Exploiting Metal-Organic-Microbial Interactions for Metal Recovery in Engineered Systems

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A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2016

Statement of Originality

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ABSTRACT

As global populations grow and become more demanding, resource limitation and the treatment of industrially generated waste constitute an escalating dual challenge. There is, however, a growing awareness of the potential of waste streams as a resource. Metal removal and recovery employing currently available physicochemical techniques are expensive, energy intensive and often unsuitable in cases of voluminous effluents containing complexing organics and low metal concentration. Immobilised biofilms have the potential to simultaneously biodegrade organics whilst in parallel immobilising and recovering metals: the focus of this research. In this study, the maximum metal uptake was 24.60 mg/g and 5.23 mg/g for Zn (II) and Mn (II), respectively, for an initial metal concentration of 80 mg/L. The biosorption of binary system was found to be competitive in nature; Zn (II) removal decreased up to 87% whereas the Mn (II) removal decreased up to 66%.

In order to improve the potential of biofilms as means of recovering resources, the mechanism by which they immobilise metals was investigated. This is the first study to investigate the potential of ultraviolet (UV) as an analytical tool for determining pollutant removal mechanisms by biofilms. A comparative

assessment of chemical (sodium azide) and physical (UV) methods of cell inactivation was undertaken to determine the relative contributions of passive and metabolically active processes on organic and metal removal. Industrial effluents typically contain both organics and metals; therefore, the effect of organics on the metal removal ability of biofilms, which is poorly understood, was elucidated to help develop biologically-based treatment strategies for treating chemically mixed wastes. Chelation had by far the most inhibitory impact on the passive metal removal mechanism, reducing the zinc biosorption by 60% in fully chelated Zn-EDTA complex system. In contrast, nonionic surfactants were the only organics that increased the rate of metal removal, whilst having no detectable impact on biosorption capacity. Hence, they are potentially good candidates for reducing metal-organic competition, assisting metal recovery in the presence of organics. The recovery of biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate (SDBS) surfactant was another novel aspect of this study. This achieved high and stable Zn (II) recovery over four successive biosorption-recovery cycles. Moreover, 94% of the Zn (II) was cumulatively recovered over six successive wash cycles, confirming that SDBS is a potential zinc recovery agent for biofilms. The findings from this study have established that the employment of biofilms to remediate chemically mixed effluents is a favourable potential alternative to energy demanding physical and chemical treatment options.

Peer-Reviewed Papers

Adapa, L.M., Azimi, Y., Singh, S., Porcelli, D., Thompson, I.P. (2016). Comparative study of chemical and physical methods for distinguishing between passive and metabolically active mechanisms of pollutant removal by biofilms. *Water Research* doi: 10.1016/j.watres.2016.06.015.

Adapa, L.M., Singh, S., Porcelli, D., Thompson, I.P. (2016). "Investigation of the interaction of metals and organics in biofilms established to treat chemically mixed industrial wastewaters." Submitted to Journal of Hazardous Materials (In review).

Adapa, L.M., Singh, S., Porcelli, D., Thompson, I.P. (2016). "A novel method of recovering biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate" (In preparation).

Conferences

Adapa, L.M., Ager, D., Thompson, I.P. “Comparative biosorption assessment of Ni (II) and Al (III) from aqueous solution by metalworking fluid acclimated bacterial biofilms.” Poster presentation at V International Conference on Environmental, Industrial and Applied Microbiology, Madrid, 2-4 October 2013.

Adapa, L.M., Porcelli, D., Thompson, I.P. Influence of coexisting organics on heavy metal removal by metalworking fluid acclimated bacterial biofilms. Abstract accepted for platform presentation at III International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, 18-21 May 2015.

This thesis is dedicated to my mother. Her support, sacrifices, encouragement, and unwavering love have sustained me through the duration of the PhD, and life.

Acknowledgements

First and foremost, many thanks to Prof. Ian Thompson for your relentless support and guidance on my thesis and for being a great mentor. Also, thank you Dr. Don Porcelli for your unfailing sharp technical and scientific advice.

I would also like to thank Dr. Yaldah Azimi, Dr. Duane Ager, Dr. Helen Townley, Dr. Errin Johnson and Dr. Kalin Dragnevski who were extremely kind in taking the time to train me on various techniques that were critical to this research. Thank you to all my Begbroke lab members who have been a pleasure to work with over the past four years, and also have become my very good friends! Cordelia R., Li Cheng S., Joan Z., Patrick T., Ana C., Fozia P., Ping S., Meg U., Dr. Naomi W., Mike M., Marimar B., Rachel M., Cindy H., Andi T., Pamela M., Anna H. Special thanks to Shivashkar S. for his brilliant scientific insights and problem-solving ability that helped me progress tremendously in my research and to Dr. Kim W., Jean T., Radhika G., Narmeen R., Sarah W. and Gunjan K. who have been my best friends. Thank you for listening to my daily rants, giving me the best advice and making me laugh on gloomy days; this truly wouldn't have been possible without you! Financial support of Singapore-Peking-Oxford Research Enterprise scholarship is gratefully acknowledged.

I owe a lot to my parents and my brother who have encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true. Thank you for giving me a chance to study in the UK despite everything. I would also like to thank my dear husband and my in-laws for taking good care of me during my write-up and supporting me to see the completion of this work, and my family in India who kindled hope in me greatly influencing my thoughts.

Above all, I owe it all to the Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

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List of Abbreviations

AAS Atomic Absorption Spectroscopy

ANOVA Analysis of Variance

ATP Adenosine Triphosphate

BOD Biological Oxygen Demand

BIT Benzisothiazolinone

BTA Benzotriazole

CFU Colony Forming Units

CMC Critical Micelle Concentration

COD Chemical Oxygen Demand

CO_2 Carbon Dioxide

CTAB Cetyl Trimethylammonium Bromide

DCCD Dicyclohexylcarbodiimide

DI Deionised

DNA Deoxyribonucleic Acid

DNP 2,4-Dinitrophenol

EDTA Ethylenediaminetetraacetic Acid

EPS Extracellular Polymeric Substance

HCl Hydrochloric Acid

HPLC High Performance Liquid Chromatography

KHP Potassium Hydrogen Phthalate

LB Luria-Bertani

MEUF Micellar Enhanced Ultrafiltration

MWF Metalworking Fluid

NF Nanofiltration

NO Nanofiltration

OD Optical Density

PBS Phosphate Buffered Saline

PER Post Exposure Recovery

PEUF Polymer Enhanced Ultrafiltration

RNA Ribonucleic Acid

RO Reverse Osmosis

SD Standard Deviation

SDBS Sodium Dodecylbenzene Sulphonate

SDS Sodium Dodecyl Sulphate

SEM Scanning Electron Microscopy

SOS Sodium Octyl Sulphate

TEA Triethanolamine

TOC Total Organic Carbon

UF Ultrafiltration

UV UltraViolet

CONTENTS

Chapter One

1. Introduction

1.1 Background and scope

The rapidly increasing global population, depleting water resources and climate change have turned fresh water into a competitive resource. Today, more than 1.1 billion people do not have access to clean drinking water and 2.6 billion lack sanitation (Montgomery and Elimelech, 2007). Industrialisation is one of the main reasons behind pollution of water resources and the increase of both organic and inorganic contaminants in supplies. Amongst these are metals which are known to be hazardous pollutants if present in industrial and domestic wastewaters above threshold limits. Indeed, their release causes major concerns in terms of deterioration of the environment, health and as a consequence this leads to heavy fines for the industries (Fomina and Gadd, 2014; Gautam et al., 2013). Pollution is a global problem, causing human sperm counts to be halved in the past 50 years (Dindyal, 2004). Furthermore, as global metal prices increase as a consequence of increasing demand, industries are directing their interest in developing recovery and recycling techniques that can reduce the amount of metal lost in their regular processes (United Nations Environment Programme, 2013). Consequently, it has become essential to develop efficient recycling technologies that remove and recover heavy metals from wastewater prior to discharging into the environment. Prevailing treatment technologies such as chemical precipitation and ion exchange are not

sustainable and are certainly very energy demanding leading to a large carbon-footprint (Jagadevan et al., 2012; Fu and Wang, 2011; Gadd, 2009). Together with ever-increasing cost of waste disposal and pressure from regulators, metal recovery approaches have to become more sustainable with a low carbon and energy footprint (Alluri et al., 2007).

There is a clear need to develop low-cost, sustainable wastewater method to recover metals and enable remediation of the organic component. Biological treatment has been exploited for over a century for water treatment and is now an emerging and attractive option for recovery of low concentrations of metals with relatively low energy demands. Biofilms offer continuous sorption-desorption system and retain relatively high cell concentrations, which occur commonly in engineered systems. They are found to be less sensitive to toxic materials and shock loadings and resistant to antibiotics at levels 500 to 5,000 times higher than those needed to kill non-biofilm bacteria (Pozo et al., 2008). Hence, biofilms were used in this study instead of free-living suspended cells. Metalworking fluids are used as a model waste to be representative of chemically mixed wastes present in engineered systems. However, no studies were performed on real MWF wastewater because chemically mixed real wastewater is typically exposed to brutal regime for a number of months or years, which results in change in its chemistry from the original concentrate and causes variation in composition depending on the application it has been used for. It is enormously challenging since these fluids are chemically complex, including the addition of toxic biocides which are added specifically to retard bio-deterioration whilst being operational. The approach investigated here is the

potential of employing bacterial biofilms to recover metals from chemically mixed wastes. In course of this research, the analytical methods to determine the underlying mechanisms responsible for metal and organic removal were developed and deployed to investigate the interactions between metal and organic pollutants to determine their impact on metal removal by biofilms. The surfactant recovery method presented here enabled recovery of metals from microbial biomass while allowing them to be used for a number of cycles, making the whole process sustainable.

1.2 Thesis layout

This thesis is composed of seven chapters including this introduction chapter. The details of each chapter are summarised below:

Chapter 2: Literature review

This chapter provides a literature review of the different aspects of this study. This starts with a brief overview on heavy metal pollution. Then, conventional metal removal technologies are summarised and their disadvantages outlined in terms of treatment of wastewaters containing low concentrations of metals. The central part of the chapter describes the emerging biological treatment- Biosorption & Bioaccumulation- and the different mechanisms of microbial removal are discussed. This is followed by brief summary of metalworking

fluids (MWFs) and their biological treatment. Biofilms and their exploitation in submerged fixed-film bioreactors are then introduced and their relative merits for the treatment are discussed in the context of chemically mixed wastes. Last but not least, the importance of metal recovery is covered along with some current recovery agents used for metal recovery from microbial biomass. The research objectives are also listed.

Chapter 3: Comparative biosorption assessment of Zn (II) and Mn (II) in single and binary aqueous solutions by bacterial biofilms

This chapter presents experimental investigations to assess the potential of employing metalworking fluid acclimated biofilms to remove Zn (II) and Mn (II) from aqueous solutions in single and binary systems. Zn (II) and Mn (II) were first selected to investigate the differences in metal removal between one that is commonly present divalent ion in metalworking fluids (MWFs) i.e. Zn (II) and one that is not i.e. Mn (II).

Chapter 4: Comparative study of chemical and physical methods for distinguishing between passive and metabolically active mechanisms of pollutant removal by biofilms

This chapter investigates the feasibility of employing UV treatment and compares this physical inhibition method of microbial metabolism with other chemical methods. These are employed to distinguish between passive and active mechanisms of metal and organic pollutants removal from wastewaters. To understand the trends in metal removal of live biofilms and those inhibited by UV, two essential metals i.e. Cu (II) and Zn (II) and one non-essential metal, one that does not play any specific role in cell growth and developments, i.e. Cd (II) were chosen.

This work has been published by Water Research:

Adapa, L.M., Azimi, Y., Singh, S., Porcelli, D., Thompson, I.P. (2016) Comparative study of chemical and physical methods for distinguishing between passive and metabolically active mechanisms of pollutant removal by biofilms. *Water Research* doi: 10.1016/j.watres.2016.06.015.

Chapter 5: Investigation of the interaction of metals and organics in biofilms established to treat chemically mixed industrial wastewaters

This chapter focuses on the individual organic components that form an integral part of MWFs and determines their impact on metal removal. Specifically, the presence of non-interacting organics on metal removal and the influence of other organics on metal chelation, toxic response of the biofilm and surface tension were investigated.

Chapter 6: A novel method of recovering biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate

This chapter introduces the utilisation of sodium dodecylbenzene sulphonate (SDBS) as a desorbing/recovery agent for Zn (II) recovery from biofilms and presents experimental evidence in batch mode to the initial pH, temperature, surfactant concentration, contact time and salt addition. This novel recovery agent is then compared to the commonly used EDTA and citric acid methods. SDBS as recovery agent outperformed the commonly used citric acid method in terms of metal recovery. A possible reason for low metal recovery observed with citric acid could be due to its biodegradable nature.

Chapter 7: Conclusions and recommendations

This final chapter provides general conclusions and recommendations for future work.

Chapter Two

2. Literature Review

2.1 Heavy metal pollution

Among the toxic substances negatively impacting the environment are heavy metals. Heavy metals have industrial, agricultural and military uses and hence, are now widely dispersed in the environment in a range of different forms (Hammaini et al., 2007; Lesmana et al., 2009; Peng et al., 2009). Over the past decades, many different definitions have been proposed for heavy metals; either based on density, atomic number or atomic weight, and some on chemical properties or toxicity. Since the term "heavy metals" has been given such a wide range of meanings, there is a lack of consensus in the literature with regards to the definition of "heavy metal". Duffus (2002) reported that no relationship was detected between density (specific gravity) and any of the various physicochemical concepts that have been used to define "heavy metals" or the toxicity attributed to "heavy metals". The author also pointed out that understanding bioavailability is the key to the assessment of the potential toxicity of metallic elements and their compounds. It is important to note that this term has never been defined by any authoritative body. Considering all these issues, a plant scientist Appenroth suggested the term "heavy metal" should be defined in relation to the position of the element in the periodic table since this position is related to the chemical properties of compounds that include the element (Appenroth, 2010). Therefore, the following three groups

from the periodic table are classified to be heavy metals: 1) transition elements; 2) rare earth elements; which are subdivided into the lanthanide series and the actinide series; 3) some elements from the p-group that are either metals or metalloids/ borderline elements.

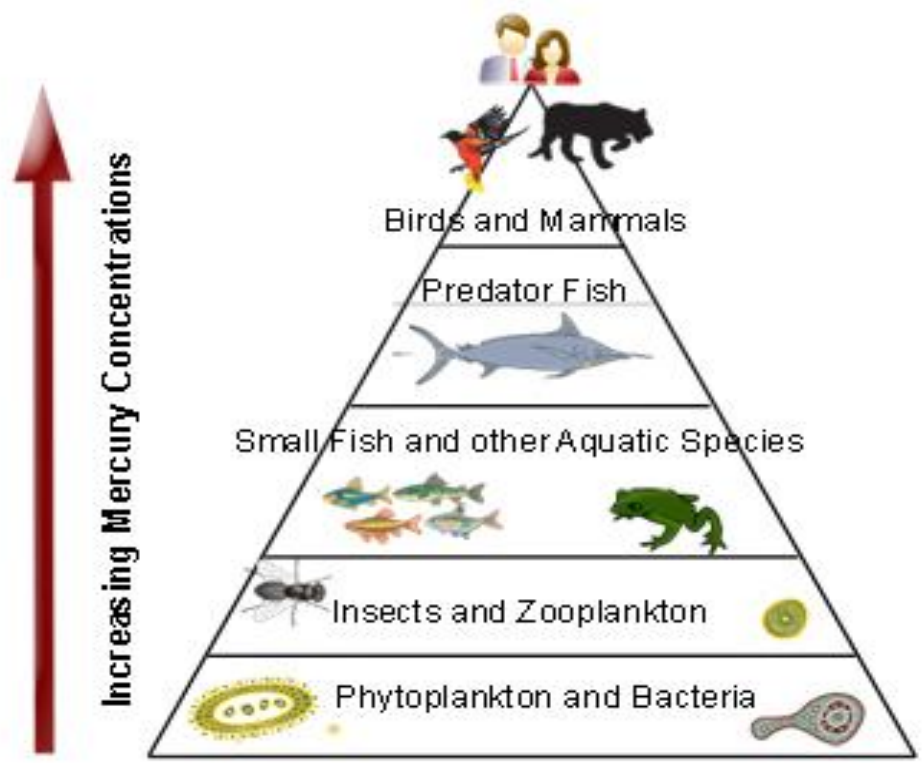


Figure 2.1 Biomagnification effect of mercury in the food chain

The non-biodegradable and persistent nature of heavy metals, their toxicity and their tendency to bioaccumulate in food chains represent one of the major environmental and health problems of our modern society (Volesky, 2001; Wang and Chen, 2006; Ucun et al., 2009; Mudhoo et al., 2012). Figure 2.1 shows the biomagnification effect of mercury in the food chain: humans are affected the most as they are at the top of the food chain because the accumulated toxic metals are at their greatest concentration. Hence, their

removal from industrial effluents is becoming an increasingly important requirement (Volesky, 2001; Fu and Wang, 2011). In addition, the increasingly strict discharge limits on toxic metals and the necessity for recycling increases the importance of being able to recover them at the end-of-pipe (Gupta et al., 2000; Macek and Mackova, 2011; Dodson et al., 2012). Some heavy metals such as Fe, Zn, Cu, Mn, Ni and Co are essential for the health of organisms as they take part in their biochemical reactions; however, they can cause various diseases and disorders in humans when present in high dosages (Chen et al., 2005; Fu and Wang, 2011). Heavy metal toxicity occurs even at low concentrations of about 0.1 and 1 mg/L. Wastewater regulations are established to minimise human and environmental exposure to hazardous chemicals (EPA, 2012). This includes limits on the types and concentrations of heavy metals that may be present in the discharge of wastewater. The metals under investigation in this study are zinc, manganese, copper and cadmium.

