flooding, the valleys surrounding the open pit were filled with waste rocks (Fig. 3.2). However, this remedy has created another serious environmental issue which is AMD formation. Waste rock dumps are one of the major AMD resources in Sarcheshmeh that has been investigated and documented widely (Shahabpour and Doorandish, 2008; Orandi, 2006; Sahraei Parizi et al., 2005). The waste rocks which are rich in pyrite, are gradually drained and release the degraded water quality drainages, highly acidic and contaminated with many heavy metals. A previous investigation on the effect of waste rock dumps on AMD production at Sarcheshmeh copper mine revealed the most contaminated sources of AMDs resulted from waste rock dumps draining. The water compositions of two main AMD resources were considered in my PhD research. The water pathway of surface waters which pass through dumps and emerge as the mentioned AMD resources are highlighted on map with the blue lines and red triangles, respectively (Fig. 3.2).
Fig. 3.2) Topographic map of Sarcheshmeh copper mine showing the open-pit, waste rock dumps and releasing points of the most contaminated AMDs

One of the main sources of surface water, Seridun River, originates from the eastern heights of the open pit and drain the huge dumps No. 26 and 11. The resulted drainage from dump No. 11 creates the most contaminated AMD in the open-pit area. The other source of AMD results from dump No 31 at the northern east of the mine pit. The AMDs from these two points are running permanently in depend of dry and rainy seasons. The AMDs from these resources, in particular from dump No. 11, are highly acidic (pH~3) and contaminated with heavy metals and metalloids included As, Co, Ni, Cr, Cu, Pb (Khorasanipour et al., 2011; Shahabpour and Doorandish, 2008, Orandi, 2007). The electron conductivity (EC) varies between 1400 to 2500 µS/cm in this AMD (Orandi, 2007).

The produced AMDs from the open-pit and waste rocks are diverted into a trench, referred to as the Total Mine Outlet (Fig. 3.2). This water passes through a lime pond to increase the pH from 2-3 to 5. The AMDs and mine wastewaters are eventually diverting to the settling and evaporation ponds.
3.1.3 Indigenous algal-microbial biofilm in AMD at Sarcheshmeh

The presence and distribution of filamentous green micro-algae, *Ulothrix gigas*, was first reported by Orandi et al. (2009, 2007), as the indicator of AMD formation at Sarcheshmeh copper mine. The green micro-algal mats were found in the acidic drainages of dump 11 and 31, independent of rainy and dry seasons. Fig. 3.3 shows the emerged AMDs from dump 11 and 31, filled with the algal-mat that were attached to the sandy substrate and long chains of filamentous micro-algae were floating submerged in the AMD. The micro-algae were covered by the bluish copper precipitates during the dry season, when the pH of these AMD increased up to ~ 5 (Orandi, 2007). Air bubbles were observed in the algal-mat where micro-algae thrived near the surface AMD, indicating active photosynthesis (Fig 3.4). These observations were the basis of this PhD research to exploit indigenous algal-microbial biofilm for AMD treatment.
Fig. 3.3 Filamentous micro-algae in released AMDs from dumps 11 and 26, in wet season (above), and dry season (below) covered by bluish copper precipitates
Fig. 3.4 Bright green filamentous micro-algae in AMD resource from dump 31 at Sarcheshmeh copper mine, arrows show the air bubbles resulting from micro-algae photosynthetic activity

### 3.2 In-vivo studies

#### 3.2.1 AMD sampling and preservation

To evaluate the physical and chemical properties of AMD, samples were collected from the most contaminated AMDs emerging from dumps 11 and 31, over a year. Sampling, preservation and analysis methodology are presented in Chapter 4.

#### 3.2.2 Microbial sampling

The inoculum for microbial study and biotreatment investigations in this study was collected from the algal-microbial consortium in dump 11. The sampling and preservation methodology is presented in Chapter 4.
3.3 *In-vitro* Studies

3.3.1 AMD analysis and Synthesis

The collected AMD samples from the field were analysed to quantify the concentration of their anions ($\text{Cl}^-$, $\text{NO}_3^-$, $\text{NO}_2^-$, $\text{PO}_4^{3-}$, $\text{SO}_4^{2-}$, $\text{CO}_3^{2-}$ and $\text{HCO}_3^-$), major cations ($\text{Na}^+$, $\text{K}^+$, $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$) and trace metals ($\text{Ag}^+$, $\text{Cu}^{2+}$, $\text{Mn}^{2+}$, $\text{Zn}^{2+}$, $\text{Ni}^{2+}$,$\text{Co}^{2+}$, $\text{Mo}^{2+}$, $\text{Pb}^{2+}$, $\text{Fe}^{2+}$, $\text{Cd}^{2+}$, $\text{Se}^{2+}$, $\text{Cr}^{3+}$, $\text{Sb}^{3+}$, $\text{Al}^{3+}$, $\text{Bi}^{3+}$ and $\text{As}^{3+}$). Sample preparation methods for the analysis and associated analytical methodology are presented in Chapter 4.

A multi-ion and acidic AMD was formulated and synthesised based on the AMD analysis. The materials and preparation methodology are presented in Chapter 4. The pH of the synthetic-AMD (Syn-AMD) was adjusted by 3 over the 10-week period of treatment to match the pH of AMD at the Sarcheshmeh copper mine (Orandi *et al.*, 2006 a, b; 2007). Additionally, the low pH favoured micro-algal growth in the AMDs (Orandi *et al.*, 2009).

3.3.2 Algal-microbial culture and maintenance

The collected algal-microbial samples were cultured and maintained in Bold Basal (BB) medium and Syn-AMD. The culture process and results are presented in Chapter 4.

3.3.3 Biofilm development assessment

Prior to the exploitation of the indigenous AMD microorganisms for biofilm development in the PRBC, the ability of these microbes to form biofilms under *in-vitro* condition was assessed. This qualification assessment was carried out by using a turf scrubber system.

For this purpose a paver was placed into a plexiglass container with the dimensions 44x26x28, on a slop with a 30 degree gradient. 10 L synthetic AMD was prepared and poured into the fish tank to reach up to the lower side of the paver. The AMD was pumped over the paver through a diffuser which was made of a propylene pipe with holes 1 cm apart. To maintain a moderate water circulation, a speed controller was used and adjusted at 4-6 amp. A plexiglass lid was used to close the trough and minimize contamination entry to the system. The tank water was aerated through two filtered inlets and vented via two filtered outlets (MILLEX Filters 0.45µm
was used, supplied by MILLIPORE Pty Ltd. Fig. 3.5 shows the schematic picture of the turf scrubber.

Fig. 3.5 Schematic picture of turf scrubber

The turf scrubber was inoculated with the algal-microbial sample. A homogeniser was used to break the algal filaments and homogenise the microbial sample. The prepared inoculum was 1L with 0.2 g/L dry weight. The synthetic AMD in the tank turned green after two weeks; however a green layer of algal-microbial biofilm was observed after 4 weeks. The paver was thoroughly covered by a thick layer of biofilm after 8 weeks (Fig. 3.6). Since the biofilm development process was carried out in a batch system, nutrients (phosphate and nitrate) were provided in excess amount (2x) per week to sustain biofilm growth. It was observed that once the initial attachment occurred, the biofilm formation proceeded and the thickness of biofilm increased considerably.

Two tubular cool white fluorescent lamps (Crompton F18T8/840) were installed above the turf scrubber to distribute light from the top. A light meter was used to measure the light intensity of the lamps, which was 440 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The light was set to a 12:12 h light: dark cycle.

Since in this research a PRBC was used for biofilm development and the discs were made from PVC, a piece of PVC was cut and attached to the paver to ensure the material favoured the algal growth. This preliminary work was conducted to ensure that the microbial sample was able to form biofilms on this terrestrial.
3.3.4 Biofilm development in photo-rotating biological contactor

The algal-microbial sample was used as an inoculum to develop an algal-microbial biofilm in a photo-rotating biological contactor. The reactor configuration, biofilm development process and nutrient uptake results are presented in Chapter 5. The algal-microbial biofilm was established and developed gradually on the discs’ surfaces in the reactor. The development processes are demonstrated in Fig. 3.7.
3.3.5 Microbial studies

The microbial diversity in the inoculum collected from mine site and the developed biofilm in the PRBC was investigated. The material and methods are presented in Chapter 6.

3.3.6 Biotreatment investigations

After biofilm development, the PRBC was drained and washed with mili-Q water as shown in Fig. 3.8 (a). Thereafter fresh Syn-AMD was introduced into the PRBC for biotreatment
investigation in batch and continuous modes (Fig. 3.8 b). The material, methods and results are presented in Chapters 7 and 8.

Fig. 3.8 PRBC preparation for biotreatment investigations, a) draining and washing the PRBC and biofilm prior to the treatment process, b) PRBC operation in batch and continuous modes

3.3.7 Electron Microscopy Analysis

In this thesis, Scanning Electron Microscopy (SEM) microanalysis was used to characterise the microbial structure of algal-microbial biofilm, elucidated in Chapter 5. Additionally, SEM analysis was applied for tracking possible changes or damages in biofilm contents after treatment process.

To chemically characterise the mineral features in the biofilm, the simultaneous application of the Energy-dispersive X-ray spectroscopy (EDS) micro-analytical system with Back-Scattered Electrons (BSE) was used. EDS spectra provide qualitative information and elemental composition, expressed as weight percentage, is semi-quantitative. The information on the species of an ion which contains a particular element is not provided by EDS analysis. For example, detected S can be presented as sulphur, sulphide or sulphate. Therefore, the
interpretation of multi-element EDS spectra is speculative, due to the absence of distinction between the elemental composition of the primary minerals (metavolcanics, sulphides) and the secondary minerals (precipitates) (Van Hullebusch et al., 2003).

In this research, SEM with BSE imaging was also used to screen the metal-rich zones in the biofilm. Electrons are back scattered in metal rich zones, thus lighting up these zones. In contrast, organic matter absorbs the electrons, resulting in black zones in the picture (Van Hullebusch et al., 2003).
3.4 References


STATEMENT OF AUTHORSHIP

Title of paper: Synthesising acid mine drainage to maintain and exploit indigenous mining micro-algae and microbial assemblies for biotreatment investigations


Sanaz Orandi (First Author)
Performed analysis on all samples, interpreted data, wrote manuscript and manuscript evaluation, and acted as corresponding author.