2.1.1 Zinc

Zinc is discharged into the global environment at an estimated yearly rate of 8.8 million metric tons (Mudhoo et al., 2012). Since zinc is essential in almost all metal polishing and metallurgical works, its ions are most ubiquitously encountered in wastewater treatment (Gautam et al., 2014). Its dual role, as an essential micro-element and as a toxic environmental factor, makes it impossible to ban the usage of zinc. Toxicity of zinc has significant concerns due to adverse health effects such as depression, circulation and neurological

disturbances and gastrointestinal distress in humans (Barakat, 2011). Excessive adsorption of zinc can also suppress copper and iron adsorption. Current EPA drinking water standards state that the maximum zinc content in drinking water should not exceed 5 mg/L (EPA, 2012).

2.1.2 Manganese

Compared to zinc, manganese has a lower drinking water limit of 0.05 mg/L (EPA, 2012). As with zinc, overexposure to manganese is known to cause neurotoxicity, cardiovascular toxicity, contact or inhalation can damage the central nervous system (Crossgrove and Zheng, 2004). Manganese is also an essential trace element that is required for the activity of several enzymes (Finley and Davis, 1999). Major sources of manganese include welding, fuel addition and ferromanganese production (Alluri et al., 2007).

2.1.3 Copper

Copper in higher doses leads to serious toxicological concerns since it can be deposited in the brain, skin, liver, pancreas and myocardium and initiates internal distress, kidney damage and anaemia (Davis et al., 2000). This trace element copper is also an essential nutrient and serves as a cofactor in respiration and thus, copper is required for aerobic metabolism (Santo et al., 2011). In addition to being one the most important heavy metal used in electroplating industries, copper is also used in other industries such as metalliferous mining, paper and pulp, specialist alloys and steels (Shetty and

Rajkumar, 2010; Gautam et al., 2014). Copper's drinking water limit is 1 mg/L (EPA, 2012).

2.1.4 Cadmium

Cadmium was selected in this study to represent one of the non-essential heavy metals required by humans, having no specific role in cell growth and development. In terms of toxicity, cadmium is one of the most toxic "Big Three" category of heavy metals with the greatest potential hazard to humans and the environment, with lead and mercury (Volesky, 2007). A disease known as "Itai-Itai" in Japan is specifically associated with cadmium poisoning, resulting in multiple fractures arising from osteomalacia. Headaches, nausea, vomiting, weakness and diarrhoea are some of the symptoms of acute cadmium poisoning (Gautam et al., 2014). Major sources of cadmium include welding, electroplating, pesticide fertilisers and CdNi batteries (Alluri et al., 2007).

2.2 Metal removal technologies

Conventional physicochemical treatment methods that are being used to remove heavy metal ions from aqueous industrial effluents include chemical precipitation, ion exchange, adsorption, membrane filtration, electrochemical treatment technologies. The selection of the optimal treatment process for certain industry is dependent on many factors such as the initial metal ion concentration, the capital and operational costs, reliability and environmental

impacts of the process (Kurniawan et al., 2006; Fu and Wang, 2011; Barakat, 2011). In this section, some of the commonly used methods are discussed.

2.2.1 Chemical precipitation

Due to its simplicity and low capital cost, chemical precipitation is the most widely used process for heavy metal wastewater treatment (EPA, 2000). Chemicals are added to the water to convert heavy metal ions into larger, insoluble precipitates that can be filtered to remove suspended contaminants from the water. The main limitation of the hydroxide purification process is that large volumes of relatively low-density sludge are produced causing dewatering and disposal problems (Barakat, 2011). Most importantly, metal precipitation is inhibited by the presence of other constituents in the wastewater, particularly complexing agents (Karra et al., 1985).

2.2.2 Chelating precipitation

It is difficult to achieve the increasingly restrictive water regulations using normal chemical precipitation processes and thus, chelating has been adopted as an alternative (Fu and Wang, 2011). Chelating agents are used to detoxify heavy metals by converting them into chemically inert forms (Sears, 2013). Although chelating agents can be very effective in the removal of heavy metals, they can also be highly toxic and difficult to dispose. For example, ethylenediaminetetraacetic acid (EDTA) is an effective chelating agent but it is

also a persistent organic pollutant, which can compromise water quality targets (Chaudhary et al., 2000; Oviedo and Rodriguez, 2003). In addition, they can also interact and bind to organic and other pollutants present in the wastewaters, adversely affecting heavy metal removal.

2.2.3 Ion exchange

Ion exchange is a process where cations from solid insoluble ion exchange resins are reversibly exchanged with the metal ions present in wastewaters. After separating the loaded resin, the metal is recovered in a more concentrated form by elution with suitable reagents. Addition of ligands that target heavy metals can improve the selectivity of the process. The resins can be loaded and regenerated many times and produce final effluents of very low heavy metal concentration. However, the wastewater requires extensive pre-treatment to avoid resin contamination and the process can be expensive, particularly when treating heavy metal pollution in low concentrations (Gupta et al., 2000). Organics and other contaminant ions will affect the metal removal, particularly if they have the same charge as the metal ions. Moreover, this does not provide any treatment for organics present in the wastewater (Fu and Wang, 2011).

2.2.4 Electrochemical removal

Electrochemical treatment involves plating-out of metal ions on a cathode surface and recovering metals in the elemental state. Rana et al. (2004) demonstrated more than 98% electrochemical removal of chromium ions from

industrial wastewater using carbon aerogel electrodes. These technologies involve relatively large capital investment and require expensive electricity supply, hence have not to date been widely applied (Barakat, 2011; Fu and Wang, 2011).

2.2.5 Adsorption

Adsorption is a mass-transfer process where atoms or molecules are transferred from the wastewater liquid phase to the surface of the solid adsorbent. The atoms are bound to the surface of the solid by physical and/or chemical interactions creating a film of the adsorbate around the surface of the adsorbent. Adsorbents can also be used for the removal of both organic and inorganic pollutants. However, the main challenge usually is to find suitable high adsorption capacity adsorbents for commercial applications. There is a huge potential for customisation of adsorbents for increased selectivity. For example, starch-stabilized zero-valent iron nanoparticles have shown to remove more chromium (VI) from groundwater when compared to other powder adsorbents (0.4 g/L nanoparticles removed 100% of 20mg/L Cr(VI)) (Shao-feng et al., 2005).

Removal of heavy metals by adsorption is growing popularity because it is low cost and can be reversible and hence, adsorbents can be used for desorption. Nanomaterials such as nanoscale zero-valent iron, Fe_2O_3, Fe_3O_4, TiO_2, SiO_2 and Al_2O_3 are the most commonly used materials that have been applied for heavy metal treatment (Mahdavi et al., 2012). Another type of

adsorbents which are industrially attractive are biopolymers because they are capable of lowering transition metal ion concentrations to sub-part per billion concentrations, and are widely available and environmentally safe (Barakat, 2011). However, the presence of oils and suspended particles reduces the efficiency of the process so pre-filtration is required.

Many researchers have worked on the use of activated carbon for heavy metal removal because of its excellent sorption capacity. However, the high cost of these materials limits their large-scale application for metal adsorption and their use has also been prohibited due to low selectivity and regeneration problems. They can be regenerated by thermal desorption of the adsorbed substances or by liquid phase extraction in the case of soluble adsorbed species. However, the former method is not very environmentally friendly and leads to partial mass loss of the adsorbent (Robinson et al., 2001; Sanghi and Bhattacharya, 2002; Crini, 2006; Asouhidou et al., 2009).

2.2.6 Coagulation- Flocculation

Coagulants such as aluminium, ferrous sulphate and ferric chloride are widely used in the conventional wastewater treatment processes. This process de-stabilises colloidal particles by adding a coagulant and results in sedimentation. Coagulation is followed by the flocculation of the unstable particles into bulky floccules to increase the particle size. pH adjustment and addition of ferric/alum salts help the coagulant to overcome the repulsive forces between particles. Some limitations of this treatment include high operational

cost due to chemical consumption and increased volume of sludge (Kurniawan et al., 2006; Fu and Wang, 2011).

2.2.7 Membrane separation

Membrane filtration describes a group of separation processes that use semi-permeable membranes to selectively remove contaminants (Chen et al., 2006). Their main advantages are that they allow permeate and retentate to be used after separation and they require less energy than conventional thermal separation processes. However, limitations include high cost, process complexity, membrane fouling and low permeate flux (Fu and Wang, 2011). Heavy metal removal can be achieved by ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Kurniawan et al., 2006). Figure 2.2 shows various types of membrane-separation processes for water treatment and their application range.

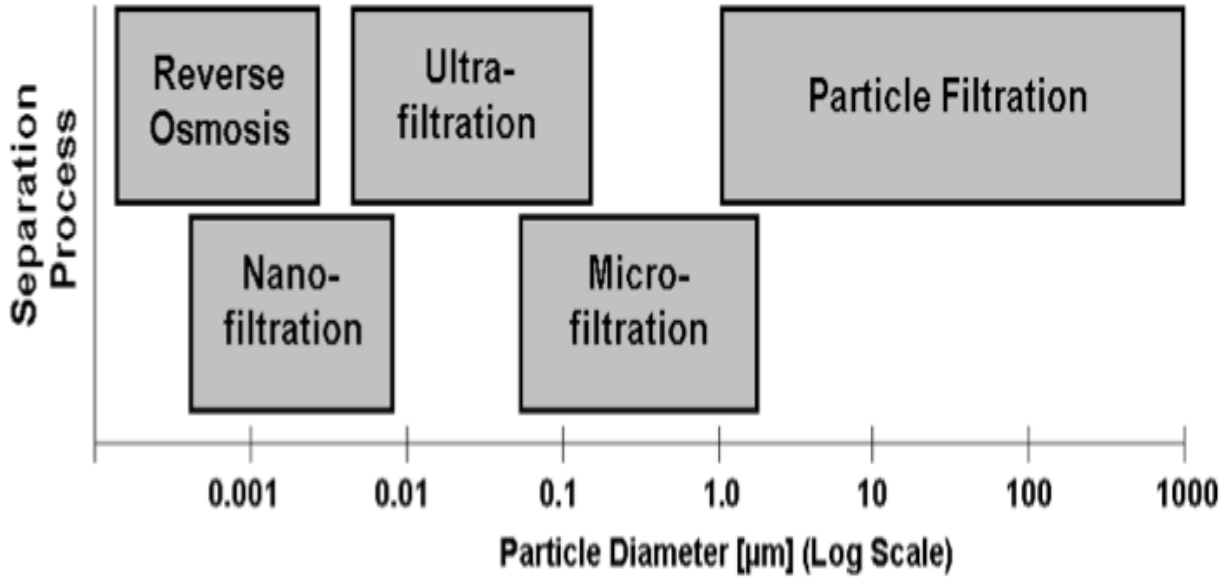


Figure 2.2 Applicability range of ultrafiltration, nanofiltration and reverse osmosis in comparison with other pressure driven processes (Chen et al., 2006)

2.2.7.1 Ultrafiltration

Ultrafiltration is used in the removal of colloidal and dissolved particles in water. Molecules larger than the pore size of the membrane (10^3-10^6 Da) are retained on the membrane and smaller molecules pass through. As heavy metal ions are of small size, complexing agents are added to form aggregates.

Micellar enhanced ultrafiltration (MEUF) has been shown to be effective for heavy metal removal from water with removal efficiencies ranging from 80 to 100% (Fu and Wang, 2011). MEUF works by introducing surfactants to the wastewater to a concentration beyond the critical micelle concentration (CMC) where the surfactants aggregate to form micelles. These bind metal ions into metal-surfactant structures and thereby increase the size of the pollutant molecules so they can be retained in the UF membrane. The removal efficiency of the MEUF process depends on factors such as operating pressure, surfactant and salt concentration and temperature. The main drawbacks of the MEUF process is that the surfactant accounts for a large proportion of the operating cost. Also, the incorrect disposal of the concentrated surfactant retained on the membrane can lead to secondary pollution (Fu and Wang, 2011; Barakat, 2011).

Polymer enhanced ultrafiltration (PEUF) has also been proposed as a feasible method to remove heavy metal ions from water with more effective retention. Water soluble polymers complex with metallic ions, resulting in higher molecular weight, thereby are retained in the filtration process. Studies have shown the PEUF process to be successful in removal of Cu (II) and Cr (III) ions

(Aroua et al., 2007; Camarillo et al., 2012). However, polymer selection for high removal efficiency can be difficult.

2.2.7.2 Nanofiltration (NO)

NO is a promising intermediate process between UF and RO for heavy metal removal. In NF, the separation mechanism involves steric (sieving) and electrical (Donnan) effects. A Donnan potential is created between the charged anions in the NF membrane and the metal co-ions in the effluent, thus creating conditions for the rejection of the latter ones. NF membranes, generally, can treat inorganic effluents with a metal concentration of 2000 mg/L, hence not suitable for treatment of trace heavy metal ions which are usually present in wastewaters (Chen et al., 2006; Kurniawan et al., 2006; Fu and Wang, 2011).

2.2.7.3 Reverse Osmosis (RO)

RO uses a semi-permeable membrane and an applied pressure to overcome osmotic pressure and to remove molecules to produce potable water. The solute is retained on the pressurised side of the membrane as water molecules are forced through the membrane. RO is a highly effective purification technique that is mainly used for desalination of seawater to drinking water. Compared with UF and NF, RO is more effective for heavy metal removal from inorganic solutions, as indicated by the rejection percentage of over 97% with metal concentrations ranging between 20 and 200 mg/L. However, its major drawbacks include high-energy consumption due to high

pumping pressures, high operational costs and the restoration of the membranes (Chen et al., 2006).

2.3 Biological treatment

Microorganisms offer an alternative to conventional physicochemical treatment methods for removing heavy metals and score highly in terms of sustainability. Biosorption and bioaccumulation are implicated in the removal of metal ions from wastewater in all biological systems which are driven by bacteria, fungi, yeast, algae activities (Kaduková & Virčíková, 2005; Das et al., 2008). These biosorbents are able to bind and concentrate dissolved metal ions out of dilute complex solutions with high efficiency and quickly, therefore they are an ideal candidate for the treatment of high volume and low concentration complex wastewaters. Such biosorbents are at least one order of magnitude (1/10) cheaper than traditional technologies, and minimise the volume of chemical and/or biological sludge to be disposed (Wang and Chen, 2006; Volesky, 2007). Moreover, biosorbents like bacteria can easily be produced using inexpensive growth media or obtained as a by-product from industry. This process has many attractive features including the removal of metals over a wide range of pH and temperature, its rapid kinetics of adsorption and desorption, possibility of biosorbent regeneration and metal recovery and low capital and operational costs. Great efforts are being made to improve biosorption process by focusing on improvement of regeneration and reuse, optimisation of biosorption process, enhancing immobilisation technology,

developing an hybrid approach combining both dead and living cells (Gupta et al., 2000; Ahalya et al., 2003; Das et al., 2008; Gadd, 2009; Macek and Mackova, 2011).

2.3.1 Bacterial removal mechanisms

The bacterial cell walls play an important role in heavy metal biosorption. Biosorption mechanisms are complex, though understood to a limited extent, which may be one or combination of physical adsorption, ion exchange, complexation and precipitation (Wang and Chen, 2006; Volesky, 2007; Macek and Mackova, 2011; Aryal and Liakopoulou-Kyriakides, 2015). Understanding the mechanism of biosorption is not only a question of academic interest, there are also practical benefits to be gained. By developing conceptual understanding that allows predictions to be made, lengthy and expensive trial and error process can be avoided. The objective of studying biosorption is obviously to optimise its application. For example, if the mechanism of biosorption is known to be largely based on ion exchange, this implies that changes in the ionic strength of the solution will affect metal uptake (Naja and Volesky, 2011). Furthermore, the choice of desorption techniques also depends on the mechanism involved. Metal binding to acidic groups, for example, can be reversed by lowering the pH and thereby protonating these groups.

Physical adsorption: Microorganisms bind metals as a result of interactions between metal ions in the solution and the negatively charged microbial surfaces by van der Waals forces (Gadd, 2009).

Ion exchange: Polysaccharides that exist on bacterial cell wall consist of counter ions such as K^+, Na^+, Ca^{2+} and Mg^{2+}, which exchange with metal ions resulting in their removal (Ahalya et al., 2003).

Complexation: Ions of heavy metals are bound to functional groups present in bacterial cell walls, such as hydroxyl, carboxyl, phosphoryl and amide groups (Gadd, 2009).

Precipitation: Metal ions removal by metabolically active cells from aqueous solution often associates with active defence system of microorganisms. They react in the presence of a toxic metal producing compounds, which favour the precipitation process. In the case of precipitation not dependent on the cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface (Ahalya et al., 2003).