Signed.............. ..................Date.........3.08.12...

David M. Lewis (Co-author)
Supervised development of work, helped in data interpretation and manuscript.

Signed..................................................Date 30/8/12
CHAPTER FIVE
STATEMENT OF AUTHORSHIP

Title of paper: Biofilm establishment and heavy metal removal capacity of an indigenous mining algal-microbial consortium in a photo-rotating biological contactor

Journal: Journal of Industrial Microbiology and Biotechnology (2012).

Sanaz Orandl (First Author)
Performed analysis on all samples, interpreted data, wrote manuscript and manuscript evaluation, and acted as corresponding author.

Signed: .......................................................... Date: 30/8/12

David M. Lewis (Co-author)
Supervised development of work, helped in data interpretation and manuscript.

Signed: .......................................................... Date: 30/8/12

Navid R. Mohelmani (Co-author)
Helped in data interpretation and manuscript.

Signed: .......................................................... Date: 30/8/12

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CHAPTER SIX
STATEMENT OF AUTHORSHIP

Title of paper: Biodiversity of indigenous algal-microbial biofilm in acid mine drainage, from field to laboratory investigations


Sanaz Orandi (First Author)
Performed analysis on all samples, interpreted data, wrote manuscript and manuscript evaluation, and acted as corresponding author.

Signed: .......................... Date: .......................... 3/19/12

Javid Amini (Co-author)
Performed molecular analysis and helped in data interpretation.

Signed: .......................... Date: .......................... 4/1/12

David M. Lewis (Co-author)
Supervised development of work, helped in data interpretation and manuscript.

Signed: .......................... Date: .......................... 3/08/12

Navid R. Mohelmani (Co-author)
Helped in editing manuscript.

Signed: .......................... Date: .......................... 3/08/12
Title of article:

Biodiversity of an indigenous algal-microbial biofilm in acid mine drainage, from field to laboratory investigations

Authors:

Sanaz Orandi¹, Amini Javid², Navid R. Moheimani³, David M. Lewis¹

Affiliation and address of authors:

¹Microalgae Engineering Research Group, School of Chemical Engineering, University of Adelaide, North Terrace Campus, SA, Australia, 5005

²Department of Microbiology, Islamic Azad University of Kerman, Iran.

³Algae R&D Center, School of Biological Sciences & Biotechnology, Murdoch University, Murdoch, WA, Australia 6150

Corresponding Author:

Sanaz Orandi

E-mail: sanaz.orandi@adelaide.edu.au

Tel: +61 8 83033959

Fax: +61 8 8303 4373
Abstract

Acid mine drainage (AMD), generally acidic and contaminated water with heavy metals, limits the biodiversity of aquatic life due to the dominance of extremophilic and resistant strains of eukaryotic and prokaryotic microorganisms. These indigenous microorganisms include green microalgae, bacteria and fungi thrive as biofilms in AMD and contribute significantly in removing metal/metalloids. However, their exploitation and contribution in AMD biotreatment systems have not been evaluated adequately. In this study, the biodiversity of an indigenous AMD biofilm, which was collected from AMD at Sarcheshmeh copper mine (Iran) and inoculated into a photo-rotating biological contactor (PRBC) for AMD treatment, was assessed. Microbial identification was carried out based on species characteristics, colonial micro-morphology and molecular analysis. The results showed the presence of autotrophic and heterotrophic microbes including filamentous and unicellular green microalgae, Klebsomidiun sp. and Chlamydomonas sp.; bacteria, Acidithiobacillus ferroxidans, Leptospirillum ferroxidans, Bacillus sp. and Pseudomonas sp.; fungi, Aspergillus sp. and Penicillium sp.; and yeasts, Rhodotorula sp. in both the collected biofilm from AMD and the PRBC. SEM-EDS analysis revealed the contribution of these microbes, in particular filamentous microalgae, in removing and accumulating heavy metals Cu, Mn, Mg, Co, Fe. The indigenous AMD algal-microbial biofilm provides a resistant biosorbent and has potential for AMD biotreatment.

Keywords Acid mine drainage, Indigenous acidophilic biofilm, Photo-rotating biological contactor, Biotreatment, microalgae
Introduction

Mining wastewater, typically acid mine drainage (AMD) has been listed as one of the most severe types of contaminated surface waters containing heavy metals (e.g. Cu, Zn, Cr, Pb) and toxic metalloids (e.g. As, Cd, Sb) (Lottermoser, 2010). High concentration of these elements and low pH (2-5) of AMD lead to considerable low biodiversity compared with the uncontaminated surface waters (Das et al., 2009a; De la pena and Barreiro, 2009). Low nutrient availability in AMD is a restricting factor for the growth of most aquatic life (Smucker and Vis, 2011; Lottermoser, 2010; Bray et al., 2008). However, extremophilic eukaryotic and prokaryotic microorganisms are commonly found in AMD, include microalgae, bacteria, fungi and yeasts, which are able to survive under extreme conditions. These microorganisms thrive in extensive biofilms in AMD resources, dominated with green microalgae e.g. *Ulothrix*, *Klebsormidium* and *Chlamydomonas* species (Das et al., 2009, a, b; Prasad, 2007; Orandi et al., 2007; Novis and Harding, 2007). The acidophilic microalgalae, in particular bright green mats of filamentous microalgalae, are indicators of AMD with high iron (Fe$^{2+}$) concentration (Valente and Gomes, 2007; Novis and Harding, 2007). Esapa et al. (2007) reported that the contribution of microalgae was more than 60% of the total biomass in the extremely acidic water of the Tintillio River in Spain. The presence of lithotrophic bacteria e.g. *Thiobacillus thiooxidans*, *Thiobacillus ferrooxidans*, *Acidithiobacillus ferroxidans* and *Leptospirillum ferroxidans*; heterotrophic bacteria e.g. *Bacillus* sp. and *Pseudomonas* sp; fungi e.g. *Penicillium* sp., *Geotrichum* sp. and *Aspergillus* sp.; and yeasts, *Rhodotorula*, *Cryptococcus*, *Tremella*, *Holtermannia*, *Leucosporidium*, and *Mrakia* was identified in AMD (Manafi et al., 2012; Brake and Hasiotis, 2010; Aguilera et al., 2010; Das et al., 2009; Bray et al., 2008; Orandi et al., 2007; Kalin, 2006; Lopez-Archilla et al., 2004). The biodiversity and role of the indigenous microorganisms in mine sites, in particular in AMD, has received significant attention by geo-microbiologists (Gadd, 2010; Esapa et al., 2007).

The extremophilic microorganisms in AMD play an important role to cleanse the AMD from metal/metalloid contaminates by absorbing the elements on/into their cells or enhancing their precipitation, which is referred to as biosorption and bio-precipitation (Gadd, 2010; Lottermoser, 2010; Prasad, 2007; Mehta and Gaur, 2005). Previous studies revealed the significant role of indigenous microbes isolated from mine sites for sequestering heavy metals compared with other isolated microbes from un-contaminated sites (Trzcinska and Skowronska, 2012; Bayramoglu et al., 2006). The indigenous AMD microorganisms are potential biosorbents that can be exploited
in AMD biotreatment systems (Orandi and Lewis, 2012a; Souza-Egipsy, 2011; Aguilera et al., 2006, 2007, 2010; Munoz and Guieysse, 2006). However, biotreatment of AMD by indigenous microbes have been limited to the exploitation of sulphate reducing bacteria (SRB) and AMD biotreatment has remained at an exploratory stage (Gadd, 2010).

Recently, Orandi et al. (2012) investigated the viability and efficiency of an indigenous algal-microbial consortium in a photo-rotating biological contactor (PRBC) for removing various metal/metalloids. The indigenous microbes were collected from AMD resources at Sarcheshmeh copper mine in Iran. A previous study reported the low pH (~3), high sulphate (>1000mg/L) and heavy metal concentration (e.g. Cu, Mn > 50mg/L) in the AMD, from which the microbial sample was collected (Orandi and Lewis, 2012a, Orandi et al., 2007). The microbial samples were used as inoculum in the PRBC and developed as an algal-microbial biofilm. The efficiency of the biofilm reactor was evaluated in both batch and continuous modes for reducing the ion content of a synthetic AMD (Orandi et al., 2012, Orandi and Lewis, 2012b). The results demonstrated the ability of the biofilm for removal of up to 80% of major elements (Cu, Mg, Mn, Zn, Ni) and trace elements (Fe, Co, Se, Sb, Cr, Ag) (Orandi and Lewis, 2012 a, b). The isolation and identification of indigenous mine microorganisms at Sarcheshmeh has mainly focused on the microbes. Seyed Bagheri and Hassani (2001) isolated the mesophilic bacteria such as Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans and Leptospirillum ferroxidans from the mine site. Manafi et al. (2012) reported two fungal genera, Aspergillus and Phialophora, which were isolated from the mine soils. Orandi et al (2007) reported the presence of the filamentous green microalga, Ulothrix gigas; fungi, Geotrichum sp. and Aspergillus sp.; and bacteria, Pseudomonas sp. and Thiobacillus sp. in the Sarcheshmeh AMDs. In the current study, the biodiversity of the microbial consortium that was collected from the most contaminated AMD resource at Sarcheshmeh is presented. Additionally, the biodiversity of these microbes which were used in the PRBC for biotreatment investigations are described. The contribution of the biofilm constituents for removing heavy metals was investigated by scanning electron microscopy and reported in the current research.
Material and methods

Study site and sample collection

Sarcheshmeh copper mine, listed as one of the giant porphyry copper deposits in the world, is located 160 km west of Kerman province, south-eastern Iran at the geographical point of 29°56′40″N and 55°52′20″E (Khorasanipour et al., 2011; Sillitoe, 2010). Environmental studies reported the degraded quality of surface waters at this mine, which resulted from AMD contamination (Orandi and Lewis, 2012; Shahabpour and Doorandish, 2008). One of the major resources of AMD originates from waste rock dumps, which are piled around mine sites (Shahabpour and Doorandish, 2008; Orandi, 2007). Algal-microbial biofilms, dominated by green mats of filamentous microalgae, fill some of drainages (Fig. 1). A microbial sample was collected from a severely acidic and heavy metal contaminated AMD for biotreatment investigations (Orandi and Lewis, 2012). The sample was successfully maintained and developed as an algal-microbial biofilm in a PRBC (Fig. 1). In this study, the microbial diversity in both field and PRBC samples was investigated. For isolation and identification, microbial samples were collected from the AMD, the suspended microorganisms in the PRBC and those immobilised as a biofilm; based on standard methods described previously (Orandi and Lewis, 2012). These samples are referred to as A, BS and BI, respectively.
Fig.1 Indigenous algal-microbial biofilms in AMD at Sarcheshmeh copper mine (above) and PRBCs (below)

**Microscopic studies for morphological observation**

The morphological characteristic of microbes in Samples A and BS were observed by light microscopy, Olympus IX50, using 60x and 100x magnifications. Scanning electron microscopy (SEM), Philips XL 30, was used to characterise the microbes in Sample BI. Sample preparation was explained previously by Orandi et al. (2012). In addition, from the prepared biofilm samples for SEM, Backscattered Electron (BSE) images were also provided to screen the metal-rich zones in the biofilm.

**Microbial isolation and enumeration**

Nutrient agar (NA) (Oxoid Ltd) and potato dextrose agar (PDA) were used for isolation of bacteria and fungi, respectively based on Eaton et al. (2005). According to the Pour Plate method,
0.1 ml of the AMD sample was aseptically mixed with 20 ml of NA and PDA. The plates were swirled gently and allowed to solidify. The NA plates were incubated at 37 °C for 24 hours for bacterial growth. The PDA plates were also incubated at 25°C for 3 days for fungal isolates. Additionally, to isolate acidophilic microbes, specific modified solid media and incubation conditions were used (Table 1) and their purpose is described (Eaton et al., 2005; Atlas, 2004). Isolated colonies were stained with iodine and safranin (Eaton et al., 2005). Additionally, to evaluate and compare the population of bacteria and fungi in the microbial samples, a series of solid media and incubation conditions were used as listed in Table 1. Media selection was based on Standard Methods for Examination of Water and Wastewater (Eaton et al., 2005; Atlas, 2004).

Table 1 Media for isolation and enumeration of bacteria, fungi and yeasts

<table>
<thead>
<tr>
<th>Media</th>
<th>Incubation conditions</th>
<th>Application purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient agar (NA)</td>
<td>37 °C for 24 hours</td>
<td>Bacterial isolation</td>
</tr>
<tr>
<td>potato dextrose agar (PDA)</td>
<td>25°C for 3 days</td>
<td>Fungi isolation</td>
</tr>
<tr>
<td>Pour Plate Culture (PPC)</td>
<td>7 days at 2-5°C, 42°C and 55°C</td>
<td>Microbial isolation at different temperatures</td>
</tr>
<tr>
<td>Sulfite Polymyxin Sulfadiazine (SPS)</td>
<td>2-4 weeks at 30 °C, anaerobic condition</td>
<td>sulphite reducing bacteria isolation</td>
</tr>
<tr>
<td>Postgate's B medium</td>
<td>2-4 weeks at 30 °C, anaerobic condition</td>
<td>Sulphate reducing bacteria (SRB) isolation</td>
</tr>
<tr>
<td>Thioglycollate broth</td>
<td>18-48 hours at 37°C, anaerobic condition</td>
<td>Anaerobic bacteria isolation</td>
</tr>
<tr>
<td>Plate Count Agar (PCA)</td>
<td>72 h at 30°C</td>
<td>Total microbial counts</td>
</tr>
<tr>
<td>Yeast extract Glucose Chloramphenicol (YGC) agar</td>
<td>3-5 days at 25 °C</td>
<td>Total count of fungi and yeasts</td>
</tr>
<tr>
<td>Plate Count Agar (PCA) in Anaerobic Jar</td>
<td>48-72 h at 37 °C, anaerobic condition</td>
<td>For anaerobic microbial count</td>
</tr>
</tbody>
</table>
Molecular Analysis

Molecular analysis was used for the identification of algal, bacterial and fungal strains in Samples A and B based on extracted DNA using specific kits for algae, fungi and bacteria (Reddy et al., 2007). For algae, DNA was extracted from 2 ml of each microbial sample using a MoBio DNA extraction kit according to the manufacturer’s instructions (MoBio Laboratories Inc, Carlsbad, CA, USA). Subsequently, a Polymerase Chain Reaction (PCR) was conducted using 2µl of the extracted DNA with 48 µl of a ready mixed KAPA taq. This method was used to amplify the gene of interest. Universal algae primers were used for PCR of the extracted DNA because of their capability to only amplify algal DNA (Reddy et al., 2007). Based on the instructions (Reddy et al., 2007; Godhe et al., 2002), PCR conditions were adjusted as the following: 95°C for 2 min; and 35 cycles of the following; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and the final stage was 72°C for 10 min. PCR amplicons (amplified DNA) were then run on a 1% agarose gel with Cybersafe, and a 1kb ladder. For sequencing, the PCR amplicons were sent to the Australian Genome Research Faculty (AGRF). Before sending, the PCR amplicons were prepared according to AGRF instructions. The results of forward and reverse sequences were combined on a CLC sequencer 6.0 and uploaded onto the National Centre for Biotechnology Information (NCBI) with a Bacic Local Alignment Search Tool (BLAST) as a FASTA file. The generated results from NCBI BLAST were used for algal identification.

Molecular analysis was also conducted for bacteria and fungi using the process described previously for algae. DNA extraction and PCR tests were conducted for bacteria using primers YT1 and YT2, based on a previous study by Yu et al. (2008). The universal primers IST 1- IST 2, IST 3 – IST 4 nested PCR were also used for fungi as described by Reddy et al. (2007).

SEM analysis

After biotreatment investigation, Energy Dispersive X-ray Spectroscopy (EDS) in conjunction with SEM was used to quantify the elemental content of the biofilm through the EDS spectra. Backscattered Electron (BSE) images were also prepared to screen the metal-rich zones in the biofilm.
Results

Microscopic studies

Microscopic observations by light microscopy and SEM revealed the presence of two genera of green microalgae in Samples A and BS. Unbranched filamentous and uni-cellular green microalgae green were found in these samples Fig.2 (a, b). These strains of microalgae contributed to the biofilm structure and their morphological characteristics were observed clearly in SEM images (Fig. 2 c, d). The filamentous green microalgae contained cylindrical and squared cells (Fig. 2 a, c). The unicellular microalgae were presented in spherical or ellipsoidal shapes. The cells were biflagellate and motile with two anterior flagella (Fig. 2 b, d).

Cells in the filamentous microalgae contained an chloroplast or plastid. Two forms of chloroplasts (plastids), round and ring-shaped, were mainly observed in the filamentous microalgae (Fig. 3 a, b). The ring-shaped chloroplasts were flat and located close to the inner face of the cells.

The cell dimensions in the microalgae filaments are reported from SEM images. The dimensions of the cylindrical cells varied between 3.9 x 3.55 and 3.89 x 2.37 µm (width x length) (Fig. 4). The width of some cylindrical cells reached 4.31 µm (Fig. 4).
Fig. 2 Filamentous and unicellular green microalgae observed by light microscopy (above) and SEM (below); a) unbranched filaments of microalgae b) Spherical and ellipsoidal unicellular microalgae; c) cylindrical cells enclosed in the microalgae filaments participated in biofilm structure; d) unicellular microalgae in biofilm, containing two anterior flagella

Fig. 3 Chloroplast morphology in filamentous microalgae, a) Round closed chloroplasts, b) Ring shaped chloroplasts
Different features of cell fragmentation in filamentous microalgae were observed in the samples (Fig. 5). Fig. 5 (a) shows an algal filament containing fragmented organelles inside a cell, and a cell with the broken cell wall, in Sample A. Fig. 5 (b) depicts the remaining cell walls after fragmentation. Similar features were also found in Sample B (Fig. 5 c, d). The fragmentated organells or cell wall could be due to reproduction processes, vegetative or asexual; in the filamentous microalgae. The H shaped cell wall could demonstrate the asexual reproduction in filamentous algae. However, these features could have also resulted from shear-force induced damage or toxicant-induced cell death (Bellinger and Sigee, 2010). Based on the observed morphological characteristics and chloroplast shapes, and possibly reproductive cells, the algal filaments were identified to be a *Klebsormidium* sp.
Fig. 5 Reproduction process of filamentous microalgae a) A filament in Sample A contained a broken cell wall and a cell with fragmented organelles, observed by 100x magnification; b) H shape wall remained after fragmentation of filaments in Sample A; c) and d) similar features for fragmentation and reproduction of microalgae in Sample BI.

Fig. 6 Dimension of uni-cellular algae, a) 6.15 x 2.94 μm, b) 3 x 7.5 μm.
The uni-cellular alga size is shown in Fig. 6 (a), 2.94 x 6.15 µm (width x length). Using the scale bar, the size of an additional uni-cell was approximately 3x7.5 µm (Fig. 5 b). The morphological properties indicated that the uni-cellular alga belongs to *Chlamydomonas* spp.

Bacteria, mainly rod-shaped and motile, were also observed in Samples A and B (Fig. 7). The SEM image of bacterial communities in the biofilm showed that the majority of bacteria were found to be rod-shaped with varying length between 797-864 nm and 1.38-2.46 µm.

![Fig. 7 Rod-shaped bacteria at length between 797-864 nm to 1.38-2.46 µm](image)

Fungi mycelium and filamentous fungi were found in both samples which were observed by light microscopy and SEM (Fig. 8) The unicellular microalgae were attached to the fungi filaments.

![Fig. 8 Fungi in Samples A and B, uni-cellular algae attached to fungi mycelium](image)
Yeast were also observed in the samples. In Fig. 9 (a) an arrow indicates a single-celled budding yeast near the unicellular microalgae in Sample A, observed by 100x magnification. The yeasts with pseudohyphae form were also observed in Sample BI (Fig. 9 b).