2.3.2 Comparison of biosorption and bioaccumulation

Large quantities of metals can be accumulated by a variety of processes dependent and independent of metabolism. Biosorption is independent of metabolism and hence, of physical nature that allows for binding contaminants mostly on the surface of the microbial cell wall. It is usually rapid and reversible and requires minimal energy for activation; in the region of 21 kJ/mol, as opposed to the activation energy required for bioaccumulation (63 kJ/mol) (Kaduková & Virčíková, 2005). Bioaccumulation occurs in the presence of living cells and hence, requires higher inputs of nutrients and energy. There is also a need to prevent from toxic effect of heavy metals on cells which may inhibit the

process. In view of this, minimal concentration of heavy metals that inhibits the growth of microorganisms should be determined prior to bioaccumulation. Due to intracellular accumulation, reuse of biosorbent and recovery of metals is rather limited (Vijayaraghavan and Yun, 2008; Aryal and Liakopoulou-Kyriakides, 2015).

In order to optimise pollutants' removal, it is essential to understand the roles and the contribution of metabolic activity relative to more passive physicochemical processes. Moreover, this understanding is also crucial for choosing appropriate desorption methods. To achieve this, researchers have used various techniques such as thermal treatments including autoclaving, exposure to low temperature and use of chemical inhibitors to inhibit microbial cells. However, these have some discrepancies that can lead to misleading physicochemical sorption measurements whereas, others, for example, chemical inhibitors are used without any investigation of their effects on cell structure or the biomass functional groups (Klein and Kennedy, 1997; Delle Site, 2001; Johnson et al., 2007; Das and Guha, 2009). These are detailed in Table 2.1.

Table 2.1 Microbial inhibition methods and their potential discrepancies

Inhibition methods	Discrepancies with the method	References
Thermal treatments through techniques such as autoclaving	Increased permeability of the cell membrane, increased surface area due to cell lysis and structural alteration of biomass and functional groups.	Tsezos and Bell, 1989; Wang and Grady, 1994; Das and Guha, 2009; Johnson et al., 2007
Exposure to low temperatures	Lowers the total kinetic energy of the system and alters the thermodynamic parameters, which affect the physicochemical removal rates.	Boyer et al., 1998; Delle Site, 2001
Use of chemical inhibitors	Unknown	Klein and Kennedy, 1997; Johnson et al., 2007; Das and Guha, 2009

2.4 Model wastewater system

2.4.1 Metalworking fluids (MWFs)

The model system employed in this study was waste MWFs, which have been selected as representative of many chemically mixed industrial effluents. These oily emulsions are commonly used as coolants and lubricants in machining processes for cutting, grinding, boring and drilling, and therefore

contain a mixture of chemicals. They are injected between the metal piece and the tool to provide lubricity in order to remove friction and control thermal distortion by removing the heat, thereby improving productivity, extending tool life, reducing power consumption, and preventing corrosion (Jagadevan et al., 2011; 2012; 2013).

Based on their different chemical compositions and uses, MWFs can be classified into four main categories: straight oils, soluble oils, synthetic and semi-synthetic. Saha and Donofrio have summarised the chemical components of MWFs including their respective roles and their respective percentage concentrations in each type of MWF in a detailed table (Saha & Donofrio, 2012). However, the exact chemical composition of a particular MWF is difficult to establish because the composition of the formulation changes temporally and spatially with use. Furthermore, whilst tools, metals and alloys are being machined, metals may dissolve into the MWF, making these MWF wastes even more hazardous. Therefore, industries are facing particular challenges in terms of treating the waste effluent in a sustainable manner (Cheng et al., 2005; Rabenstein et al., 2009; Teli et al., 2014).

2.4.2 Disposal of operationally exhausted MWFs

MWF becomes less effective after prolong use because of thermal degradation and contamination by microorganisms, and therefore they must be replaced periodically and the spent liquid has to be disposed safely. Hence, the disposal of operationally exhausted MWF places a significant burden on the environment, leading to surface water, soil and groundwater pollution, air

pollution and consequently, agricultural product and food contamination. They are representative of chemically mixed effluents which are generated in significant quantities by engineering industries during machining and other processes and are also challenging to treat sustainably due to their high pollution load (chemical oxygen demand) and toxicity (Jagadevan et al., 2013; Teli et al., 2014). Moreover due to the tightening environmental regulations around the world, the typical means of disposing spent MWFs through incineration is no longer viable (van der Gast et al., 2004b; Jagadevan et al., 2013). Studies have suggested that the disposal costs of MWFs can be over ten times the cost of their purchase and specifically estimated to be £16 million within the United Kingdom alone (Cheng et al., 2005; 2006). Their composition is 95% water and rest composed of chemicals, some of which are summarised below in Table 2.2.

The impact of using MWFs on safety, health and environment is also significant. Workers can be exposed to MWFs in two ways: 1) through skin contact by exposure to splashes during immersion of the machine tool and while handling parts, tools and equipment covered with MWFs and 2) by inhalation of thermal degradation products and of aerosols composed of diverse chemicals, microbial organisms and their respective toxins (Cheng et al., 2005; Howell et al., 2006).

The commonly employed methods to remediate these MWF wastewaters, which include coagulation, membrane separation, incineration or disposal into landfills, are energy intensive and not so sustainable options. Therefore, biological route is an increasingly attractive option because of its effectiveness

and relatively low energy demands. This is however very challenging because MWFs are chemically complex, composed of hazardous biocides which retard microbial deterioration when the fluids are operational (Jagadevan et al., 2013).

Table 2.2 Typical organic components present in MWF formulations.

Components	Purpose in MWFs	References
Emulsifiers and surfactants	Added to water-based fluids to disperse oil drops in the fluid and to reduce the fluid surface tension and critical for producing a stable oil-in-water emulsion.	Skerlos et al. 2008 Negm et al., 2015 Anderson et al., 2003
Corrosion inhibitors	Create a physical barrier, in the form of a dense hydrophobic, monolayer of chemisorbed surfactant molecules, which prevent access of water and oxygen and attack by acids, and peroxides on the metal surface.	Jagadevan et al., 2013
Biocides	Inhibit microbial growth.	Rizvi, 2003
Alkaline reserve compounds	Sodium hydroxide and ethanolamines are added to keep the pH of MWF between 8.5 and 9: this controls bacterial growth, maintains rust protection and retains emulsion stability.	Anderson et al., 2003 Rizvi, 2003
Extreme pressure agents and anti-weld agents	React with metal surfaces at high temperature and pressure to create a protective layer: to guard against welding of the work piece with the metal tool in heavy duty machining.	Anderson et al., 2003

2.4.3 Biological treatment of MWF wastewater

Researchers have investigated the biological treatment of waste MWF (van der Gast et al., 2002; 2003; 2004a; Jagadevan et al., 2011; 2012; 2013; Rabenstein et al., 2009; Kim et al., 1994; Muszynski et al., 2007). Studies reported relatively good removal efficiencies (60-90%) on chemical oxygen demand (COD) and biological oxygen demand (BOD) when researchers isolated the aerobic bacteria from spent MWFs and used the strains to degrade their MWF samples (van der Gast et al., 2004b, Connolly et al., 2006). Muszynski and Lebkowska considered the immobilisation of the isolates on PVC foam carriers for MWF treatment obtaining 87% COD, 97% BOD, 98% of petroleum ether extractable organic and more than 96% of total content of hydrocarbons (Muszynski and Ledkowska, 2005).

Typically zinc, manganese and copper may either be dissolved or present as micro-particulates (less than 100μm) in wastewater from metal cutting facilities and applications (Burke & Gaines, 2006). Since these heavy metals pose a major risk to the environment and human health, it is crucial to remove them from spent MWF before disposal. Biological treatment may offer the option to simultaneously degrade the organic load while simultaneously remove the metals from MWF waste streams; this hasn't been studied to date.

2.4.4 Interaction of metals and organics

The presence of both metals and organics together in chemically mixed waste makes their biological treatment challenging because their interaction can

have a synergistic impact in terms of toxicity. Moreover, certain organics also form strong complexes with the metal ions in solution. For example, ethylenediaminetetraacetic acid (EDTA) is added to some process solutions to sequester metal ions. Successful treatment of effluents of this type to achieve legislative compliance will depend upon whether the heavy metals affect the process of degradation of the organic species and whether the presence of organic molecules hinders the process of removal of heavy metals. Industries for which this type of effluent occurs include metallurgy, cleaning, agricultural and textile (Chaudhary et al., 2000).

Diels et al. (1995) have demonstrated simultaneous recovery of heavy metals and degradation of xenobiotics in a reactor compartmentalised by separation membrane, which also acted as an immobilisation support for bacteria. However, in both aerobic and anaerobic biological treatment systems, the metal toxicity inhibits or delays microbial biodegradation of organic compounds (Said and Lewis, 1991; Kuo and Genthner, 1996; Olaniran et al., 2011; 2013). This inhibition can be explained by Paracelsus' famous phrase, for which he is considered the father of toxicology, which states that the effect of a particular compound is dose dependent and that higher exposures to a hazardous compound will therefore generate higher risks (Pagel, 1982; Langman & Kapur, 2006). The general trend indicates progressive increase in inhibition as the concentration of bio-available metal in co-contaminated system rises; however, addition of metals has also been observed to stimulate activity in some cases (Sandrin & Maier, 2002; 2003). When metals inhibit biodegradation, their effects are not always dose-dependent and there is

evidence for two semi-dose dependent patterns of metal effects on organic biodegradation (Sandrin & Hoffman, 2007): (i) low metal concentration stimulates biodegradation until a maximum level of stimulation is reached and thereafter, metal toxicity increases with increasing metal concentration; (ii) low metal concentration increasingly inhibits activity until a maximum level of inhibition is reached and thereafter, metal toxicity decreases with increasing metal concentration. The impact of metals, linked to their bioavailability and toxicity, on the biodegradation of organic pollutants has been extensively studied and appropriate strategies to allow effective biodegradation of targeted organic pollutants have been well documented (Sandrin and Maier, 2002 & 2003; Gadd, 2010). Said and Lewis even quantified the inhibitory effects of heavy metals on aerobic microbial degradation rates of organic chemicals (Said and Lewis, 1991). However, the effects of organic compounds on metal removal from aqueous solutions or mixed effluents have received little attention. Hence, the need to investigate the influence of co-contaminating organics on metal removal arises, particularly in industrial chemically mixed wastes.

2.5 Biofilms

2.5.1 Emerging alternative for the treatment of co-contaminated waste waters

Many industrial waste streams are co-contaminated with both organics and heavy metal components and there is growing drive to treat these using sustainable approaches. Microbial communities, particularly in the form of biofilms, are usually detrimental in industries where they lead to fouling and

blockages. However, they have recognised potential for remediating both organics and metals in wastewater (Singh and Cameotra, 2004; Singh et al., 2006; Edwards and Kjellerup, 2013). Consequently, there is growing interest in exploiting their potential in industrial applications since conventional treatment methods for dealing with hazardous effluent score poorly in terms of sustainability (Jagadevan et al., 2012). Biofilm formation is a natural process where microbial cells attach to a support or form flocs/aggregates without the use of chemicals and form thick layers of cells known as "biofilms" with cell concentrations as high as 74 g/L (Qureshi et al., 2005). In the remediation process, microorganisms remove organic pollutants through biosorption and active biodegradation while simultaneously accumulating heavy metals intracellularly or by immobilising them on the cell surface (Malik, 2004; Aksu, 2005; Vijayaraghavan and Yun, 2008). Biofilm reactors are increasingly used to treat industrial effluents because they are able to retain relatively high biomass concentrations, which are less sensitive to shock loadings and toxic materials. This ability to retain high biomass concentrations reduces reactor size, area requirement and capital costs of the biofilm reactor plant, when compared to suspended growth reactor system. Immobilised biomass also offers the continuous sorption-desorption system (Gupta et al., 2000; Toner et al., 2005).

Exploiting biofilms composed of mixed communities is a long established cornerstone of municipal treatment systems, however their deployment in treating industrial waste waters, which are more toxic and recalcitrant, is far less accepted as serious treatment options. If biofilms are going to become a serious option in terms of providing a robust, reliable and sustainable treatment, it is

essential to have better understanding of how they function and their ability to deal with the challenging conditions of chemical mixtures. Currently, our understanding of how the immobilisation of metals by biofilms may be influenced by the presence of co-contaminating organics is very limited. To optimise biofilms to deal with such challenges reflective of the real world, it is essential to develop effective methodologies to enable understanding of their functioning in chemically mixed exposures. And also, there is a need to understand the interactions of the various organics present on metal removal and the effect of metals on the biodegradation of organics.

2.5.2 Biofilm formation

There are four stages to the development of a mature biofilm: initial attachment, irreversible attachment by the production of extracellular polymeric substances (EPS), early development and maturation of biofilm architecture (Figure 2.3). Free-floating or planktonic bacteria can become attached to a submerged surface within minutes and begin to produce slimy extracellular polymeric substances (EPS) and to colonise the surface. EPS production allows the emerging biofilm community to develop a complex, three-dimensional structure that is influenced by a variety of environmental factors. Biofilms can then propagate through detachment of small or large clumps of cells, or by a type of "seeding dispersal" that releases individual cells. Either type of detachment allows bacteria to attach to a surface or to a biofilm downstream of the original community (O'Toole et al., 2000; Stoodley et al., 2002).

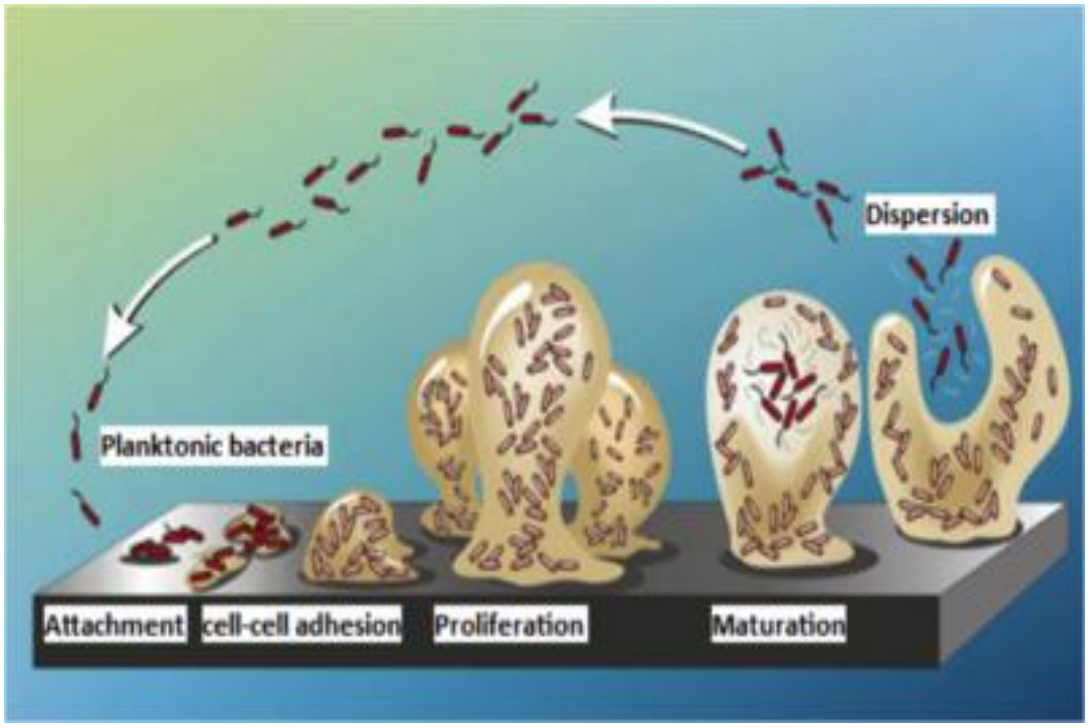


Figure 2.3 Illustration of the main stages of biofilm development: 1) Reversible attachment of cells to the surface 2) Irreversible attachment of the cells on the surface as a result of EPS production 3) Maturation of biofilm architecture 4) Dispersion of single cells from the biofilm (Stoodley et al., 2002).

2.5.3 Importance of the immobilisation matrix

The surface onto which cells will attach has an important impact on biofilm formation. Rough surfaces tend to enhance biofilm formation because shear forces are lower near a rough surface, and there is larger surface area to which cells can adhere. Their formation also increases with the hydrophobicity of the surface material. Biofilms form much more rapidly on Teflon and other plastics than glass or metal. This is possibly due to the differences in hydrophobicity of the surfaces and ionic charges (Naja and Volesky, 2011).

Microbial cells are basically small particles, with low density, poor mechanical strength and little rigidity. Hence important issues of concern include solid-liquid separation problems, possible biomass swelling, inability to regenerate and development of high pressure drop in the column mode. Immobilisation techniques have exhibited greater potential with benefits including the control of particle size, ease of separation and the reuse and regeneration of the used biomass (Hu and Reeves, 1997). The choice of immobilisation matrix is a key factor in the environmental application of immobilised biomass. The polymeric matrix determines the mechanical strength and chemical resistance of the final biosorbent particle to be utilised for successive sorption-desorption cycles. Furthermore, a successful immobilisation matrix should allow most active binding sites to have access to the solute, which means large surface area.

The immobilisation material used in Chapter 3 is EXPO-NET's BIO-BLOCK® (Figure 2.4), made of polyethylene material, which is widely used in industry for biological trickling filters. These cylinders have a large specific surface area ($300 m^2/m^3$) with 65 % of hollow space and suitable surface topology (www.expo-net.dk). However, due to uneven growth of biofilms on these structures which were important for microscopic analysis, a new supporting material was used for the remaining chapters. A welded stainless steel mesh with 0.81 mm wire diameter was initially tested and results showed similar biosorption metal removal (mg/g biofilm) with both types of immobilisation matrices. Unlike the polyethylene material, this metal mesh gave an even biofilm coverage which was necessary for performing microscopic analysis for

the remaining chapters of the study. Biofilms grown on these two types of immobilisation matrices are shown in Figure 2.4.