Fig. 9 Yeasts in Samples A and B, a) a single-celled budding yeast; b) pseudohyphae form of yeasts in biofilm structure

**Results from isolation and enumeration of bacteria and fungi**

Colonies of fungi were isolated from the microbial samples on the PDA plates. The characteristics of colonies are reported in Table 2. The four fungi strains, *Penicillium* sp, *Mucor* sp, *Aspergillus* sp and *Cladosporium* sp were identified and based on the colonial morphologies and characteristics listed in the Table 2 (Ellis *et al.*, 2007). These fungi were common in Samples A and B except *Mucor* sp and *Cladosporium* sp which were found only in Sample A. The orange colonies of the yeasts, *Rhodotorula* sp. were found in both samples. The genus *Rhodotorula* is characterised by the combination of red or yellow colonies resulted from carotenoid pigments. These carotenoids serve in photo-protection absorbing cell-damaging wavelengths (Ellis *et al.*, 2007).
Table 2 Isolated fungi and yeasts on potato dextrose agar plate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Characteristics of colonies</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, BI, BS</td>
<td>Gray in centre and white in margin</td>
<td><em>Penicillium</em> sp</td>
</tr>
<tr>
<td>A</td>
<td>White fluffy</td>
<td><em>Mucor</em> sp</td>
</tr>
<tr>
<td>A, BI, BS</td>
<td>Green, brown in centre</td>
<td><em>Aspergillus</em> sp</td>
</tr>
<tr>
<td>A</td>
<td>Greeny-black (olivious-black)</td>
<td><em>Cladosporium</em> sp</td>
</tr>
<tr>
<td>A, BI, BS</td>
<td>Orange</td>
<td><em>Rhodotorula</em> sp</td>
</tr>
</tbody>
</table>

Bacterial colonies were also isolated on the Nutrient Agar plates. The majority of colonies were coloured creamy and white. The isolated colonies were stained and microscopic analysis showed three genera to be Gram-negative and one genus Gram-positive bacteria. The bacteria were rod and spiral shaped, and some of them had endospores. The dendrites form of bacteria was also observed on the plates. *Bacillus* sp has flagella and grows in dendrite form (Madigan and Martinko, 2005). One of the agar plates changed colour to green which was due to the presence of *Pseudomonas* sp. These bacteria produce pyocyanin, which is a green water soluble pigment (Madigan and Martinko, 2005). Pink and orange colonies of yeasts were also found on the NA plates. No growth was observed in the PPC, SPS, Postgate’s B medium and PCA in anaerobic jars. These results demonstrated that the thermophilic, sulphite and sulphate reducing bacteria were not present in the samples. However, surface growth on Thioglycollate medium was recorded which demonstrated the presence of anaerobic bacteria in the samples.

The isolation and enumeration results are summarised in Table 3. The enumeration results for total counts from Plate Count Agar (PCA) showed the population of microorganisms in Sample B, which was approximately 10 times more than the results for Samples A and BS. These results are attributed to the higher concentration of microbes in the biofilm structure.

The enumeration results for total count of fungi and yeasts on YGC agar showed a higher population, 100 times more, in Sample A compared with Samples BI and BS. These results illustrated the different environmental conditions in the AMD and PRBC, which affected the fungi and yeast population.
Table 3 Enumeration results on PCA and TGC media, counts are presented as CFU/ml

<table>
<thead>
<tr>
<th>Samples</th>
<th>Media and conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA</td>
</tr>
<tr>
<td>A</td>
<td>$2.1 \times 10^4$</td>
</tr>
<tr>
<td>BS</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>BI</td>
<td>$4.2 \times 10^5$</td>
</tr>
</tbody>
</table>

The distinct physiological characteristics of bacteria contribute in their identification. However, molecular analysis has been used more recently for their identification and classification (Baker et al., 2009; Escobar et al., 2008).

**Molecular Analysis**

NCIB BLAST results showed that the sequence of isolated algae from Samples A was 94-96% similar to *Chlamydomas geitleri* whilst samples BS and BI were 76-87% similar to *Chlamydomonas pseudopertusa*. These results indicated the existence of *Chlamydomonas* spp. in all samples. However, the results did not show the presence of filamentous micro-algal species, which could be due to the thick cell wall of these microorganisms, as rigid cell walls affect the DNA extraction stage. For example, Erkelens et al., (2012) investigated fungal communities in TNT contaminated soils. The findings showed that the extraction of fungal DNA from soil was not optimised due to the thick cell wall of fungal spores.

Fig. 10 shows the post PCR amplicons which were attached to the universal algal primers and were ran on agarose gel. The numbers 1-6, which are printed above each lane, show the DNA related to the microbial samples. Lanes 2, 3 and 4 are related to Samples A, BS and BI and Lanes 1, 5, 6 were related to the positive control samples. The image obtained from the gel showed a light band for all microbial samples, indicated with an arrow. These bands indicate the existence of a single algal species.
The PCR results for bacteria and fungi are listed in Table 4. The results indicated the presence of *Acidithiobacillus ferroxidans*, *Leptospirillum ferroxidans* and *Bacillus subtilis* in all samples. *Pseudomonas xanthomarina* was only reported in Samples A and BI. This result showed the prevalence nature of *Pseudomonas* for biofilm formation which was found both in the AMD biofilm and the developed biofilm in the PRBC. Biofilms favored occurrence of this bacterium to tolerate the extreme conditions of AMD. *Pseudomonas* sp. are well known for growth in biofilms (Halan et al., 2011). *Bacillus licheniformis* were also only isolated and identified from Samples BS and BI. Different conditions in the field, *in-vivo*, and in *in-vitro* environments affects the microbial diversity.

The results for identification of fungi are listed in Table 5. The majority of fungal strains were present in all samples. However, *Mucor circinelloides* was found only in Sample A whereas *Verticillium* sp. and *Monosporium verticillium* were found only in the Samples BS and BI.
Table 4 PCR results for bacteria in the microbial samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Isolated Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Acidithiobacillus ferroxidans, Leptospirillum ferroxidans, Bacillus subtilis,</em></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas xanthomarina</em></td>
</tr>
<tr>
<td>BS</td>
<td><em>Acidithiobacillus ferroxidans, Leptospirillum ferroxidans, Bacillus Subtilis,</em></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus lichniformis, Pseudomonas xanthomarina</em></td>
</tr>
<tr>
<td>BI</td>
<td><em>Acidithiobacillus ferroxidans, Leptospirillum ferroxidans, Bacillus Subtilis,</em></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus lichniformis</em></td>
</tr>
</tbody>
</table>

Table 5 PCR results for fungi in the microbial samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Isolated Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Cladosporium cladosporiodes, Aspergillus nidulans, Penicillium spinulosum,</em></td>
</tr>
<tr>
<td></td>
<td><em>Penicillium citreongium, Mucor circinelloides, Trichoderma pseudokoningii</em></td>
</tr>
<tr>
<td>BS</td>
<td><em>Cladosporium cladosporiodes, Aspergillus nidulans, Penicillium spinulosum</em></td>
</tr>
<tr>
<td></td>
<td><em>Penicillium citreongium, Verticillium sp., Monosporium-verticillium sp.</em></td>
</tr>
<tr>
<td>BI</td>
<td><em>Cladosporium cladosporiodes, Aspergillus nidulans, Penicillium spinulosum</em></td>
</tr>
<tr>
<td></td>
<td><em>Penicillium citreongium, Verticillium sp., Monosporium-verticillium sp.</em></td>
</tr>
</tbody>
</table>

SEM analysis results

The SEM images, which were prepared from Sample BI, illustrated the fine-grained deposits which were mainly precipitated on the cell walls of filamentous microalga (Fig. 11).

The SEM-EDS spectra revealed the presence of heavy metals in and/or on the biofilm. The semi-quantitative data from the spectra showed precipitated elements included heavy metals and metalloids e.g Mg, Mn, Fe, Co, Cu, Ni, Zn. One spectrum with related semi-quantitative data is depicted in Fig. 12 as an example of typical spectra. Cu and Fe were removed considerably compared with the other elements. The presence of adapted microbial strains to the higher concentration of Cu in the Sarcheshmeh copper mine and also iron oxidising bacteria in the
PRBC attributes to higher precipitation of these elements (Orandi and Lewis, 2012; Orandi et al., 2012)

The SEM-BSE imaging allowed the evaluation of metal-rich distribution within Sample BI (Fig. 13). The light or white zones in the images indicated metal rich zones in the biofilm where as the dark zones depicted organic matter zones. The filamentous microalgae, particularly their inner parts, and bacteria were denoted with the white colours compared with their dark adjacent background.

Fig. 11 SEM images presenting precipitates on Sample BI, in particular filamentous microalgae cell wall

Fig. 12 SEM-EDS image presenting the metal content in Sample BI

<table>
<thead>
<tr>
<th>Elements</th>
<th>Na</th>
<th>Mg</th>
<th>Mn</th>
<th>Fe</th>
<th>Co</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values %</td>
<td>1.7</td>
<td>0.3</td>
<td>0.9</td>
<td>31.6</td>
<td>1.5</td>
<td>15.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Fig. 12 SEM-EDS image presenting the metal content in Sample BI
Discussion

In this study, an indigenous algal-microbial biofilm which was collected from an AMD and used in a PRBC for biotreatment investigations was investigated. For the biotreatment study, it was necessary to identify the microbial strains present in the AMD as resistant and extremophilic strains. Additionally, the compatibility of these microbes under in-vitro condition and during treatment process was required. The results of the reported study showed the presence of autotrophs which included phototrophic and chemo-litho-trophic; and heterotrophic microorganisms in the microbial samples. The biodiversity of the microbes in the AMD and PRBC was similar.

A previous study reported the filamentous green microalgae, *Ulothrix gigas*, in AMD resources at Sarcheshmeh copper mine (Orandi et al., 2007). However, based on the morphological features of the algal filaments, chloroplasts and reproductive cells and also according to the recent documents reporting the difference of *Ulothrix* and *Klebsormidium* (Rindi et al., 2012, Rindi et al., 2008, Mikhailyuk et al., 2008; Novis and Harding, 2007), the microalgae was identified to be a *Klebsormidium* sp. Additionally, the presence of *Chlamydomonas* sp. was verified based on the documented literature and the typical morphology observed in this investigation. The existence of this micro-alga was also identified from the PCR results.