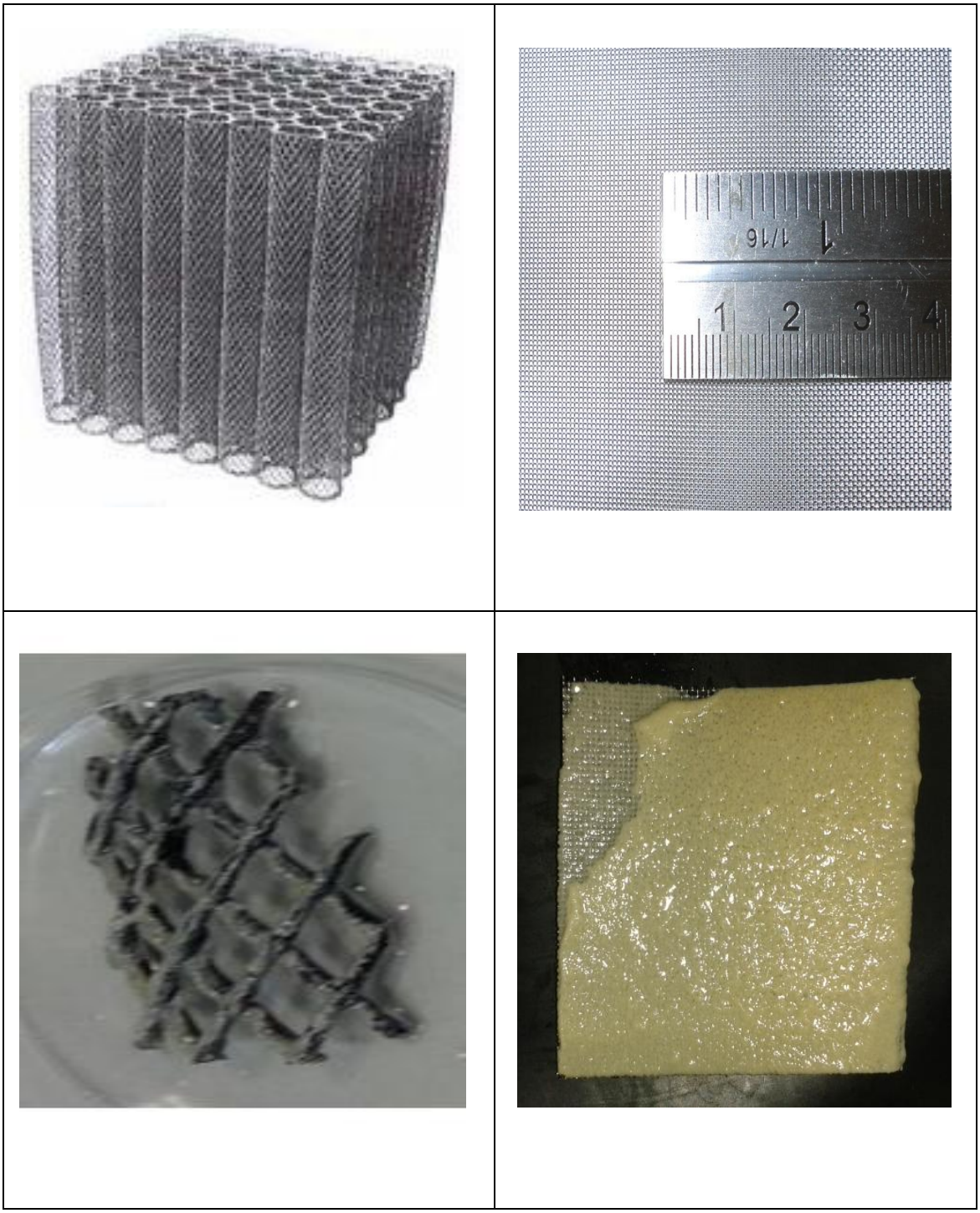


Figure 2.4 Two types of immobilisation matrices for biofilm formation: Expo-net's Bio-blok® (top left) and stainless steel mesh (top right). Uneven biofilm formation on Bio-blok (bottom left) and even biofilm formation on stainless steel mesh (bottom right).

(Available at: www.expo-net.dk and www.streme.co.uk)

2.5.4 Use of mixed community vs. single strains

The use of microbial mixed cultures has proved to be more suitable for bioremediation than pure cultures because of the following two reasons. Firstly, their biodiversity is known to enhance environmental survival, making mixed community cultures more resistant to toxic compounds and extreme conditions like spikes of pH or high metal concentration, which are often encountered with industrial wastewater. Secondly, there is an increase in the number of catabolic pathways available for the biodegradation of the environmental pollutants, increasing the efficiency of pollutant removal/degradation thus providing a more realistic picture of real waste streams (Malik, 2004; Smith et al., 2005; Cheng et al., 2005). However, most biosorption studies to date have been conducted using single species of bacteria only (Toner et al., 2005; Johnson et al., 2007; Das and Guha, 2009; Guo et al., 2012). Biofilms formed from mixed community are even more advantageous as the exo-polymer content of the biofilms are able to entrap dispersed solids and also biosorb dissolved metals. It has also been shown that the positive interaction between constituent species can also facilitate the survival of sensitive strains (Bradshaw et al., 1998). However, the cultures need to be acclimated and stabilised before their applications because for example, specialised species enriched from spent MWF have the ability degrade almost all components of MWF effluent, including synthetic ones. They are also much more resilient than the microorganisms used in sewage treatment works, because they are adapted to the highly polluting and toxic nature of the MWF effluent (van der Gast et al., 2003; Thompson et al., 2005).

2.5.5 Submerged fixed-film bioreactors

In this system, biofilms are grown on immobilisation matrices submerged in the bioreactor tank, and generated specifically for treating metal-organic contaminated wastes. The organic material in the wastewater is degraded by aerobic microorganisms present in the biofilm, whereas metals are immobilised on the surface of the biofilm or taken in either by intracellular accumulation and/or passive diffusion. Immobilisation allows desorption of metals and regeneration of the biofilm to develop a sustainable process. Diffusers are used to provide air bubbles for both aeration and turbulence, latter of which prevents the excessive biofilm growth. Some advantages associated with fixed-film over suspended-growth processes include (Singh and Cameotra, 2004; Qureshi et al., 2005; Singh et al., 2006; Edwards and Kjellerup, 2013):

- Reduced operating and energy costs;
- High volumetric biomass concentrations allowing to have smaller reactor volume;
- Resistance to short-term toxic loads;
- Operational simplicity; and
- Reduced sludge.

2.6 Metal recovery

2.6.1 The future

With the EU legislation on 20% reduction in carbon emissions by 2020 compared to 1990 levels, the key driver is "low carbon technology" which is driving technological change (www.theccc.org.uk). Global sources of metals such as zinc, copper, cadmium resources are not only depleting at a remarkable rate as seen in Figure 2.5 but we are also quickly dispersing them throughout our environment, making it more costly and difficult to recover them (Dodson et al., 2012). In conjunction with environmental issues, there are monetary costs associated with the loss of metals.

Figure 2.6 shows the forecast change in annual average prices of different metals in 2011; of which tin, zinc and copper, which are all heavy metals, were expected to rise by 24%, 12% and 10%, respectively (Norgate and Rankin, 2002). Moreover, carbon tax is also shown to have the greatest effect on the price of aluminium, followed by steel, zinc, lead, copper and nickel in that order (Figure 2.7) (The Economist, 2011). Thus, the increasingly imposition of carbon tax combined with the accelerated rise in metal prices and depletion of metal resources further intensifies the need for metal recovery. The good thing about metals is that they can be recycled repeatedly without altering its properties. The recovery, recycling and reuse of metals from waste streams is a cost effective and environmentally beneficial route to valuable materials, which can fulfil the needs of the current and the future generation.

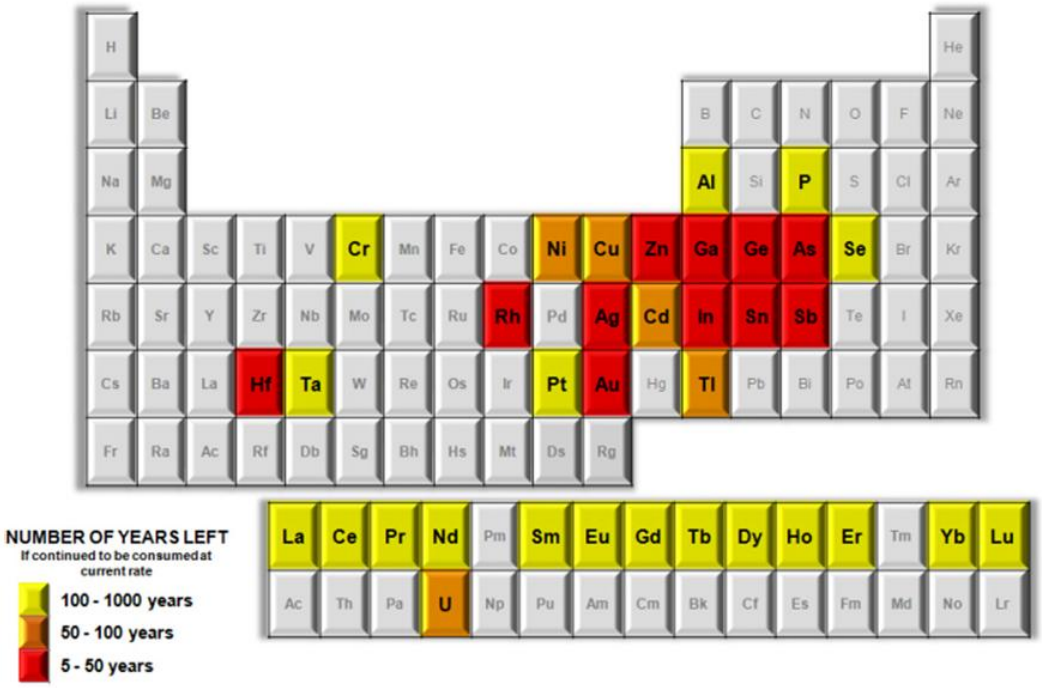


Figure 2.5 Number of years remaining of metal reserves if consumption and disposal continues at present rate (Rhodes, 2008; Dodson et al., 2012)

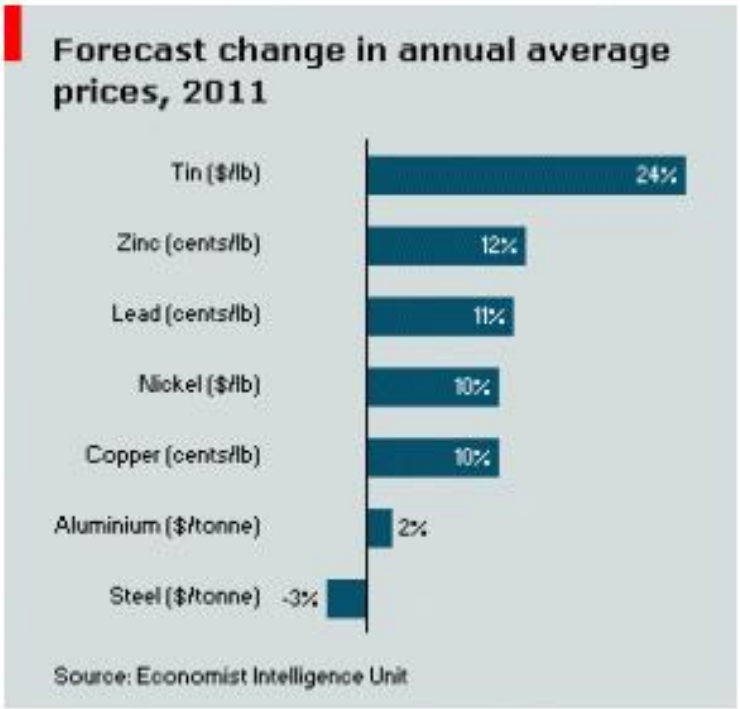


Figure 2.6 Forecast change in average annual metal prices (The Economist, 2011)

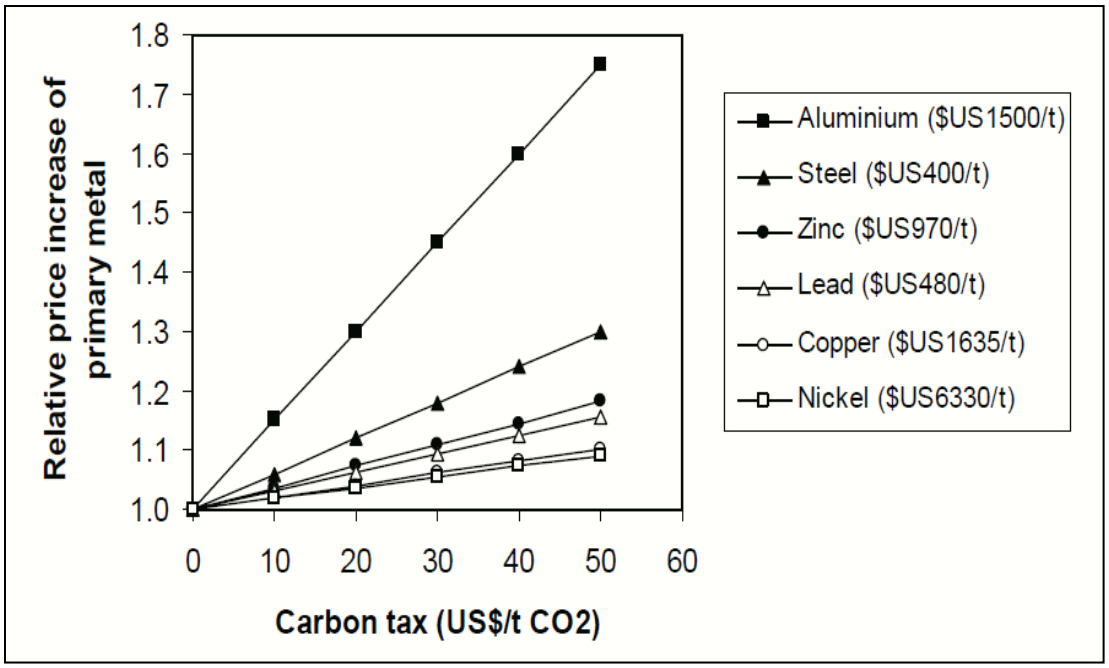


Figure 2.7 Effect of a carbon tax on metal prices (Norgate and Rankin, 2002)

2.6.2 Biosorption as a potential metal recovery method

There are several novel methods in their infancy with great potential for metal recovery, such as the use of hyperaccumulating plants, biosorption using biomass and bacterial leaching. To achieve efficient metal recovery through biosorption process, metals must be effectively desorbed and concentrated (Volesky, 2001; Dodson et al., 2012). The elution of copper sorbed on the cells of *M. luteus* has been shown to be more than 90% by washing with 0.05 M sulphuric acid within five minutes (Leung et al., 2000). In another study, copper

and zinc desorption efficiencies of 95.3% and 83.8%, respectively, were achieved by non-living cells of *Pseudomonas putida* CZ1 using 0.1 M HCl (Chen et al., 2005). These results indicate the potential for a low cost continuous metal adsorption-desorption system, making the recovery of metals from the liquid phase an economically viable solution.

2.6.3 Criteria for good desorption/recovery agent

The purpose of the recovery process after biosorption is to desorb the metals and to regenerate the biomass to be used in subsequent biosorption and recovery cycles. Desorption plays an important role, especially when the biomass preparation and generation is costly, because it allows to decrease the process cost and also reduces the dependency of the process on a continuous supply of biomass. Hence, the desorption/recovery agent needs to be carefully selected, which strongly depends on the type of biomass and the mechanism of biosorption (Vijayaraghavan and Yun, 2008). For example, if the main mechanism responsible is electrostatic attraction, then it would be logical to make the surface of the biosorbent negative using alkaline solutions to repel the negatively charged biosorbent. Hence once again, there is an important need to understand the different mechanisms by which metals are removed by the biomass and for this, effective techniques to quantify the different proportions of metal removal by each mechanism must be developed and made available to researchers. In this way, novel and more effective desorption agents can be researched, potentially making the whole biosorption process more economical

and sustainable. Another important factor is the amount of the recovery agent, which should be as low as practically possible to obtain the maximum metal recovery in the smallest possible amount with minimum number of washes (Volesky, 2001). The following is a list of criteria that the desorption/recovery agent must meet:

- Non-damaging to the biomass so that both the biosorption and the recovery efficiency do not decrease over subsequent cycles;
- Cheap and reusable to minimise process costs;
- Environmentally friendly to minimise any external costs on the environment, humans and the society and to be acceptable by legislation; and
- Effective (Veglio and Beolchini, 1997; Gadd, 2009).

2.6.4 Current desorption/recovery agents used for metal recovery from microbial biomass

Dilute mineral acids (HCl, H_2SO_4, HNO_3), organic acids (citric, acetic, lactic) and complexing agents (EDTA, thiosulphate, etc.) are commonly used in the literature to recover metals (Alluri et al., 2007; Hammaini et al., 2007; Shetty and Rajkumar, 2010; Lata et al., 2015). Desorption studies till date have been conducted using single species and activated sludge; none using biofilms (Vijayaraghavan and Yun, 2008). Many researchers also perform desorption studies for only one cycle, however this information is incomplete and gives no information on the reusability of the biofilm (Vijayaraghavan and Yun, 2008).

Table 2.3 Commonly employed agents for recovering metals from microbial biomass and their weaknesses.

Recovery agents	Description of the study	Weaknesses	References
Hydrochloric acid (HCl)	Desorption experiments from activated sludge showed that HCl was a good eluent for the five metals tested, particularly at low pH values (1 and 2).	Aggressive effect exerted by HCl on molecules of biological tissues, did not allow for reuse of biomass.	Hammaini et al., 2007
Acid, alkaline and potassium thiocyanate	High cobalt desorption efficiencies were found from *Ascophyllum nodosum* using these agents at various pH values.	Electron micrographs of the algal biomass showed some changes in cellular structure after desorption.	Kucayak and Volesky, 1989
Citric acid	44% copper desorption by metal resistant *Pseudomonas sp.* during the first cycle, which was significantly decreased during subsequent cycles.	Relatively lower metal desorption compared to other recovery agents and its damaging effect on the biomass.	Shetty and Rajkumar, 2010
Ethylenediaminet-etraacetic acid (EDTA)	89-95% zinc ions were desorbed with 0.1 M EDTA from immobilised *Spirulina platensis,*	EDTA found to be persistent and remains in nature for 15 years	Gaur and Dhankhar, 2009

	which allowed the reuse of biomass in three biosorption-desorption cycles without considerable loss in biosorption capacity. 100% recovery of Pb, Cu, Cd, Zn, Ni from activated sludge at 1 mM was found, with the reuse of biomass for three subsequent-desorption cycles.	following disposal. It is actually banned in parts of the USA, severely restricted in some countries and carefully controlled in others (Chaudhary et al., 2000; Oviedo and Rodriguez, 2003).	Hammaini et al., 2007

Table 2.3 summarises common metal recovery agents used in microbial biosorption studies along with their weaknesses and limitations. Some studies focusing on metal recovery from microbial biomass using acids and alkaline have demonstrated cellular destruction, which reduced the biosorption and desorption capacities in subsequent cycles. On the other hand, organic acids like citric acid gave lower recovery amounts. In contrast, EDTA has good recovery agent characteristics but the main problem is that it is persistent in the environment and therefore, has been banned in USA and several other countries (Chaudhary et al., 2000; Oviedo and Rodriguez, 2003). Hence, there is scope to research a novel recovery agent to desorb metals from microbial biomass.

2.6.5 Use of surfactants as metal recovery agents

Surfactants are commonly used for the recovery of metals from contaminated soils because of their excellent surface active properties, anionic nature and low toxicity (Wuana et al., 2011; Peng et al., 2009). Hence, they are a preferred option compared to complexing agents for site remediation (Ramamurthy et al., 2008). The major users of surfactants have traditionally been petroleum users, where they are used for enhanced oil removal applications. Removal by surfactants takes place either by increasing the solubility of petroleum components or by lowering the interfacial tension to enhance mobility of the petroleum (Mulligan et al., 2001).

Surfactants are amphiphilic compounds, which mean that their molecules contain both water soluble (hydrophilic) and water insoluble (hydrophobic) portions. The water solubility of the surfactants is due to the hydrophilic portion, while the hydrophobic portion tends to concentrate at the air-water interfaces or in the centre of micelles, reducing the surface tension of the solution. For example, sodium dodecyl sulphate (SDS) has the dodecyl chain which has very low water solubility and the sulphate group with high water solubility. Their key ability is to form micelles: surfactant molecules can self-assemble into dynamic clusters at lower surface and interfacial tensions (Figure 2.8). This characteristic allows them to act as a detergent and have properties such as emulsifiers, foaming and dispersing agents (Mulligan et al., 2001; West and Harwell, 1992). Micelle formation takes place above a critical concentration of surfactant monomers, known as the critical micelle concentration (CMC) which is unique to

each surfactant (Mulligan et al., 2001; West and Harwell, 1992; Bustamante et al., 2012).

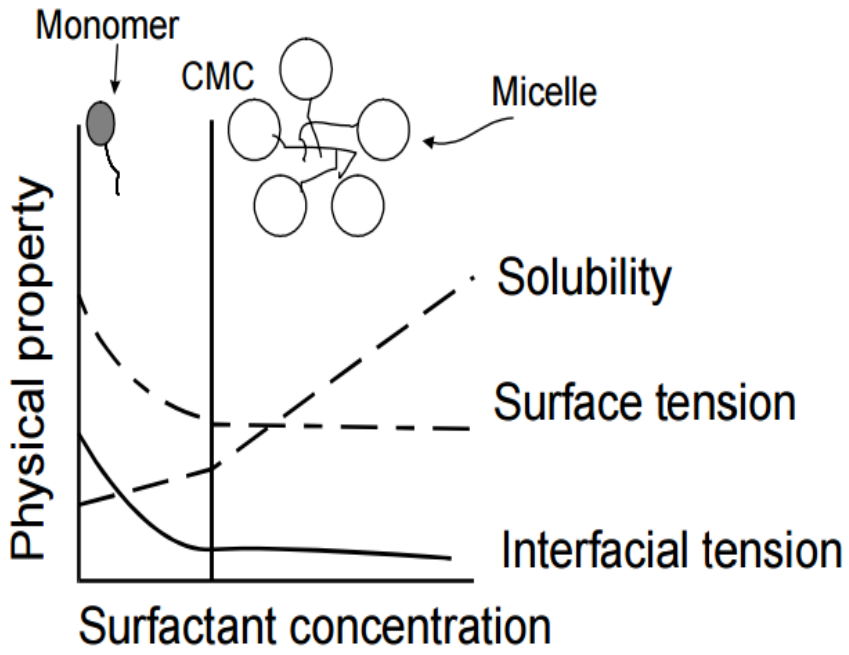


Figure 2.8 Schematic diagram of the variation of surface tension, interfacial and contaminant solubility with surfactant concentration (Mulligan et al., 2001)

Several researchers reported enhanced electrokinetic remediation efficiency of hydrophobic organic compounds in soil by introducing surfactants (Yuan and Weng, 2006; Karagunduz et al., 2007). Moreover, researchers have shown that the surfactants could be used to enhance the heavy metals desorption from soil and sludge (Mulligan et al., 2001; Ramamurthy et al., 2008), potentially due to the hydrogen bonding and electrostatic forces between the surfactants and metals. In soil remediation, chemical (SDS) and natural (humic acid) surfactants have been used to enhance the electrokinetic removal of cadmium from contaminated soil (Giannis et al., 2007).

Surprisingly, no published study to date has reported on investigating employing surfactants to recover metals from bacteria, after biosorption. A possible explanation for this void could be that most surfactants, like sodium dodecyl sulphate (SDS), are known to prevent the formation of biofilm and to kill bacteria (Ridgway et al., 1996; Li et al., 2013). The main factors that should be considered when selecting surfactants include effectiveness, cost, public and regulatory perception, biodegradability and degradation products, toxicity to humans, animals and plants and ability to recycle. Obviously, the first consideration is that the surfactant is efficient in removing the pollutant. This can be established either by previous experience or by laboratory studies prior to the field-scale demonstrations. Most importantly, surfactants must be recovered and reused for the process to be economic (Mulligan et al., 2001; Lata et al., 2015).

2.7 Research Objectives

Metal-organic-microbial interactions have huge potential in terms of sustainable metal recovery in engineered systems. The general metabolic activities of microbes affect metal distribution and bioavailability, while simultaneously with the potential to biodegrade some organics. Although the presence of multiple competing ions is more frequent than the existence of a single contaminant in the real world, only a few detailed studies have dealt with the topics of competition and interference related to heavy metal and organic pollutants removal.

The overall aim of this study was to exploit the interactions between microbes, metals and organics to recover metals from engineered systems whilst in parallel degrading the organic components. Metalworking fluids (MWFs) were used as a model representative of chemically mixed wastes in this study. Firstly, the ability of MWF acclimated bacterial biofilms to remove zinc and manganese when present singly and in combination in aqueous solutions were investigated and compared to free-living suspended cells. Freundlich and Langmuir models were employed to describe the biosorption equilibrium and pseudo first and second order for kinetic modelling and estimating the sorption rates. In order to find the dominant mechanism of pollutant removal, chemical and physical inhibition methods of microbial metabolism were compared, with UV being introduced for the first time as an analytical tool. Surfactants, corrosion inhibitors, biocides, chelators are commonly used in MWFs to stabilise pH or inhibit corrosion, and thus play a critical and essential role in machining processes. Thus their ubiquitous presence in MWF waste streams make it essential to understand how they might affect biofilm specifically used in end-of-pipe treatment for immobilisation of metals. The effects of such organic compounds on metal immobilisation efficiency and their possible influence on general metal behaviour in the system were thus studied. An additional aim was to find the most efficient way in terms of recovery and biofilm regeneration to remove the biosorbed metals from the microbial biofilms. Surfactants were employed for the first time as potential metal recovery agents from microbial biofilms. Batch equilibrium experiments

were conducted to optimise the process and then compared to commonly used chelating agents and organics acids for metal recovery.

To this end, the research objectives were as follows:

1. To study the equilibrium and kinetics of Zn (II) and Mn (II) removal by metalworking fluid acclimated biofilms from aqueous solutions when present individually and in combination.

2. To compare chemical and physical methods of microbial inhibition for distinguishing between passive and active mechanisms in biofilms removing metal and organic pollutants and to assess their relative merits.

3. To determine the dominant mechanism of removal for three metal (Zn (II), Cu (II), Cd (II)) and two organic (triethanolamine and benzotriazole) pollutants using chemical and physical inhibition methods and establishing their relative merits and discrepancies.

4. To explore metal-organic interactions and to study their impact on the biotreatment of chemically mixed wastewaters.

5. To investigate surfactants as potential recovery agents to desorb metals from microbial biomass and to compare sodium dodecylbenene sulphonate (SDBS) with the commonly employed EDTA and citric acid to determine the most efficient and sustainable Zn (II) recovery agent from microbial biofilms.

Chapter Three

3. Comparative Biosorption Assessment of Zn (II) and Mn (II) in Single and Binary Aqueous Solutions by Bacterial Biofilms.

3.1 Introduction

Biosorption of metals by immobilised microbial systems is being increasingly used for the removal of metals from industrial effluents (Singh et al., 2006; Rani et al., 2011; Edwards and Kjellerup, 2013). The growing appeal is that the approach is sustainable. Metals are sequestered by different parts of either live or dead microbial cells via various physicochemical processes such as absorption, adsorption, ion exchange, surface complexation and precipitation (Gadd, 2009; Wang and Chen, 2009). The major advantages of the biosorption technology are its selectivity and effectiveness in reducing the concentration of metal ions when present in very low levels, its rapid kinetics of adsorption and desorption as well as significant low capital and operational costs (Macek and Mackova, 2011). Furthermore, adsorbed metals can be easily recovered from the biomass by many chemical and physical methods, leading to potentially repeated use of the biomass and better process economy (Gautam et al., 2013).

Bacteria are exploited in this process because of their small size, ability to grow under controlled conditions and adaptability to a wide range of

environmental conditions. Mixed communities of bacteria are typically present in real contaminated sites and therefore provide a more stable and robust approach than single strains.

Although few have investigated the comparative assessment of biosorption between different metals, none have focused on the behaviour of Zn (II) and Mn (II) in multi-metal systems. Vijayaraghavan et al. (2012) reported that the presence of Cd (II) severely affected Al (III) uptake by an inactive brown seaweed, *Turbinaria conoides,* when investigating biosorption of the two metals in both single and binary systems (Vijayaraghavan and Gupta, 2012). In single systems, they proposed high ionic electronegativity and ionic radii favoured Cd (II) biosorption better than Al (III) biosorption. In another study, variation of adsorption of lead and other heavy metals by biofilms sampled from Nanhu Lake were also explained due to the differences in atomic radii and standard electrode potential; the latter of which was positively correlated to the electronegativity of a metal (Dong et al., 2003). However, Lim et al. (2008) indicated that there were other factors that could also play important roles in the process: the adsorption of Cu (II) onto sawdust was found to be greater than Zn (II), even though the ionic radius of Zn (II) is larger than Cu (II) (Lim et al., 2008).

The influence of metals on organics degradation has been extensively studied (Sandrin and Maier, 2003; Sandrin and Hoffman, 2007; Olaniran et al., 2011). However, there are currently no published papers of the influence of organics on the ability of biofilms to immobilise metals, an important factor in terms of real world exploitation where waste streams are chemically mixed.

Therefore, this chapter is an initial study which provides insight into the biofilms' metal immobilisation ability in the absence of contaminants and organics.

Biofilms formed from metalworking fluid (MWF) acclimated bacteria were used in this study to investigate its biosorption capacities for removal of divalent Zn (II) and Mn (II) cations from aqueous solutions as an initial step to successfully remove heavy metals from MWF waste streams. The effects of various parameters such as initial temperature, initial pH, initial metal ion concentration and contact time were investigated. Primarily, the metal removal ability of MWF acclimated bacterial biofilms to remove zinc and manganese when present individually in aqueous solution system was compared and their interaction in binary systems was then studied. Freundlich and Langmuir models were used to describe the biosorption equilibrium and pseudo second order was used for kinetic modelling. Metal immobilisation by biofilms was then compared to the free-living cells of the same bacterial consortia and also to heat treated and autoclaved cells. SEM characterisation and toxicity tests were also performed to gain further insight into the biosorption trends observed for these two metal ions.

3.2 Materials and Methods

3.2.1 Description of bioreactor system

Suspensions of a mixed consortium bacteria were freeze-dried to ensure uniform inoculum was employed for each experiment. The consortium was supplied by Microbial Solutions Ltd. (a small company specialised in the disposal of metalworking fluids) and originated from an aged oil-based metalworking fluid (MWF) bioreactor adapted to treating Castrol CoolEdge BI. The seed consortium and method used to develop this reactor was described by van der Gast and Thompson (van der Gast and Thompson, 2004a). MWF was selected as a model effluent since 95% of it is water, with the remainder of the composition being organics and metals, and there is a growing need to develop sustainable methods for its end of life treatment (van der Gast et al., 2004b). Before freeze-drying, the culture was centrifuged and the resulting cell pellet was resuspended in sterile *Mist dessicans,* which is 1:3 (v/v) solution of Luria-Bertani (LB) broth, with 10 g/L D-glucose anhydrous (Fisher Scientific), in horse serum. Two mL of this was then dispensed into sterile freeze-drying vial, frozen at -80^{O}C overnight, and freeze-dried for 24 hours at -55^{O}C.

Two mL of phosphate buffered saline (PBS) solution was used to resuspend a freeze-dried inoculum before it was used. The biofilms were grown using two vials of the freeze-dried stocks in a 4 L bioreactor with 800 mL autoclaved M9 minimal medium and 1% v/v fresh MWF (CoolEdge BI concentrate provided by Castrol) at 25^{O}C with continuous air flow over 9 days. The bioreactor was

decanted and refilled every 3 days with fresh MWF. The MWF was diluted (5% v/v) to form an emulsified solution before it is used in operation.

Thirty polyethylene strips (immobilisation matrix for biofilms) of 2 grams each were cut out and suspended on a thin plastic tube in a plastic reactor, namely a bioreactor (Figure 3.1). These were cut from commercially available biological filter medium, with the trade name of BIO-BLOK 300® (Expo-Net Bio-Blok). This is used to grow biofilms in industrial systems and is produced with a modified surface structure that ensures rapid development of microbial growth, has a specific surface area of 300 m^2/m^3, and an area of flow of 50 % with hollow space of approximately 65 %.

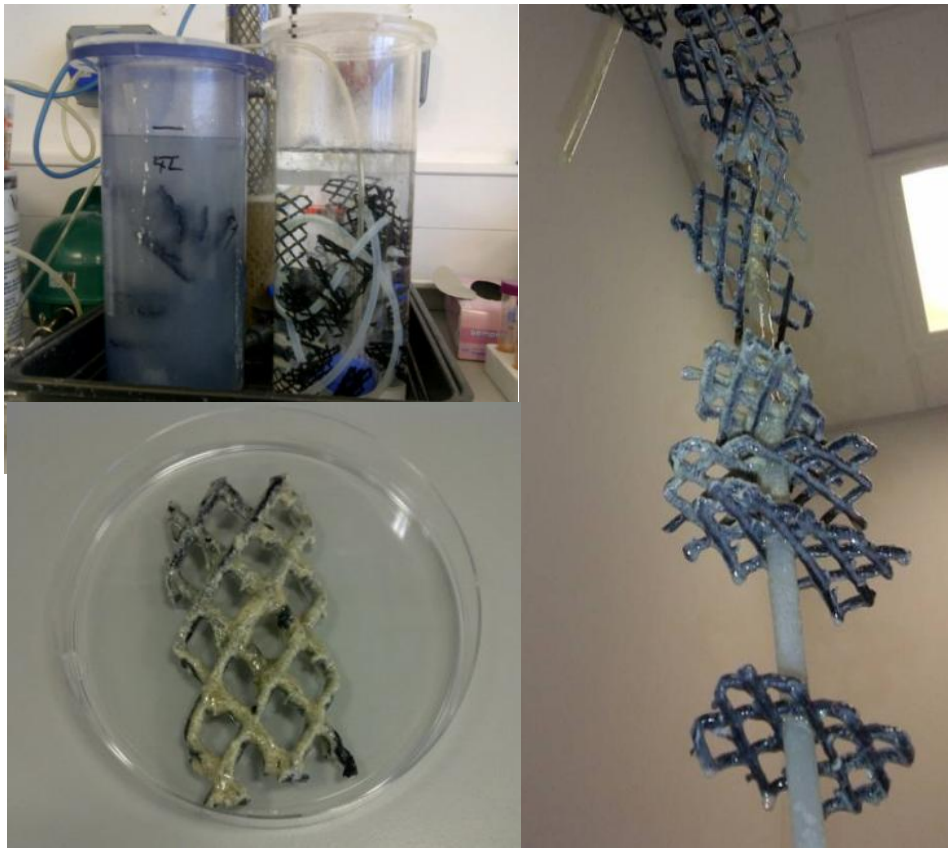


Figure 3.1 Polyethylene strips (right), bioreactor (left top) and biofilm grown on single polyethylene strip (left bottom)

3.2.2 Analytical methods

All chemicals were analytical reagent grade supplied from Sigma-Aldrich. Zn (II) and Mn (II) stock solutions (each at 1 g/L concentration) were prepared by suspending the appropriate amounts of $ZnSO_4.7H_2O$ and $MnSO_4.4H_2O$ in deionised (DI) water. These are ubiquitous constituents of MWFs, but were further diluted to obtain concentrations for practical use. pH measurements were performed with a Jenway pH ion meter 3354 which was calibrated using buffer standard solutions of pH 4.0, 7.0 and 10.0. Aliquots of 0.1 M HNO_3 or 0.1 M NaOH were used to adjust the pH over 1.0-9.0. After adjusting the pH, metal ion concentrations were verified to ensure that there was no significant change. Samples collected in all experiments were centrifuged using Megafuge11R Thermo Scientific, at 12,000 rpm for 15 min to minimise biofilm content in samples.