The identified bacterial community in the microbial samples indicated the existence of the chemo-litho-trophic iron and iron-sulphur oxidizing bacteria, *Acidithiobacillus ferroxidans*,...
Leptospirillum ferroxidans; and heterotrophic bacteria, Pseudomonas sp. and Bacillus sp. These bacteria were all Gram-negative except Bacillus sp which are a Gram-positive bacterium. The importancetance of these bacteria in adsorption/absorption of various elements, in particular metals, has been documented and emphasised extensively (Gadd, 2010; Gadd, 2004). Bacillus spp, Pseudomonas sp, fungi and yeasts were the identified heterotrophic species among the microbial community. Generally, Bacillus spp can be found in acidic, neutral and alkaline waters. They are resistance bacteria and the cells produce oval endospores that can stay dormant for extended periods (Alcaraz, 2010). The extensive presence of fungi in all samples indicated their resistant nature under low pH. The fungi and yeast contributed to the formation of biofilm structure significantly.

The presence of acidophilic/extremophilic bacteria, such as the two genera of Acidithiobacillus and Leptospirillum, in mine sites has been documented (Novis and Harding, 2007). Acidithiobacillus ferroxidans and Leptospirillum ferroxidans are the dominant autotrophic iron- and sulphur-oxidizing and; iron-oxidizing bacteria, respectively (Yang et al., 2008, Cabrera et al., 2005). Acidithiobacillus ferrooxidans is a Gram negative rod-shaped bacterium. The cells are non-sporing and occur in single or occasionally in pairs or chains, depending on growth conditions. Highly motile with a single flagellum and non motile strains have been reported. The optimal pH level for the growth is 1.5 – 2.5 (Valdes et al., 2008). Leptospirillum ferroxidans is Gram-negative and has spiral-shaped cells, 0.3-0.5 microns wide and 0.9-3.0 microns in length. Acidithiobacillus ferrooxidans and Leptospirillum ferroxidans are strictly chemo-litho-autotrophic (Escobar et al., 2008). The acidophilic strains of Bacillus were also reported from mine sites (Raja and Omine, 2012). This heterotrophic bacterium is Gram-positive and rod-shaped and able to form tough and protective endospores, allowing the organism to tolerate the extreme conditions of AMD. It is mostly reported as an obligate aerobic (Madigan and Martinko, 2005). Pseudomonas is a Gram-negative bacterium that can be found in many environments, included mine sites (Novis and Harding, 2007; Das et al., 2009 a,b). It has rod-shaped cells that are able to produce EPS under extreme condition of AMD. This ability highlights of Pseudomonas role for biofilm formation (Palleroni, 2010).

The single-celled budding form of Rhodotorula sp., were isolated and identified in the AMD. However, the yeasts were formed as pseudohyphae in the biofilm. Yeasts generally grow as an oval, budding yeast, but in some yeasts e.g. Rhodotorula mucilaginosa and Candida albicans the budding yeast may elongate and remain attached producing filament-like structures.
called pseudohyphae under certain culture conditions. The pseudohyphae help yeast to invade deeper after it colonizes in biofilms (Kaeberlein, 2010; Arroyo-Lopez et al., 2009).

The indigenous AMD microbial consortium, in particular filamentous green microalgae, presents a resistant and capable biosorbent for biotreatment of metal bearing wastewaters such as AMD.

**Acknowledgments**

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CHAPTER SEVEN
STATEMENT OF AUTHORSHIP

Title of paper: Biosorption of heavy metals in a photo-rotating biological contactor - a batch process study


Sanaz Orandi (First Author)
Performed analysis on all samples, interpreted data, wrote manuscript and manuscript evaluation, and acted as corresponding author.

Signed.............  Date.............30/8/12

David M. Lewis (Co-author)
Supervised development of work, helped in data interpretation and manuscript.

Signed......  Date 30/8/12
*Applied Microbiology and Biotechnology, v. 97(11), pp. 5113-5123*

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[http://doi.org/10.1007/s00253-012-4316-5](http://doi.org/10.1007/s00253-012-4316-5)
CHAPTER EIGHT
STATEMENT OF AUTHORSHIP

Title of paper: Metal/metalloids removal from acid mine drainage by indigenous mine biofilm in a photo-rotating biological contactor—a continuous process study


Sanaz Orandi (First Author)

Performed analysis on all samples, interpreted data, wrote manuscript and manuscript evaluation, and acted as corresponding author.

Signed........... .......................... Date ........... 30/8/12

David M. Lewis (Co-author)

Supervised development of work, helped in data interpretation and manuscript.

Signed..... .......................... Date .........................

30/8/12
Title of article:

Metal/metalloid removal from acid mine drainage by indigenous algal-microbial biofilm in a photo-rotating biological contactor—a continuous process study

Authors:

Sanaz Orandi and David M. Lewis

Affiliation and address of authors:

Micro-algae Engineering Research Group, School of Chemical Engineering, University of Adelaide, North Terrace Campus, 5005 SA, Australia

Corresponding Author:

Sanaz Orandi

E-mail: sanaz.orandi@adelaide.edu.au

Tel: +61 8 83033959

Fax: +61 8 8303 4373
Abstract

Indigenous biofilms isolated from acid mine drainage (AMD), that are dominated by filamentous green micro-algae, cab be exploited for immobilisation/biosorption and mobilisation of metal/metalloids which can be used in bioremediation. However, there is limited knowledge related to the process of metal removal within these biofilms and the ability to exploit these in a continuous treatment process. The reported research focuses on the exploitation of indigenous AMD biofilms for removing/immobilising metals from AMD in a scalable biotreatment system for potential use at mine sites. In this research an indigenous algal-microbial biofilm, dominated by the filamentous green micro-alga Klebsormidium sp. was developed in a photo-rotating biological contactor (PRBC), and used to investigate the removal of 16 elements, including heavy metals and metalloids from a multi-ion synthetic AMD. The PRBC was operated continuously with a 24h hydraulic residence time (HRT) over a 10-week period. Water analysis was conducted on a daily basis for the first 28 days and then weekly. The daily results demonstrated the ability of the algal-microbial biofilm to remove 30–70 % of the major elements (Cu, Mg, Mn, Zn, Ni, Ca, Na) and 10-90% of the trace elements (Ag, Mo, Co, Al, Se, Sb, Cr, Pb and Fe) from the synthetic AMD. The removal efficiency for each element was not consistent as three desorption stages occurred within 28 days, which was followed by increased removal. This work revealed the effect of the immobilisation and mobilisation potential of indigenous microbes. Biotreatment with indigenous AMD algal-microbial biofilm offers a sustainable approach for primary treatment of AMD at mine sites.

**Keywords:** Acid mine drainage, Indigenous AMD biofilm, Micro-algae, Photo-rotating biological contactor, Biosorption

**Abbreviations:** Acid Mine Drainage (AMD), Photo-Rotating Biological Contactor (PRBC), Synthetic AMD (Syn-AMD)
1. Introduction

Mining wastewater, typically acid mine drainage (AMD) has been listed as one of the most severe types of contaminated surface waters containing heavy metals (e.g. Cu, Zn, Cr, Pb) and toxic metalloids (e.g. As, Cd, Sb) that threat aquatic life and human health by carrying these elements into water resources (Nganje et al., 2010; Lottermoser, 2010, Gadd, 2010; Ahluwalia and Goyal, 2007; Malik, 2004). Conventional treatment techniques, mainly neutralisation, have been ineffective or expensive for removing the recalcitrant elements e.g. Mn. Additionally, huge volumes of contaminated sludge is produced with conventional treatment methods (Wang, 2011; Silverira et al., 2009; Das et al., 2008; Ahalya et al, 2003). The alternative technique of biotreatment/bioremediation using live microbial cells, particularly immobilised biofilms in bioreactors, may provide an effective technique for reducing the dissolved ion contents of AMD. However, resistant strain of microbes that tolerate low pH and high concentrations of heavy metals and toxic metalloids in AMD is required for biotreatment trials (Das et al., 2009; De la Pena and Barreiro, 2009).

Indigenous microorganisms can thrive in algal-microbial biofilms found in AMD resources (Aguilera et al. 2008; Aguilera 2006). Various studies report the extremophilic green microalgae, bacteria, fungi and yeasts found in AMD biofilms (Orandi et al., 2009; Orandi et al., 2007; Espana et al., 2007). These microbes are able to accumulate heavy metals on their cell surface through different mechanisms which immobilise ions including biosorption and biomineralisation (Gadd, 2010; Gadd, 2004). The indigenous microorganisms found in AMDS are potential biosorbents for removing metal/metalloids from AMD (Aguilera et al. 2008; Aguilera, 2006). The benefit of these microbes has not been exploited in a practical biotreatment system at mine sites (Gadd, 2010; Gadd, 2009; Tsezos, 2007). There is limited information relating to metal removal trends of these microbes and their efficiency over an extended period for the design of an effective system for AMD biotreatment.

Immobilisation mechanisms of microorganisms may promote solubilisation of metals in some circumstances by shifting the equilibrium to release more metal into solution, which is referred to as mobilisation (Gadd, 2010; Gadd, 2004). Mobilisation mechanisms provide natural refurbishment of microbial cells and enable them to withstand harsh environments found in AMD and maintains healthy biofilm (Malik, 2004). However, the outcome of this mechanism affects
the potential removal efficiency of a biotreatment system. Therefore, it is required to understand the trend of metal adsorption/desorption prior to design biotreatment systems.

In a previous study Orandi and Lewis, (2012b) demonstrated the potential of indigenous algal-microbial biofilms for removing various elements at different initial concentrations from AMD in a batch-process study. To develop commercially relevant biotreatment systems for mine sites, it is required to design a continous system. In the current study, the efficiency of an indigenous AMD algal-microbial biofilm, which was collected from Sarcheshmeh copper mine in Iran, was evaluated for reducing the metal/metalloid ion concentrations in AMD. These indigenous biosorbents were immobilised and developed as a biofilm in a photo-rotating biological contactor to investigate metal/metalloid removal trends over a prolonged continous operation.