Metal concentrations were determined employing an atomic absorption spectroscopy (Agilent 240FS AA). All the determinations were performed after optimising the instrument using the below-indicated parameters (Table 3.1). Before measurement, the heavy metal solutions were diluted with DI water to ensure that the heavy metal concentration in the sample was linearly dependent to the absorbance detected. Negligible removal of metals was detected when using blank 2 grams polyethylene strips (without biofilms).

Table 3.1 AAS setting parameters

Metal	**Wavelength**	**Flame**	**Mode**
Zinc	213.9 nm	Air/acetylene	Linear origin
Manganese	279.5 nm	Air/acetylene	Linear origin

The metal removal efficiency (R) and metal removal (Q_e) was calculated as follows (Volesky, 2007):

$$R = \frac{(C_0 - C_e)}{C_0} \times 100$$

$$Q_e = V(C_0 - C_e)/M$$

Where Q_e is the metal removal (mg/g biofilm); C_0 and C_e are the initial and equilibrium metal concentrations in solution (mg/L) respectively; V is the solution volume (L); and M is the biofilm dry weight (g). The biofilms grown on polyethylene strips were rinsed twice with deionised (DI) water, then dried at 70^{o}C for 4 hours to constant weight to obtain the dry weight of the biofilm.

Calculation of zinc and manganese speciation in aqueous solution as a function of total salt concentration and solution pH was performed using the Visual Minteq program (version 3.0) (Pinto et al., 2011). This works with an extensive thermodynamic database for the calculation of metal speciation, solubility and equilibrium. Data were calculated at atmospheric CO_2 ($p(CO_2)$=38.5 Pa).

3.2.3 Biofilm and free-cells sorption experiments

The factors that affected the metal removal by MWF acclimated biofilms were examined in batch mode operation by exposing the biofilm strips to 200 mL of the metal ion solution. Biofilms on the polyethylene strips were placed in 250mL Erlenmeyer flasks for the removal experiments. All batch experiments were carried out using a shaking incubator employing pre-determined conditions of temperature, pH, contact time and initial metal ion concentration.

The effect of biofilm aging was first evaluated by measuring the metal removal by biofilms grown for 6, 9 and 12 days. Biofilm detachment (%) was calculated at the end of the biosorption experiment using the dry weight of the biofilm after biosorption. This was subtracted from the average dry weight of biofilm grown on a single polyethylene strip to determine the degree of detachment. The average dry weight of the biofilm in a given bioreactor was determined by randomly sampling 10 of 30 biofilm strips in each bioreactor, and the dry weight was taken to be representative of all the strips in the reactor.

The effect of temperature (25-50^{o}C) and pH on the removal of Zn (II) and Mn (II) was evaluated at a range of pH values 4-8. On reaching equilibrium, the samples were centrifuged and the supernatant liquid analysed for residual metal ions. The influence of the contact time (0.5-24 hrs) on the metal removal was performed in order to quantify the equilibrium removal. The experimental procedure was similar to that of the pH studies. Metal solutions ranging from 1 to 80 mg/L were prepared and used to study the effect of initial metal ion concentration on biosorption. Single and binary metal experiments of Zn (II) and

Mn (II) were carried out at 120 rpm, pH 6.0 ± 0.2 and temperature 30^{o}C ± 0.5 ^{o}C. The binary metal solutions (Zn-Mn and Mn-Zn) were prepared with a primary metal concentration fixed at 20 mg/L, and the secondary metal concentration varied from 1, 10, 20, 40 and 80 mg/L.

In the case of bacterial free-living cells, the optical density of the bacteria within the flask was first measured by extracting 1 mL sample and centrifuging it at 12000 rpm for 15 min. The supernatant was disposed and the resultant cell pellet was washed twice with PBS, before being resuspended in DI water. The samples were placed in a 2 mL UV-Vis cuvette, and the optical density was determined using a UV-2450 UV-Vis spectrophotometer (Shimadzu Ltd., Manchester UK) at 590nm. The dry weight of the cell pellet in 1 mL suspension was measured by washing twice with DI water and then drying at 70^{o}C for 1 hour. Two classes of treated cells were used: 1) autoclaved for 15 min at 126^{o}C and 2) dried at 70 ^{o}C for 12 h in an incubator.

3.2.4 Zeta potential measurement

The bacterial cell surface is dynamic and responds to environmental parameter and fluctuations. The components of the bacterial cell wall and its interaction with surrounding media lead to an electrical charge. The charge represents the electrical potential at the shear plane between a cell wall and its suspending solution. This surface charge characteristic of MWF acclimated bacterial biofilm cells at different pH values were determined via zeta potential measurement by a Zetasizer (Malvern Zetasizer Nano Z, UK). Zeta potential

was calculated from their electrophoretic mobility by Helmholtz-Smoluchowski's equation (Wilson et al., 2001). In all cases, the measurements were repeated five times at 30 ± 0.5ºC. The instrument was allowed to equilibrate for 2 min before a set of three measurements were taken. Capillary cells were used for all samples and were thoroughly washed with ethanol and with three subsequent flushes of the respective solution before a new sample was measured (Janjaroen et al., 2013). Biofilms were prepared as cell suspensions, and their OD_{590} was adjusted to 0.1 using buffer solution (PBS). The pH of the suspension was adjusted in the range of pH 4 to 7 by the addition of appropriate volumes of concentrated 0.1M HNO_3 or 0.1M NaOH solutions.

3.2.5 SEM Characterisation

Biofilm strips were fixed using 2.5% glutaraldehyde for 1 hour, and then subsequently washed with buffer solution. They were then immersed in 1% osmium tetroxide for 30 min and then, dehydrated using ethanol in the following concentration series: 30%-50%-70%-90% ethanol, allowing for 10 min between each step. Samples were then dehydrated 3 times with absolute ethanol, allowing 20 min between each wash. After dehydration, the samples were immersed in hexamethyldisilazane (Sigma-Aldrich) for 3 min, before being left to dry overnight. Gold coating, using a Q150R ES sputter coater under a current of 20 mA for 120 secs, was first performed and then SEM images were taken using a JEOL JSM-6390 SEM with the voltage set at 5 kV.

3.2.6 Toxicity test

Toxicity was assayed using a bioluminescence based bacterial sensor in a two stage test, involving exposure and post exposure recovery. *Escherichia coli* HB101 containing pUCD *lux* genes, which produce light only in the absence of contaminants, enable it to act as a biosensor of toxicity and provide a sensitive measure of contaminant concentration (Mwinyihija, 2011). The HB 101 *Escherichia coli* biosensor strains were received from Aberdeen University, who utilised soil bacteria engineered to bioluminescence by insertion of *lux* genes (Paton et al., 1995). Compared to other techniques, the bacterial bioassay method is the most effective method due to its ease of conduct, quick response, cost-effectiveness, enabling application to a wide range of pollutants and has the key advantage of providing a measure of bioavailability rather than just concentration.

For the toxicity test by biosensor, plates were prepared by adding 180 µL of metal solution samples into the 96 vials plate (Nunc brand). The inocula was prepared by adding 5 mL of an overnight culture to 100 mL of sterile lysogeny broth-Miller with 1 g/L glucose (LBG) with 50 µg/mL ampicillin. The antibiotics were added to specifically select for bacterial cells that contain the pUCD607 plasmid after growth, which is responsible for luminescence. The culture was incubated at 37°C and 150 rpm agitation for 8 hours until the cells reached mid-exponential growth phase. The cells were washed in PBS of which, 20 µl were inoculated into each well of the 96 vial plates. Relative light units were measured and recorded after 15 min of exposure using a microtitre spectrophotometer (Synergy HT, BioTek).

Post recovery test was undertaken employing the Miles & Misra (Miles et al., 1938) plate count method to enumerate colonies after exposure to the test compounds. The aim was to determine the recovery rate of the biosensor after exposure to the test compound. After 15 min of exposure, 100 uL of the aqueous solution and bacteria were taken and diluted 6 times using 900 uL PBS and then regrown on LB Agar plates. The plates were grown in a static incubator at 35^{o}C for 18 hours. The colonies were counted and recorded manually. LB Agar plates were made using LB Miller agar (40 g/L, Fisher Scientific) in DI water with additional D-glucose anhydrous (5g/L, Fisher Scientific). Then, sterile ampicillin stock was added after the liquid agar was autoclaved (Classic, Prestige Medical) and only after the temperature of the liquid dropped down to approximately 40^{0}C. Once, it was well-mixed, approximately 25 ml of the agar was poured into the petri dishes and left for 2 days on the bench to dry.

Degradability test was done similarly to the toxicity test. However, the inoculum was taken from an active aerobic bioreactor for treating MWFs, which contains a mixed community instead of single species. Bioreactor communities are diverse and dynamic; therefore, it is likely that the community species composition and the relative abundances vary between inoculations on different days. However, given the diversity of species and the functional redundancy of bacteria, overall community function is maintained (van der Gast et al., 2004b). The cells were washed in 0.01M PBS before inoculated into the plate (Sterilin vitro) and the optical density was recorded at 590 nm every 30 minutes for 24 hours.

All three tests were undertaken in triplicate for two metal solutions (Zn (II) and Mn (II)) individually at five different concentrations (1, 10, 20, 40, 80 mg/L) and also 9 possible combinations of two metals. All processes were performed under aseptic conditions and in triplicates.

3.2.7 Biosorption isotherms

The amount of metal removal is one of the most important characteristics of a biosorbent. The equilibrium study on the biosorbent has provided sufficient information on the metal removal capacity of the biosorption. The biosorption isotherm indicates how the adsorbed metal ions distribute between the liquid and the solid phase when the biosorption process reaches equilibrium i.e. when there is no more change in the metal concentration (Volesky, 2007). Also, it can be used to compare the biosorption capacities of the biosorbent for different metal ions. The equilibrium biosorption data is analysed by fitting them to different isotherm models in order to select a suitable model that can be used for design purposes.

The Langmuir isotherm model estimates the maximum adsorption capacity produced from complete monolayer coverage on the adsorbent surface and can be represented as (Gadd, 2009; Mudhoo et al., 2012):

$$Q_e = \frac{Q_{max} b C_e}{1 + b C_e}$$

This can also be represented in the following linear form (Gadd, 2009):

$$\frac{C_e}{Q_e} = \frac{1}{bQ_{max}} + \frac{C_e}{Q_{max}}$$

Where C_e is the equilibrium concentration of the metal (mg/L), Q_e the metal removal (mg/g biofilm), Q_{max} and b are Langmuir constants related to monolayer adsorption capacity and affinity of adsorbent towards adsorbate, respectively. It is valid for monolayer adsorption on specific homogenous sites containing a finite number of identical sites and assumes uniform energies of adsorption on the surface and no transmigration of sorbate in the plane of the surface (Vijayaraghavan and Yun, 2008). The essential characteristics of the Langmuir isotherm may be expressed in terms of dimensionless constant called the separation factor or equilibrium parameter (R_L), which is defined by the following equation:

$$R_L = \frac{1}{1 + bC_0}$$

Where b is the Langmuir constant and C_0 is the initial concentration of each element. The value of R_L indicates the type of the isotherm to be either unfavourable (R_L>1), linear (R_L=1), favourable (0<R_L<1) or irreversible (R_L=0).

The Freundlich isotherm equation is purely empirical based on sorption on a heterogeneous surface with interaction between adsorbed molecules. It is assumed that stronger binding sites are initially occupied, with the binding strength decreasing with increasing degree of site occupation (Vijayaraghavan and Yun, 2008). It is commonly presented as:

$$Q_{eq} = K_F C_{eq}^{1/n}$$

This can be linearised as:

$$\log Q_e = \log K_F + \frac{1}{n}\log C_e$$

Where K_F ((mg/g)(L/mg)$^{1/n}$) is the Freundlich constant indicative of relative adsorption capacity of the adsorbent related to the bonding energy and can be defined as the adsorption or distribution coefficient. It represents the quantity of element adsorbed onto adsorbent for unit equilibrium concentration. The constant n gives an indication of how favourable the adsorption process is. The slope 1/n is a measure of adsorption intensity or surface heterogeneity. If n=1, then biosorption is linear; if n<1, then biosorption is a chemical process; if n>1, then biosorption is a physical process.

3.2.8 Kinetic studies

Pseudo first and pseudo second order models are widely used to represent kinetic data in batch reactors by neglecting mass transfer effects. Pseudo second order model predicts the behaviour over the entire contact time range, which is different to the pseudo first order model and is in agreement with biosorption mechanism being the rate-controlling step. The pseudo second order rate equation is expressed as (Oliveira et al., 2011):

$$\frac{dQ_t}{dt} = k_2(Q_{eq} - Q_t)^2$$

Integration of above equation (boundary conditions: t = 0, q = 0 and t = t, q = q) can be expressed in the linear form:

$$\frac{t}{Q_t} = \frac{t}{Q_{eq}} + \frac{1}{k_2 Q_{eq}^2} = \frac{t}{Q_{eq}} + \frac{1}{V_0}$$

Where k_2 is the pseudo second order rate constant (g/mg*min) and v_0 is the initial biosorption velocity (mg/g*min). Parameters Q_{eq} and k_2 can be determined by plotting t/Q_t against t. Pseudo second order model is generally more appropriate than pseudo first order model to represent the kinetic data of heavy metal biosorption in batch reactors (Macek and Mackova, 2011).

3.2.9 Statistical analysis

All experiments were conducted in at least three replicates and the data represent the mean values ± standard error. T-test and ANOVA followed by Tukey's test were applied when appropriate, and P values less than 0.05 and 0.01 were considered statistically significant.

3.3 Results and Discussion

3.3.1 Effect of biofilm aging on zinc removal

The growth of a biofilm is the result of a complex process that involves the transport of organic and inorganic molecules and microbial cells to the surface, a subsequent adsorption to the surface and finally an irreversible attachment aided by the production of extracellular polymeric substance (EPS) (Davey and O'toole, 2000; Donlan, 2002). The EPS plays a vital role in the removal of heavy metals because its presence can correspond to an increase in the number and type of functional groups (Baker et al., 2010). Often the composition and the quantity of the EPS vary depending on the type of microorganisms, age of the biofilms and the different environmental conditions under which the biofilms exist. Therefore, it was important to study the effect of biofilm aging on heavy metal removal.