2. Material and methods

2.1. Synthetic AMD

A synthetic AMD (Syn-AMD) was formulated using the AMD analysis from Sarcheshmeh copper mine. The Syn-AMD contained cations including major (Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\)) and trace metals (Ag\(^+\), Cu\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Mo\(^{2+}\), Pb\(^{2+}\), Fe\(^{2+}\), Cd\(^{2+}\), Se\(^{2+}\), Cr\(^{3+}\), Sb\(^{3+}\), Al\(^{3+}\), Bi\(^{3+}\) and As\(^{3+}\)) and anions (Cl\(^-\), NO\(_3^-\), PO\(_4^{3-}\), SO\(_4^{2-}\), CO\(_3^{2-}\)) as explained previously (Orandi and Lewis, 2012a). The pH of Syn-AMD was adjusted to 3 for the continous process investigation (Orandi and Lewis, 2012a).

2.2. Photo-Rotating Biological Contactor

A laboratory scale photo-rotating biological contactor (PRBC) was constructed and operated continuously as described previously (Orandi et al., 2012). The PRBC was single stage and contained 16 polyvinyl chloride (PVC) discs which were partially submerged (40%). The Syn-AMD, 15 l, was introduced to the PRBC at the flow rate of 10ml/min, providing a 24 h hydraulic residence time (HRT) over a 10-week period. The HRT was adjusted to 24h based on the results from a previous study by Costley and Wallis (2000), who studied the influence of various HRTs on the efficiency of an RBC for metal accumulation. They quantified the removal of Cu, Zn and
Cd with a HRT of 3, 6, 12 and 24h. Each HRT was implemented for a total of 24h. Their results showed that a longer HRT of 24 h was associated with greater metal removal.

2.3. Algal-microbial biofilm
An algal-microbial consortium was collected from AMD at Sarcheshmeh copper mine and used as inoculum in the PRBC. The algal-microbial biofilm was developed on the disc’s surfaces as described previously by Orandi et al., (2012). The biofilm was composed of filamentous green micro-alga Klebsormidium sp.; bacteria, Acidithiobacillus ferroxidance, Pseudomonas spp; and fungi, Aspergillus sp. and Penicillium sp. (Orandi et al., 2007).

2.3. Biotreatment investigation- water analysis
To evaluate the efficiency of the biotreatment system over the continuous period, the PRBC was drained and refilled with milliQ water. The PRBC was then operated for 2 h to remove any trace of previously used Syn-AMD from the biofilm and the PRBC’s trough, prior to continuous operation. After the washing process, the PRBC was drained and refilled with fresh Syn-AMD.

During the continuous process, water sampling was conducted on daily and weekly basis over the 10-week period. The water samples were collected from the inlet and outlet of the PRBC and analysed within 2 days. The first sample collection was carried out within the first 24h immediately after the PRBC operation and then after 5, 10, 30, 60, 120, 180, 360, 720 and 1440 minutes to monitor the removal efficiency of the system and evaluate the effectiveness of continuous operation for removing various elements. After the first 24h, the samples were collected daily for 28 days to investigate the elemental removal trend in the PRBC. After this period, water samples were collected three times per week until the end of the 10-week period to evaluate the efficiency and sustainability of the indigenous algal-microbial biofilm in the PRBC. The results were recorded as weekly average removals. In this research, the removal efficiency of the system was calculated and recorded as a percentage of removed elements from the Syn-AMD, comparing the concentrations of each element in the inlet and outlet.

The sampling procedure and preparation for analysis by ICP-MS were demonstrated previously (Orandi et al., 2012). In the continuous process, water samples were analysed for the major and
trace elements including Cu$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Na$^{+}$, Ca$^{2+}$ and Fe$^{2+}$, Al$^{3+}$, Cr$^{3+}$, Pb$^{2+}$, Co$^{2+}$, Mo$^{2+}$, Se$^{2+}$, Ag$^{+}$, respectively.

Additionally, during continuous operation, the physical properties of the water samples were recorded and included temperature, pH, Eh, EC and DO, which were recorded on a daily basis with a water quality logger (TPS-90 FL).

2.4. Biofilm digestion and analysis

After the 10-week treatment process, three biofilm samples were randomly removed from the PRBC’s discs using a spatula and their dry weight was recorded. The dried samples were powdered with a mortar and pestle and digested in 10ml HNO$_3$ based on the method reported by Costely and Wallis (2001). The digested samples were diluted to 2x and 500x with a 2% HNO$_3$ solution and analysed using ICP-MS. The average of the results was recorded for each element, representing the percentage of the major and trace elements associated with the total mass of algal-microbial biofilm in the PRBC.

2.5. SEM analysis

During the continuous process, SEM images were taken from the algal-microbial biofilm. The procedure for sample preparation was previously explained (Orandi et al., 2012). Additionally, Energy Dispersive X-ray Spectroscopy (EDS) in conjunction with SEM was used to provide EDS spectra to evaluate the semi quantitative data from the chemical composition of the algal-microbial biofilm. The simultaneous application of the EDS micro-analytical system with Back Scattered Electron (BSE) images serves to chemically characterise the mineral features in biofilms (Van Hullebusch et al., 2003). From the prepared biofilm samples for SEM-BSE images were also provided to screen the metal-rich zones in the biofilm.
3. Results

3.1. Water analysis results within the first HRT (24h)

The water analysis results for the first 24h of continuous PRBC operation for the major and trace elements are presented in Figures 1 and 2, respectively.

The results for the major elements showed an increasing removal rate from 20 to 40%, after the first 5 min of PRBC operation (Fig.1). Major elements were removed in the order of: Ni (39.9%) > Mn (37.7%) > Cu (36.3%) > Mg (34.1%) > Ca (26.7%) > Zn (23.7%) > Na (20.3%). However, a decreasing removal was observed after 10 min for all major elements, followed by a slightly increased removal. The removal efficiency of the major elements increased up to 25-45% by 60 min. The removal rate pattern fluctuated significantly after 60 min until 720 min (12h), where a decreased removal occurred for the major elements. The maximum removal was recorded after 1440 min (24 h). At this point, the major elements were removed in the order of: Ca (54.6%) > Cu (52.2%) > Mn (50.2%) > Mg (47.08%) > Na (46.5%) > Ni (45.9%) > Zn (45.5%).

![Fig.1 Removal percentages of major elements within the first 24 h](image)

The water analysis results for the trace elements showed that the elements were removed after 5 min in the order of: Pb (38.8%) > Ag (30.08%) > Fe (29.1%) > Se (27%) > Mo (22.8%) > Sb (8.8%) >
Al (5.8%) > Cr (3.9%) > Co (3.5%) (Fig. 2). The removal efficiency of these elements decreased after 10 min, followed by increased levels up to 15-65%, which were recorded after 60 min of PRBC operation. After 60 min, the removal capacity for Ag and Pb increased significantly where it reached steady-state for Mo and Fe. Ag and Pb were removed up to 80-90%, at 720 min (12h). The removal efficiency for Se, Pb, Cr, Al and Co decreased between 60-720 min, and Co showed a negative level which meant desorption occurred.

At the completion time of the first HRT, 1440 min (24 h), the maximum removals were achieved for all the trace elements in the order of: Ag (97.7%) > Pb (85.1%) > Fe (68.8%) > Mo (60.6%) > Se (46.6%) > Sb (35.1%) > Cr (32.2%) > Al (10.9%) > Co (10.8%).

![Fig. 2 Removal percentages of trace elements over the first 24h](image)

After a HRT of 24 h, the recorded values for the physical properties of the treated AMD showed that the pH increased from 3.3 to 3.8 and EC decreased from 1875 to 1286 µS/cm. The water temperature in inlet and outlet was recorded as 18 and 20°C, respectively. The recorded values for Eh and DO were 370mV and 4.6 mg/L, respectively.
3.2. Adsorption and desorption cycles over continuous operation

The results for daily water analysis are presented as graphs for the major and trace elements in Figures 3 and 4, respectively. Four distinct adsorption cycles followed by three distinct desorption cycles were observed over the 28-day experiment.

In the first adsorption cycle, the major elements (Na, Mg, Ca, Cu, Mn, Ni, Zn) were adsorbed up to 40-50% after the first 24h, which was the first day of continuous operation. The adsorption efficiency of the PRBC for these elements increased significantly up to 50-80% by the end of the second day (Fig.3). However, the first desorption cycle occurred in the third day of operation and the decreased levels of adsorption were recorded for the majority of major elements, down to 10-30%, over the subsequent three days. Ca and Na were desorbed up to 17-20% in the first desorption cycle.

In the second cycle of adsorption and after the fifth day, the majority of the major elements were adsorbed dramatically up to 60-70%. The adsorption efficiency for the major elements remained between 30-60%, over the next seven days of continuous operation. As shown in Figure 3, higher adsorption (40-70%) were recorded for Cu, Mg, Mn and Ni where as lower values (20-40%) were recorded for Ca, Na and Zn, within the first 2 weeks of continuous operation.

The second desorption cycle occurred after two weeks, where the majority of elements were adsorbed to less than 10%. Cu, Mn and Ca adsorption decreased to 5-20% at this stage. However, the removal efficiency for the major elements rose significantly within one day, similar to the first adsorption cycle. The adsorption efficiency values for these elements remained between 30-60%, followed by a third desorption cycle, where most of elements in particular Cu, Mn and Ca were desorbed similarly to that of the second desorption cycle.

The fourth adsorption cycle occurred within the fourth week of experiment and the major elements, particularly Cu, was adsorbed up to 70%. The lowest adsorption (40%) was recorded for Ni and Zn in this cycle.

The daily analysis results for the trace elements followed a similar trend as for the major elements and three distinct desorption cycles were observed over the 28-day experiment (Fig.4).
However, numbers of trace elements were removed over the experiment period. The adsorption efficiency for the different trace elements varied broadly. Ag showed the highest adsorption over the 4-week period whereas the lowest adsorption was achieved for Al and Co. Ag was adsorbed between 80% and 90%, whilst the maximum adsorption for Co and Al was recorded as 20%. Overally, Al was desorbed rather than being adsorbed during the continous operation. Mo, Fe and Pb were adsorbed up to 60% during the same period. The adsorption trends for Se, Cr and Sb were less than the former elements.

The pH was maintained at 3± 0.5 in the inlet; however, it inadvertently decreased to less than 3 (2.8) during the third week. The recorded pH in the outlet showed an increase of 0.5 for most of the measurements in particular during the adsorption cycles. A decrease in pH correlated with the desorption cycles, and EC reduced up to 650 µS/cm over the adsorption cycles whereas, an increase of 50 µS/cm was recorded over desorption cycles.