The amount of zinc removal (mg/g biofilm) increased with biofilm age for both initial Zn (II) concentrations: 10 mg/L and 80 mg/L (Figure 3.2). However, the focus of the study was to develop a sustainable metal recovery system. Hence, the biofilm detachment at the end of the batch biosorption experiments was also another important factor that needed attention. Even though greater amount of Zn (II) was removed (mg/g biofilm) on Day 12, the biofilm detachment (%) was also much greater compared to Day 6 and Day 9, which meant that overall less zinc was recovered at the end of the process. Therefore in subsequent studies, biofilms were grown on polyethylene strips for only 9

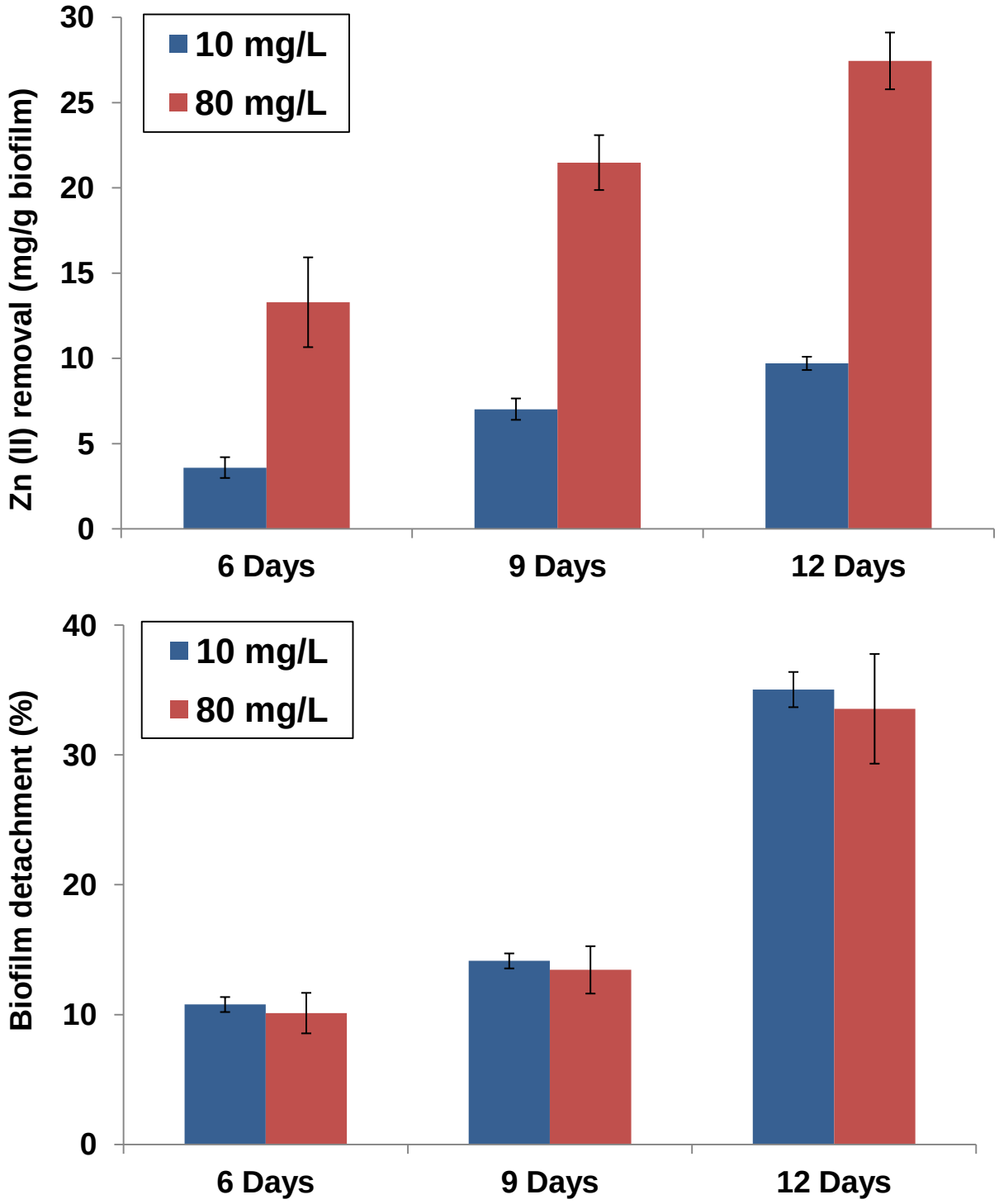


Figure 3.2 A) Effect of biofilm aging on Zn (II) [Co= 10 and 80 mg/L] removal (mg/g dry biofilm) by immobilised MWF acclimated bacterial biofilms (above) B) Biofilm detachment (%) after zinc removal (below) (120 rpm, 30^{o}C, pH 6, 300 min). Error bars represent the standard deviation of triplicate measurements.

days, thereby providing a balance between the zinc removal (mg/g biofilm) and the biofilm detachment (%) from the polyethylene strips.

3.3.2 Effect of temperature on zinc removal

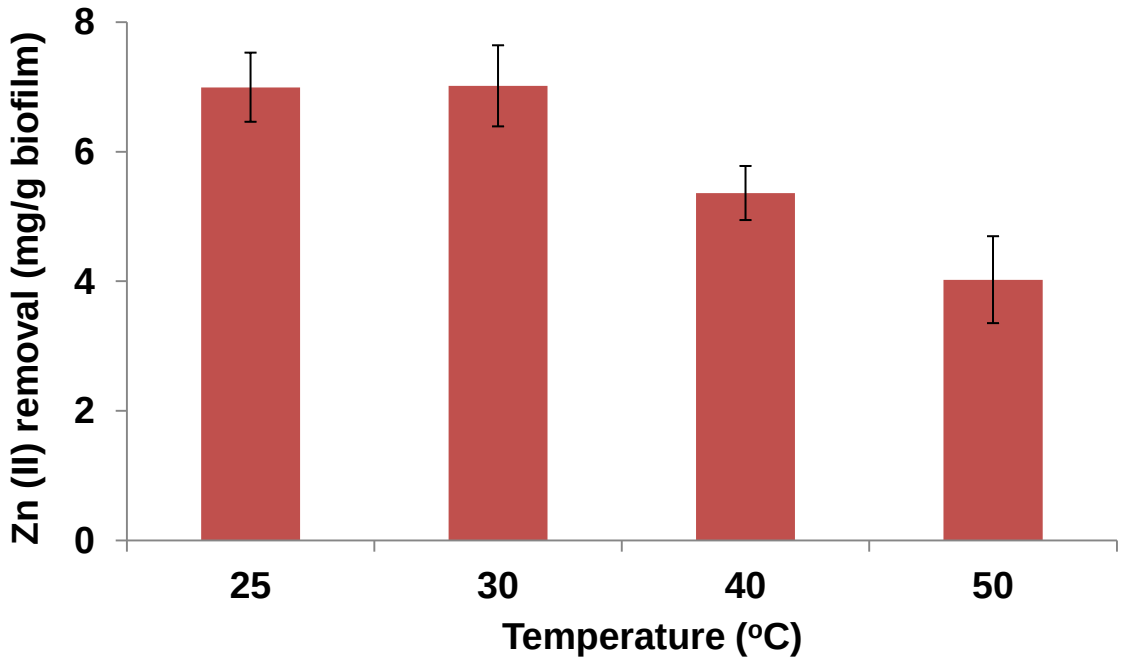


Figure 3.3 Effect of temperature on Zn (II) removal (mg/g biofilm) by immobilised MWF acclimated bacterial biofilms (120 rpm, pH 6, 10 mg/L initial Zn (II) concentration, 9 days biofilm age, 300 min). Error bars represent the standard deviation of triplicate measurements.

Zinc removal (mg/g biofilm) by MWF bacterial biofilms was temperature dependent (Figure 3.3). Temperature changes are known to affect a number of factors which are important in heavy metal ion uptake. Figure 3.3 shows that the maximum zinc removal of 7.02 mg/g biofilm (± 0.63) occurred at 30^{o}C. The zinc removal was identical at 25^{o}C and 30^{o}C ($P > 0.05$). However, the zinc removal

decreased to 5.36 mg/g (± 0.42) at 40^{o}C and to 4.02 mg/g (± 0.67) at 50^{o}C. Thus, all further experiments in this study were performed at 30^{o}C. The reason for the decrease in zinc removal was due to the increased detachment of the biofilm from the surface of the matrix with increasing temperature. However, no change in zinc removal was observed between 25^{o}C and 30^{o}C as the original consortium is known to grow best around these temperatures.

3.3.3 Effect of initial pH

Biosorption is strongly pH dependent and different metals are known to have different optimal operating pH values. Initial pH controls the surface charge and the surface properties of the biofilm by way of functional group dissociation and also metal speciation (Oliveira et al., 2011). Zn (II) and Mn (II) removal (mg/g biofilm) at initial metal ion concentration of 10 mg/L at pH values ranging from pH 4 to 8 are presented in Figure 3.4 A. Zinc removal increased from 4.47 mg/g (± 0.54) at pH 4 to 7.16 mg/g (± 0.49) at pH 6, then decreased to 4.73 mg/g at pH 7 (± 0.39). Mn (II) removal revealed similar trends: increasing from 1.13 mg/g (± 0.06) at pH 4 to 1.57 mg/g (± 0.12) at pH 6 and then decreasing to 1.30 mg/g (± 0.06) at pH 7. The effect of pH on the zeta potential of MWF acclimated bacterial biofilms in buffer solution (PBS) is illustrated in Figure 3.4 B. Zeta potential is a measure of the electrokinetic potential between the charge associated with the surface of a particle and the liquid medium in which it is dispersed (Hunter, 1986). It can also be described as the difference in electric potential between the stern layer (stationary layer of fluid on the outside of the

particle) and a point in the bulk dispersion medium. Zeta potential was measured for biofilms to study the surface charge because in many conditions, it reflects the surface charge of a particle, especially in low ionic strength conditions. In terms of metal removal, the electrostatic attraction of the positive metal ions and the negative bacterial surface should lead to increased metal removal with more negative bacterial surface. As the pH increased, the zeta potential of the biofilm cell suspension became more negative until pH 6 (-43.3mV ± 5.9) and then became less negative at pH 7 (-32.6mV ± 4.7). Similarly, Zinc removal increased from 4.47 mg/g (± 0.54) at pH 4 to 7.16 mg/g (± 0.49) at pH 6, then decreased to 4.73 mg/g at pH 7 (± 0.39). Mn (II) removal revealed similar trends: increasing from 1.13 mg/g (± 0.06) at pH 4 to 1.57 mg/g (± 0.12) at pH 6 and then decreasing to 1.30 mg/g (± 0.06) at pH 7. Therefore, a possible explanation for the biosorption trend at different pH values could be as explained below. As the pH of the solution increased to pH 6, the number of protons dissociated from the functional groups present on the cell wall increased, thereby providing more available negative groups on the biofilm. This increased electrostatic attraction between the positively charged metal ions and negatively charged biofilm leading to an increase in the metal removal (mg/g biofilm).

Figure 3.5 shows the speciation of zinc and manganese calculated as a function of total salt concentration and solution pH using the Visual Minteq program, which works with extensive thermodynamic database for the calculation of metal speciation, solubility and equilibrium. Data were calculated at atmospheric CO_2 (p(CO2)=38.5 Pa). Results show that at pH 6, more than

97% of zinc and manganese were present in the form Zn^{2+} and Mn^{2+} ions, respectively (Figure 3.5). The influence of pH was not studied beyond pH 8 because at higher pH values, precipitation of metals occurs by the formation of metal hydroxides, such as $Zn(OH)_2$ from pH 7 onwards, and $Mn(OH)_2$ from pH 8 onwards. Furthermore, lower pH values were also avoided because the hydrogen ions present in solution would displace or competitively exclude the zinc and manganese ions from the biofilms' active sites in low pH solutions, resulting in lower metal removal. The optimal initial pH value for Zn (II) and Mn (II) removal by immobilised MWF acclimated bacterial biofilms were both observed at pH 6 and therefore, all further experiments including binary system experiments were performed at pH 6.

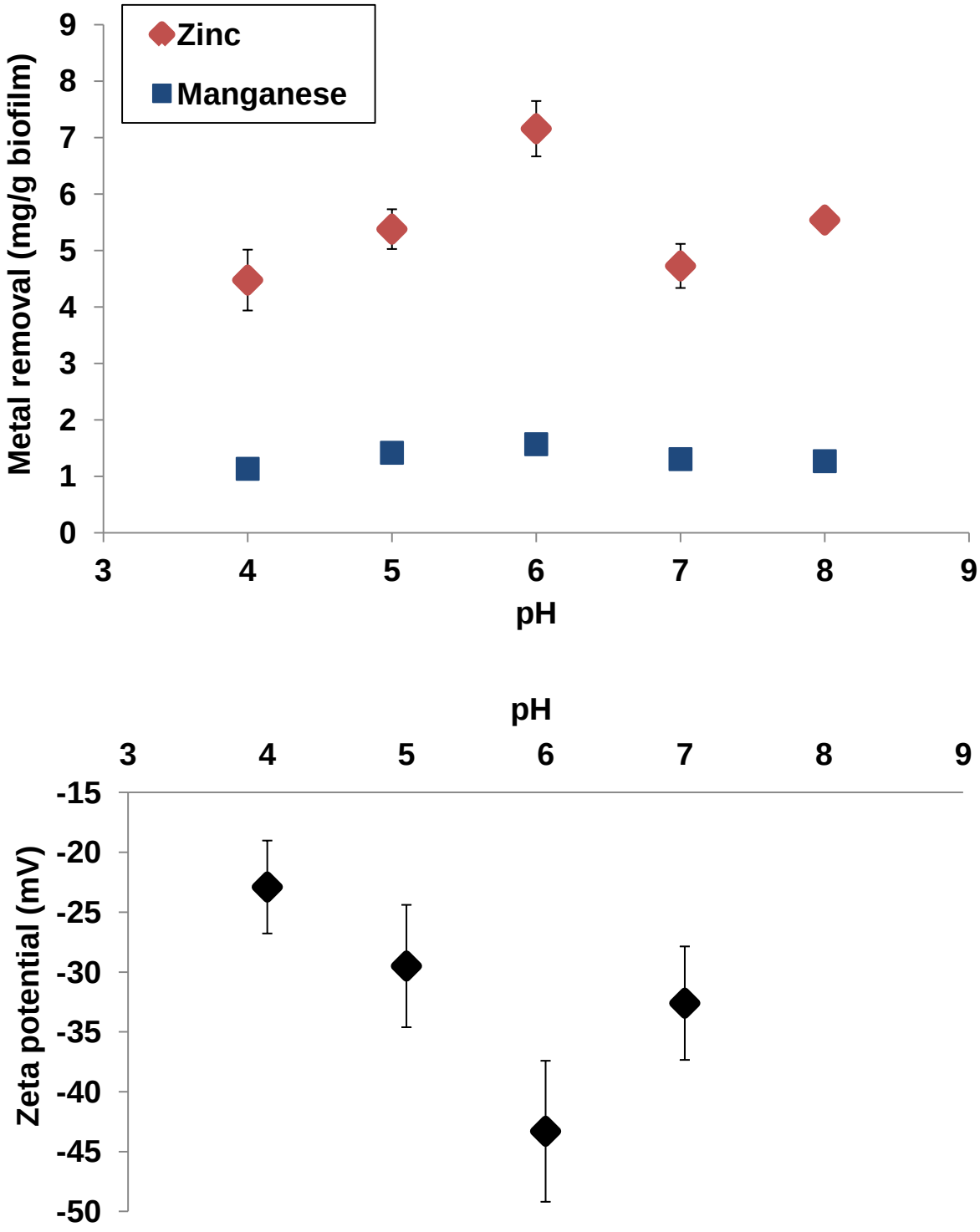


Figure 3.4 A) Effect of pH on Zn (II) and Mn (II) removal by immobilised MWF acclimated bacterial biofilms (120 rpm, 30^{o}C , 10 mg/L initial Zn (II) and Mn (II) concentration, 9 days biofilm age, 300 min) B) Effect of pH on zeta potential of MWF acclimated bacterial biofilm in PBS. Error bars represent the standard deviation of triplicate measurements.

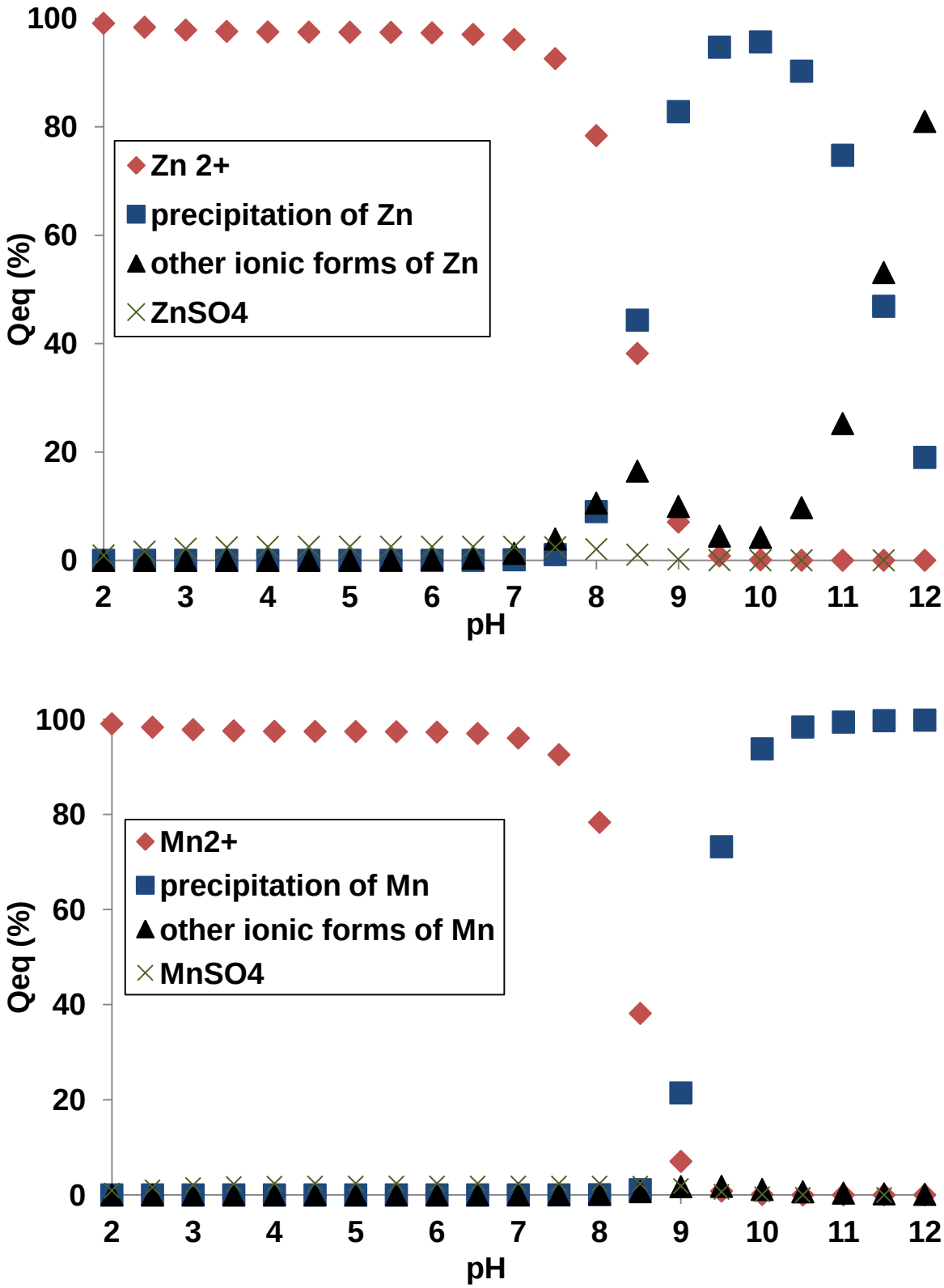


Figure 3.5 Theoretical zinc (top) and manganese (bottom) speciation in solution, calculated using Visual MINTEQ version 3.0 with initial conditions: 24 mg/L ZnSO4 and 27 mg/L MnSo4, T = 30^{o}C, pCO2=38.5 Pa.