During the period of experiment, water temperatures in the inlet and outlet were between 19-21 °C and 21-23°C, respectively. The PRBC was operated at ambient temperature and light irradiance was the only source of heat that resulted with a 2°C increase in temperature in the Syn-AMD in the inlet compared with the treated water in the outlet of the PRBC. Eh and DO fluctuated between 320-480 mV and 4.2-5.7mg/L, respectively.
Fig. 3 adsorption and desorption cycles for major elements over 28-day continuous operation

Fig. 4 Daily removal percentages for trace elements over 28-day continuous operation
The weekly water analysis results indicated that maximum adsorption was achieved for Cu and Mg (40-50%) over the measurement period. The adsorption percentages for other major elements occurred in the order of: Ni (30-45%) > Na (25-45%) > Mn (20-40%) > Ca and Zn (20-35%). The weekly adsorption results varied within a small range for the majority of trace elements including Ag, Fe, Pb, Mo and Cr, whereas for the other elements: Se, Sb, Co and Al changed within a considerable range. The weekly adsorption percentage of these elements were recorded in the order of: Ag (80-98%) > Fe (45-55%) > Pb (40-55%) > Mo (40-50%) > Cr (25-40%) > Se (20-50%) > Sb (15-50%) > Co (3-15%) > Al (-40-20%). The physical properties of the water samples were comparable to the recordings over the daily measurements.

3.3. Results of algal-microbial biofilm analysis

The results of the algal-microbial biofilm analysis showed the major elements that accumulated in the biofilm were in the order of: Cu (1.4%) > Mg (0.9%) > Na (0.6%) > Ni (0.6%) > Ca (0.5%) > Mn (0.5%) > Zn (0.3%) > Si (0.02%) > K (0.001%). The biofilm contained Cu and the other major elements at 1% and 0.3-0.9%, respectively. Si and K were also detected in the biofilm as these elements were included in the chemicals which were used for preparing Syn- AMD. Accumulated trace elements in the biofilm structure in the order of: Fe (3.40%) > Cr (0.25%) > Ag (0.17%) > Pb (0.14%) > Se (0.04%) > Mo (0.03%) > Sb (0.013%) > Co (0.004) > Al (0.001%). Considerable quantity of Fe (3.5%) was measured in the biomass. Based on the biofilm analysis, the major and trace elements accounted for 10% of the dry weight of the biofilm in the PRBC.

The SEM images illustrate the fine-grained deposits formed on the algal-microbial biofilm, in particular on filamentous micro-algae (Fig. 5). The SEM-BSE images also allowed the evaluation of a metal-rich distribution within the algal-microbial biofilm. The light or white zones in the images indicated the metal rich zones in the biofilm whereas the dark zones depicted the organic matter zones (Fig. 6). The filamentous micro-algae, particularly their inner parts, were denoted with the white colours compared with their dark adjacent background.
Fig. 5 SEM images representing the precipitates on the algal-microbial biofilm after biotreatment process

Fig. 6 SEM-BSE images representing metal rich zones as lighter area in the algal-microbial biofilm
The SEM-EDS spectra revealed the presence of heavy metals in/on the biofilm. One example SEM-EDS spectrum is shown as a representative metal/metalloids adsorption on the biofilm (Fig. 7). The semi-quantitative data of accumulated elements in/on the main biofilm’s components included filamentous micro-algae, bacteria and fungi zone are reported in Table 1. Filamentous micro-algae showed adsorption of elements in the order of: Fe (6.8%) > Cu (3.6%) > Mg (1.6%) > Na (1.1%) > Ni (0.8%) > Zn (0.5%) > Mn (0.3%) > Co (0.05%). Elements found in the bacterial zone were in the order of: Fe (13.4%) > Cu (7.64%) > Na (3.44%) > Mg (2.8%) > Zn (0.95%) > Mn (0.33%) > Co (0.3%). The fungal zones contained metal/metalloids in the order of: Fe (8.03%) > Cu (4.78%) > Mg (3.6%) > Na (2.06%) > Ni (1.4%) > Zn (0.71%) > Mn (0.45%) > Co (0.1%). Ni adsorption in fungi was greater than for the other zones.

Table 1 Semi-quantitative data from SEM-EDS spectrums of the main biofilm’s components

<table>
<thead>
<tr>
<th>Detected Zone</th>
<th>Na</th>
<th>Mg</th>
<th>Mn</th>
<th>Fe</th>
<th>Co</th>
<th>Cu</th>
<th>Zn</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-algae</td>
<td>1.13</td>
<td>1.06</td>
<td>0.34</td>
<td>6.84</td>
<td>0.05</td>
<td>3.66</td>
<td>0.52</td>
<td>0.80</td>
</tr>
<tr>
<td>Bacteria</td>
<td>3.44</td>
<td>2.80</td>
<td>0.33</td>
<td>13.4</td>
<td>0.30</td>
<td>7.64</td>
<td>0.95</td>
<td>0.35</td>
</tr>
<tr>
<td>Fungi</td>
<td>2.06</td>
<td>3.60</td>
<td>0.45</td>
<td>8.03</td>
<td>0.10</td>
<td>4.78</td>
<td>0.71</td>
<td>1.42</td>
</tr>
</tbody>
</table>
4. Discussion

The indigenous AMD biofilms have been adapted to survive and grow in the metal/metalloid contaminated water and adsorb these metal/metalloids (Gadd, 2010).

The water analysis results within the first 24h period of continuous operation illustrated the removal pattern of elements from the Syn-AMD. Major elements were adsorbed between 20-40% from the Syn-AMD within the first 5 min, followed by a gradual increase of adsorption by the end of the first 24h period. Trace elements followed a similar removal pattern to major elements, in particular Ag, Pb, Fe, Mo and Se. This result demonstrated the two main steps of biosorption, which are active and passive processes (Das et al., 2008). The first step usually occurs within 5-10 min (Das et al., 2008), which was similarly observed in this experiment. The gradual increase of adsorption could also be attributed to the second step of biosorption by the algal-microbial biofilm (e.g. Bayramoglu et al., 2006; Costely and Wallis, 2001). The results for continuous operation denoted the effect of a longer treatment period on removal efficiency as the maximum removal for the majority of elements was obtained after 24h.

The daily water analysis over the 28-day period signified the potential and effectiveness of the indigenous algal-microbial biofilm for adsorbing or immobilising the majority of elements including recalcitrant elements e.g. Mn and Ni. The obtained results were significant when considering the limiting factors for adsorption of metal/metalloids in the Syn-AMD, such as low pH, high concentrations of Ca, Na and SO\(_4\) and co-ion effects, which can adversely affect the biotreatment results as shown for batch process (Orandi and Lewis, 2012b).

Generally, the adsorption (immobilisation) /desorption (mobilisation) cycles for the major and trace elements were similar within 28 days. The adsorption cycles could occur due to the microbial cell interactions including bio-precipitation, physical adsorption, ion exchange, intercellular accumulation and complexation, which participate in the immobilisation of elements and reducing the free ions in solutions. The observed desorption cycle also could be due to the mobilisation mechanisms of microbial cells such as chelation (Gadd, 2010; Gadd, 2004; Ahalya, 2003). Environmental conditions affect microbial strains and their response, for example
Penicillium sp. was reported as a potential strain for removing heavy metals (Leitao, 2009) where as another study reported its’ application for bioleaching of heavy metals (Deng, 2012).

The 28-day analysis demonstrated the effect of complex processes for the removal potential by the algal-microbial biofilm in the PRBC operated continuously. The results revealed the periods of biosorption and desorption which mostly occurred over 7 and 3 days, respectively. The biosorption results fluctuated within ± 10% for most of the elements where as the desorption results showed a release of 40%. The comparison between the elemental removal and pH recordings showed that increased values of pH occurred during the biosorption period from 0.2-0.5 whereas decreased values of pH, up to 0.2, were recorded in the desorption stages. These results demonstrate the significant role of mobilisation mechanisms, particularly bioleaching which microorganisms are able to acidify their environment and rid their cells of accumulated heavy metals. Additionally, acidification occurred due the low pH (~3) of the Syn-AMD that provided protons (H⁺) and out-competed the other ions in binding to the microbial cells and resulted in the release of the ions. Espana et al. (2007) studied the indigenous AMD biofilm in Rio Tinto, Spain. They stated that the tolerant species such as micro-algae survive by reducing proton influx (H⁺ ions) which coincides with increased proton pumping limiting available cell surface binding sites for metal ions.

During the 28-day period, the greater adsorption was achieved for the major elements, 30-70%, whereas the less and broad adsorption, 10-70%, was recorded for the trace elements. The adsorption efficiency for most of the elements was constant during the adsorption cycles. The higher initial concentrations of the major elements (3-100 mg/L) compared with the trace elements (0.005-1 mg/L), attributed to the aforementioned results (Orandi and Lewis, 2012a). Additionally, the adsorption capacity of microorganisms depends on their selectivity and preference for different elements. These reasons are discussed previously in Orandi and Lewis (2012b).

It was shown that pH played the most dominant role in the biotreatment results. The higher acidity (pH~2.8) of the Syn-AMD over the third week affected the biotreatment results as 10% less removal occurred with the major elements. The reduced level was comparable for some of the trace elements, in particular for Fe (Fig.4).
The comparison between the EC variations and elemental removal trends demonstrated that the EC decreased, up to 650 µS/cm, during the biosorption period whereas increased levels of EC were observed during desorption processes. The relationship between elemental removal variations and decreased EC and increased pH values, provides a predictive scale for estimating removal efficiency of the system by measuring pH and EC. For example, the increased level of pH by 0.5 and decreased EC > 500µS/cm occurred when the most major element removal was obtained on the days 2, 7, 11 and etc (Fig. 3).

The recorded DO varied between 4.2-5.7mg/L in the PRBC solution. Espana et al. (2007) measured the oxygen level of water where the green algal mat thrived in AMD sources in the Tinto River, Spain. The oxygen content of 3.6–5.9 mg/L was reported, which was ~ 40%–70% of the saturated level. This sub-saturated level of DO resulted from the diffusion of atmospheric oxygen and photosynthetic activity of green algal communities (Espana et al., 2007). The DO values reported from the PRBC investigation was attributed to the discs’ rotation, which mixed the Syn-AMD with the atmosphere; and algal photosynthesis which, increased the oxygen diffusion into the system versus the heterotrophic microorganisms that consumed oxygen.

The results from the weekly analysis of water samples showed the potential of the system for removing the majority of elements from the Syn-AMD over a prolonged continuous period. Additionally, the consistent adsorption capacity for the most elements demonstrated the sustainability of the system for remediation efficiency which can be exploited in mine sites for AMD biotreatment. The natural desorption processes of the algal-microbial biofilm is also one of the advantages of biotreatment by live cells that maintain a refurbishing biofilm for removing elements prior to the saturation level (Malik, 2004).

The major and trace elements particularly Cu, Ni, Mn, Mg, Fe, Mo, Cr, and Ag were removed considerably throughout the 28-day experiment. Cu, Ni, Mn and Mg were found in the Sarcheshmeh copper mine’s AMDs as the major contaminants. The indigenous biofilms in these AMDs have adapted to survive and grow within this range of contamination and also are able to absorb and select these elements from their environment. This reason is also attributed to the higher adsorption of Ag and Mo. These elements are found to be concentrated in the
Sarcheshmeh ore minerals (Aftabi and Atapour, 2011). Ag, is a precious elements, but it is also one of the most toxic heavy metals for aquatic life (Gadd and Griffith, 1978). In the reported study, the greatest removal percentage for Ag signifies the efficiency of the indigenous algal-microbial biofilm for adsorbing and accumulating this element with being non-toxic to the biofilm.

The results from the biofilm digestion showed that 10 % of the dry weight of the algal-microbial biofilm was composed of the major and trace elements. Aguilera et al., (2008) studied a several extremophilic biofilm samples collected from contaminated waters with AMD. They observed the biofilms by low temperature scanning electron microscopy (LTSEM) which revealed the mineral adsorption in the matrix of EPS and onto the cell walls. EPS in all biofilm samples was mainly composed of carbohydrates and heavy metals, reaching up to 16% of the biofilm dry weight. In the reported research, the elemental content of the developed algal-microbial biofilm in vitro was similar to the reported data from indigenous AMD biofilm in vivo by Aguilera et al., (2008).

The biofilm analysis showed the accumulated major elements, in particular Cu and Mg, in an order that was comparable with the adsorption results from the PRBC operation. The trace elements accumulated in a different order as Fe accumulated the most and Ag moved to the third in the sequence (Fe> Cr> Ag> Pb> Se> Mo> Sb> Co> Al). The higher initial concentration of Fe (0.7 mg/L) and Cr (0.1 mg/L) in the Syn-AMD compared with Ag (0.02 mg/L) was attributed to this observation. Co and Al adsorbed less than the other trace elements, which was comparable with the 28-day experiment results.

Al and Co do not have a significant role in metabolic function and the toxic effect of these elements can adversely affect cellular function (Costley and Wallis, 2001), whereas Fe is an essential element for microbial growth. Espana et al. (2007) stated that the available free Fe ions in the source of released AMD from mine waste dumps is one of main reasons for algal growth. Additionally, the autotrophic mine bacteria that were present in the algal-microbial biofilm assimilate Fe for their energy requirements. Thus Fe was found to be accumulated in the biofilm more than the other elements. This result was also found in the semi-quantitative data of the SEM
images. The highest peak and values belonged to Fe and Cu and this result was observed in the different components of the biofilm including filamentous green micro-algae, bacteria and fungi.

The SEM images demonstrated the significant role of the filamentous micro-alga, Klebsormidium sp., for accumulation and precipitation of elements on their cell walls. Previous studies (Van Hullebusch et al., 2003) reported fine-grained metal deposits closely associated with bacterial cells and a mineral network structure adjacent to the cyanobacterial walls (Ascaso et al., 2002) which demonstrate bio-precipitation of metal/metalloids in microbial biofilms.

5. Conclusion
The reported study provides a novel approach to exploit indigenous AMD microorganisms as effective biosorbents for removing a variety of metals/metalloids from a multi-ion synthetic AMD. This paper presents the efficiency and potential of immobilised resistant algal-bacterial biofilm and PRBCs to be used for the primary treatment of AMD at mining sites under severe acidic conditions. The combination of biosorption and desorption processes in the biotreatment system can be adjusted by application of sequential PRBCs. The achievements through the laboratory scale provided the prerequisite information for scaling up system for future work.

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References


CHAPTER NINE
CHAPTER 9: Summary and Conclusions

9.1 Introduction
The principal achievement of the work reported in this thesis was the development of an applicable biotreatment system in mine sites for reducing the elemental content of AMD. Selection of a resistant and potential biosorbent was a major step in this research that was achieved with the exploitation of indigenous AMD microorganisms, in particular micro-algae. The indigenous biosorbents are naturally found in AMD resources, adapted to low pH and nutrients. The indigenous biosorbent was immobilised in a PRBC as an algal-microbial biofilm resembling their natural habitat, and reported results demonstrate the potential of the developed biofilm system for the primary treatment of AMD, where severe AMD (≤3) is released in mine sites.

9.2 Syn-AMD development
The first challenge of my PhD research was to maintain and culture the indigenous AMD microbial consortium in vitro, which was achieved by AMD synthesis. A Syn-AMD was developed and successfully used for the microbial culture (presented in Chapter 4). Additionally, to validate the biotreatment experiments in my research for further study in mine sites, the realistic composition of AMD was required. Thus, the Syn-AMD was composed of main cations and anions that are commonly found in AMD resources, included nutrient requirements NO₃, NH₄ and PO₄, and Glucose for autotrophic and heterotrophic microbes, respectively. The concentration of each element/mineral for the Syn-AMD was calculated based on the AMD analysis and the achieved results demonstrated close concentrations which are shown in Fig. 9.1. AMDs from many of metalliferrous mine sites contain the same components within the same concentrations. Therefore the formulated multi-ion Syn-AMD presents the general complex recipe applicable for any biotreatment investigation.
Fig. 9.1 Averaged results from AMD and Syn-AMD analysis, presenting the close concentration of each component in both solutions
9.3 Algal-microbial biofilm development

The second and more time consuming challenge in this research was the biofilm development in the PRBC. The algal-microbial biofilm was successfully established and covered the PRBC discs’ surfaces gradually after being treated with extra nutrients (Presented in Chapter 5). The phototrophic strains of indigenous filamentous and unicellular green micro-algae, *Klebsomidium* sp. and *Chlamydomonas* sp. were found in the biofilm structure, under *in vitro* conditions. Additionally, the biofilm was composed of chemolithotrophic iron and iron-sulphur oxidizing bacteria, *Acidithiobacillus ferrooxidance* and *Leptospirillum ferroxidans*, and heterotrophic bacteria, fungi and yeasts, *Bacillus* sp., *Pseudomonas* sp., *Aspergillus* sp., *Penicillium* sp., *Rhodotorula* sp. These microbes were also identified in the collected AMD samples (Chapter 6). Therefore, the indigenous microbes were incorporated in biotreatment investigation in this research *in vitro*. The developed biofilm mass did not changed significantly throughout the experimental period. Detachment was observed after treatment when the PRBC was operating with a solution containing only nutrients. This observation indicated that the importance of major/trace elements on the growth and maintenance of the algal-microbial biofilm. However, after adding these elements the biofilm re-established which highlighted the resistant nature of biofilm that supplied a permanent inoculum resource.

9.4 Elemental removal achievements through batch and continuous processes

The aforementioned microbes involved in the biofilm structure have been documented as significant potential biosorbents (Gadd, 2010; Leitao, 2009; Das *et al.*, 2009; Valdes *et al.*, 2008; Gadd, 2007; Aguilera *et al.*, 2006; Nourbakhsh *et al.*, 2002; Kapoor *et al.*, 1999). However, the absence of information on metal uptake efficiencies by these microbes as an integrated form of biofilm has not resulted in an applicable treatment system for mine sites (Gadd, 2010, 2009, Ahalya, 2003). Recent reviews state that the investigation of metal uptake efficacy by the microbial biomass is essential for the industrial application of biosorption, as it gives information about the equilibrium of the process which is necessary for the design of the measurement plant (Ahalya, Gadd, 2010, 2009, 2003).

Biotreatment investigations resulting from my PhD research provided clarity for the adsorption efficiency of metal/metalloids by the indigenous mine microbes during prolonged treatment period (Presented in Chapters 7 and 8). The weekly results obtained from continuous operation
are presented as graphs shown in Fig. 9.2 that demonstrate the weekly average of adsorption/desorption cycles in the system for various elements. Average adsorption for the major elements was in the range of 20 to 50% (Fig. 9.2a) whereas for the trace elements were within the broad range of 10 to 100% (Fig. 9.2b). The most removed major and trace elements, Cu, Mg, Ag and Fe are also listed as significant contaminants in AMD from Sarcheshmeh copper mine. Thus the exploitation of indigenous microbes benefits the presence of Cu-Mg-Ag-Fe-selective population in biofilm.

Fig. 9.2 Weekly averaged adsorption/desorption for major and trace elements
9.5 Suggestions for future work

Biotreatment results indicated the applicability of the biofilm-PRBC system for the primary treatment of AMD. However, prior to the examination of the system in pilot scale, there is recommended work that must be accomplished including:

1. This research demonstrated the efficiency of a PRBC for removing most of elements from Syn-AMD. However, it is essential to examine the efficiency of system using specific AMD at actual mine sites.

2. Results of this study were achieved with a single stage PRBC. To improve the removal efficiency, in particular for the elements such as Zn, Mn, Mo, Cr, Pb, Se, Sb, Co and Al with lower adsorption (~20%), a series of sequential PRBCs must be designed and used. Additionally, sequential reactors could supplement the adsorption results where desorption occurred.

3. Rotational speed supporting the growth of algal-microbial consortium was optimised through this work. However, most effective rotational speed for elemental removal is also required to be evaluated.

4. The recovery of elements from biofilms, in particular precious elements e.g. Ag must be investigated.
9.6 References


*International Mine Water Symposium, Bunbury, Western Australia, pp. 231-236*

**NOTE:**
This publication is included on pages 153-158 in the print copy of the thesis held in the University of Adelaide Library.