During biosorption, the pH of the reaction mixture tends to change due to the chemical interaction between the biosorbent and the solution. The chemical constituents of a biosorbent and the nature of the biosorption mechanism are mainly responsible for any pH change (Davey and O'Toole, 2000). In this study, changes of pH value at the end of the biosorption of zinc and manganese ions were also recorded. The pH values measured at the end of the experiments shifted to pH 7 for zinc and to pH 6.5 for manganese regardless of initial pH conditions. Typically, pH should be controlled over the entire contact period until equilibrium is reached (Volesky, 2007).

3.3.4 Effect of contact time and initial metal ion concentration

Figure 3.6 shows the effect of contact time on metal removal (mg/g biofilm) for 1 mg/L of zinc and manganese in single metal systems. For all concentrations, the removal of metal ions appeared to take place in two steps: a very rapid initial sorption over few minutes, followed by a long period of much slower removal. This trend was observed for all concentrations in both metal systems: the biosorption capacity increased with increasing contact time and the major biosorption process was achieved in the first 90 min in all cases. For all initial metal concentrations, the biosorption reached equilibrium within 300 min for both zinc and manganese single systems. In industrial applications, this rapid biosorption phenomenon is advantageous since the shorter contact time effectively allows for reduced equipment and process cost (Aryal and Liakopoulou-Kyriakides, 2015).

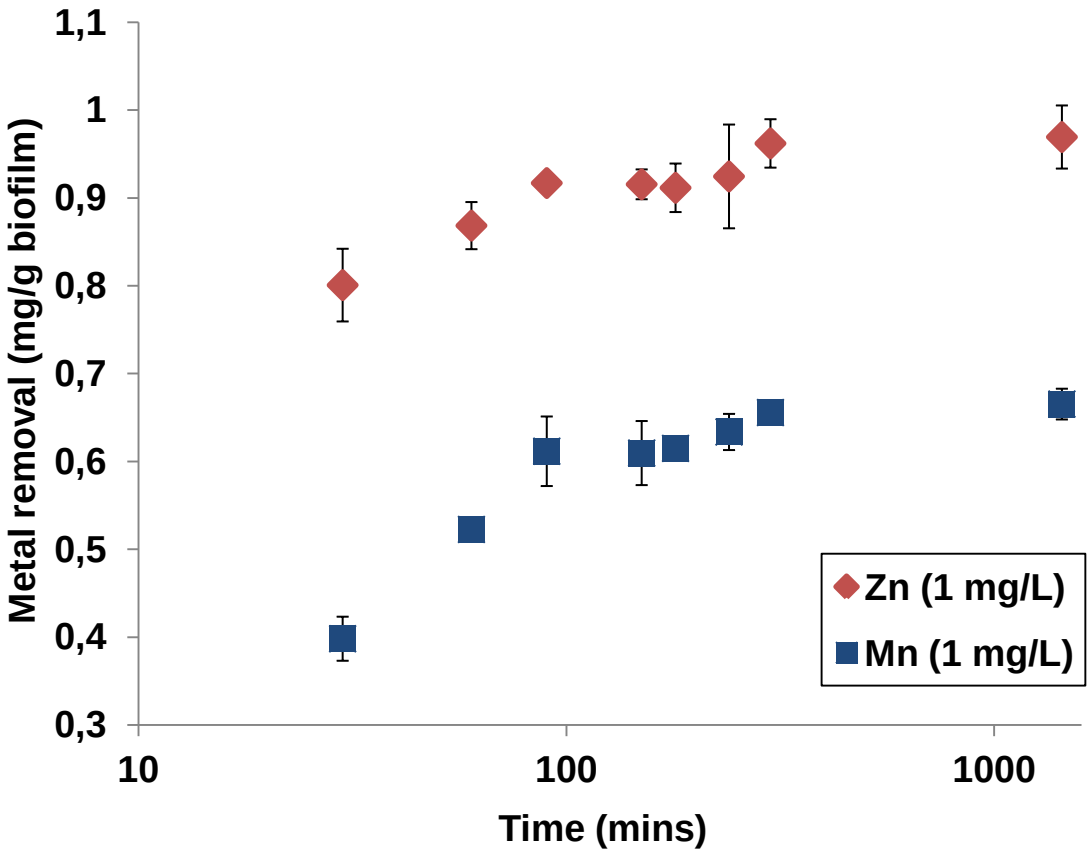


Figure 3.6 The effect of contact time on Zn (II) and Mn (II) removal, respectively, by immobilised MWF acclimated bacterial biofilms (120 rpm, 30^{o}C, pH 6, 1 mg/L initial Zn (II) and Mn (II) concentrations, 9 days biofilm age). Error bars represent the standard deviation of triplicate measurements.

The fast removal rate in the first 90 min may have been the result of huge driving force between the positively charged metal ions and the negatively charged binding sites available on the biofilm surface. With the gradual occupancy of these sites, the process became slower eventually resulting in no change in concentration. The metals tend to accumulate on the biofilm until equilibrium was reached, at which time no further accumulation occurred. Therefore, 300 min was chosen to be long enough for the process to establish equilibrium in subsequent experiments.

The effect of initial metal ion concentration on metal removal efficiency (%) and metal removal (mg/g biofilm) at equilibrium contact time of 300 min is shown in Figure 3.7. As the initial metal ion concentration increased from 1 mg/L to 80 mg/L, the zinc removal efficiency of the biofilm decreased from 79.15% (± 1.64%) to 25.10% (± 3.72%), whilst the zinc removal (mg/g biofilm) increased from 0.96 mg/g (± 0.03) to 24.60 mg/g (± 3.46). Similar trends were observed for manganese. Manganese removal efficiency by biofilm decreased from 50.76% (± 1.17%) to 5.40% (± 0.28%) with increasing concentration of manganese from 1 mg/L to 80 mg/L, whereas the manganese removal (mg/g biofilm) increased from 0.66 mg/g (± 0.01) to 5.23 mg/g (± 0.28). The higher Zn (II) removal compared to Mn (II) could be related to the property of microbial community growing in the biofilm. Microbes exposed to toxic metals and organics for long periods of time develop adaptation and resistance strategies, which could lead to improved resistance to metal and improved biodegradation organics and biosorption of metals. This is particularly true in the case where the community might be acclimated to a particular metal then, its removal would be greater compared to one where the community is not acclimated. In this case, Zn (II) is a commonly found metal in waste MWFs, but not Mn (II), and the original consortium for the biofilm formation in this study originated from an aged oil-based MWF bioreactor adapted to treating waste MWF. Hence, one possible explanation for the increased Zn (II) removal compared to Mn (II) could be due to the acclimatisation of biofilm community to Zn (II) during biofilm formation.

It can be seen that the zinc removal efficiency was inversely related to the initial metal ion concentration. A possible explanation could be due the increase in the number of ions competing for the fixed number of available binding sites in biofilms and thus, at higher metal concentrations, more metal ions were left in solution due to the saturation of binding sites. At the same time, higher initial metal ion concentrations provided a greater driving force to overcome all mass transfer resistances between the metal solution and the biofilm. In addition, the number of collisions between the metal ions and the biofilm active binding sites increased with increasing initial metal ion concentration and thus, the metal removal could also have increased (Chen et al., 2005).

From Figure 3.7, it can be seen that immobilised MWF acclimated bacterial biofilms biosorbed 4 times more zinc than manganese at the initial metal ion concentration of 80 mg/L. The affinity of the biomass towards a particular ion can be correlated with its electronegativity and ionic radii (Dong et al., 2003; Vijayaraghavan and Gupta, 2012). As ion exchange is possibly one of the major mechanisms accounting for the biosorption process, it is expected that more electronegative metals would demonstrate a greater adsorption tendency. The electronegativity of zinc (1.65) is more than that of manganese (1.55), thereby having greater affinity towards binding sites.

Ionic radii also influence metal ion binding. Metal ions with larger ionic radius have smaller hydrated radius and consequently lower hydration energy. The lower the hydrated radius, the better the metal ion binds to the biosorbent surface compared to the higher one (Vijayaraghavan and Gupta, 2012; Nassar, 2012). Among the metal ions studied, the ionic radii of zinc and manganese are

74 pm and 83 pm, respectively. As manganese has the greater ionic radius, one would expect it to have the greater removal. However, the results of this study indicate that the biosorption of zinc ions was greater than that of manganese, implying that other factors such as molecular mass or even toxicity could also play a crucial role in the biosorption process.

Congruent with the literature, immobilisation of the metal ions on the biofilm in this study might not be the result of just one single mechanism taking part in the biosorption process, but several (Sag et al., 2002; Lim et al., 2008). Sag et al., 2002 also state that it is generally complicated to find a common rule to identify how metal properties affect the competitive biosorption, as the observed behaviour may result from a combination of different factors. Moreover, the characteristics of biosorbents and the variations in composition and structure of the biosorbent surface, when changing physicochemical parameters of the solution, also affect the order of metal biosorption preference (Sag et al., 2002). Moreover as mentioned above, Zn (II) is commonly present in waste MWFs but not Mn (II), and since the biofilms in this study were grown in waste MWF, it very likely they were acclimated during growth and hence have higher metal removal for Zn (II) compared to Mn (II).

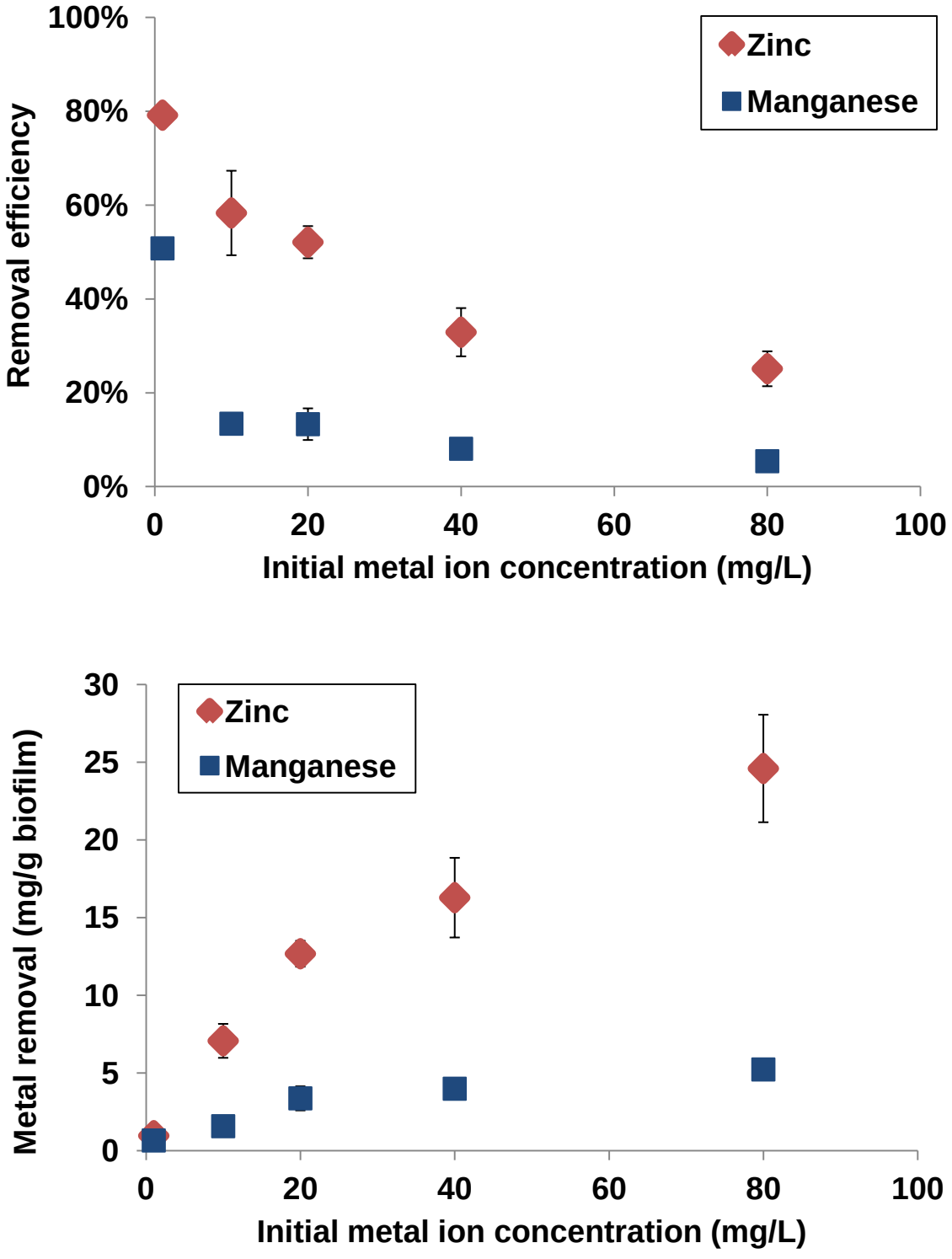


Figure 3.7 The effect of initial metal ion concentration on zinc and manganese removal efficiency (%) (top) and metal removal (mg/ g biofilm) (bottom) by immobilised MWF acclimated bacterial biofilm (C_O = 1, 10, 20, 40 and 80 mg/L for both zinc and manganese, pH 6, 120 rpm, 300 min, 30^{O}C) Error bars represent the standard deviation of triplicate measurements.

3.3.5 Biosorption isotherm

Firstly, the Langmuir model was used and the model parameters, the maximum biosorption capacity Q_{max} and the affinity constant b, are listed in Table 3.2. The maximum biosorption capacity Q_{max} is a good measure for comparing different metals for the same biosorbent, although they are dependent on experimental conditions such as solution pH and temperature. The affinity constant b corresponding to the initial gradient is important, which evaluates biosorbent performance by indicating the biosorbent affinity at low metal concentrations.

Table 3.2 Langmuir and Freundlich parameters for the biosorption of Zn (II) and Mn (II) in single systems by immobilised MWF acclimated bacterial biofilm

Model		**Zn (II)**	**Mn (II)**
Langmuir	**Q_{max} (mg/g)**	27.17	6.01
	b (L/g)	0.091	0.067
	R^2	0.95	0.94
Freundlich	**K_F (mg/g)(L/mg)$^{1/n}$**	2.73	0.82
	n_F	1.74	2.34
	R^2	0.98	0.97

In agreement with the experimental data, the Langmuir model also predicted that the immobilised MWF acclimated bacterial biofilm showed more preference towards zinc. As shown in Table 3.2, the maximum removal of Zn (II) ions was approximately 4.5 times higher than that of Mn (II). The affinity constant b of Zn (II) ions (0.091) was higher than that of Mn (II) ions (0.067) as well. This clearly indicates the high affinity of biosorbent towards Zn (II) ions. Values of R_L were

found to be in the range of 0.16 to 0.94 and 0.12 to 0.92 for Zn (II) and Mn (II), respectively. Therefore, immobilised MWF acclimated bacterial biofilms were found to be favourable for biosorption of Zn (II) and Mn (II) under conditions used in this project.

The equilibrium data was then presented by the Freundlich model. The Freundlich parameter K_F and n_F are also listed in Table 3.2. The K_F is the Freundlich constant indicative of the relative biosorption capacity of the biosorbent. It represents the quantity of metal biosorbed onto biosorbent for unit equilibrium concentration (Vijayaraghavan and Yun, 2008). The K_F of Zn (II) ions were more than 3.3 times than that of Mn (II), which agreed with the prediction of the Langmuir model. The values of n were found to be larger than 1. This indicated the physical biosorption characteristic of metal ions onto biofilm. Besides this, higher value of n for Mn (II) compared to Zn (II) also indicated a stronger bond between Mn (II) and the biofilm.

The isotherm experimental results fitted well for both Langmuir and Freundlich models with correlation coefficients R^2 above 0.9. However, Freundlich model seemed to be the better fitting model for the experimental data. It is noteworthy that Langmuir model assumes that biosorption occurs on a homogeneous surface; while Freundlich model describes biosorption where the biosorbent has a heterogeneous surface with biosorption sites that have different biosorption energies. Figure 3.8 showed biosorption isotherms of zinc and manganese with Freundlich model fitting. In both cases favourable isotherms were observed. In conclusion, the immobilised MWF acclimated

bacterial biofilms were preferential to Zn (II) ions removal compared to Mn (II) ions.

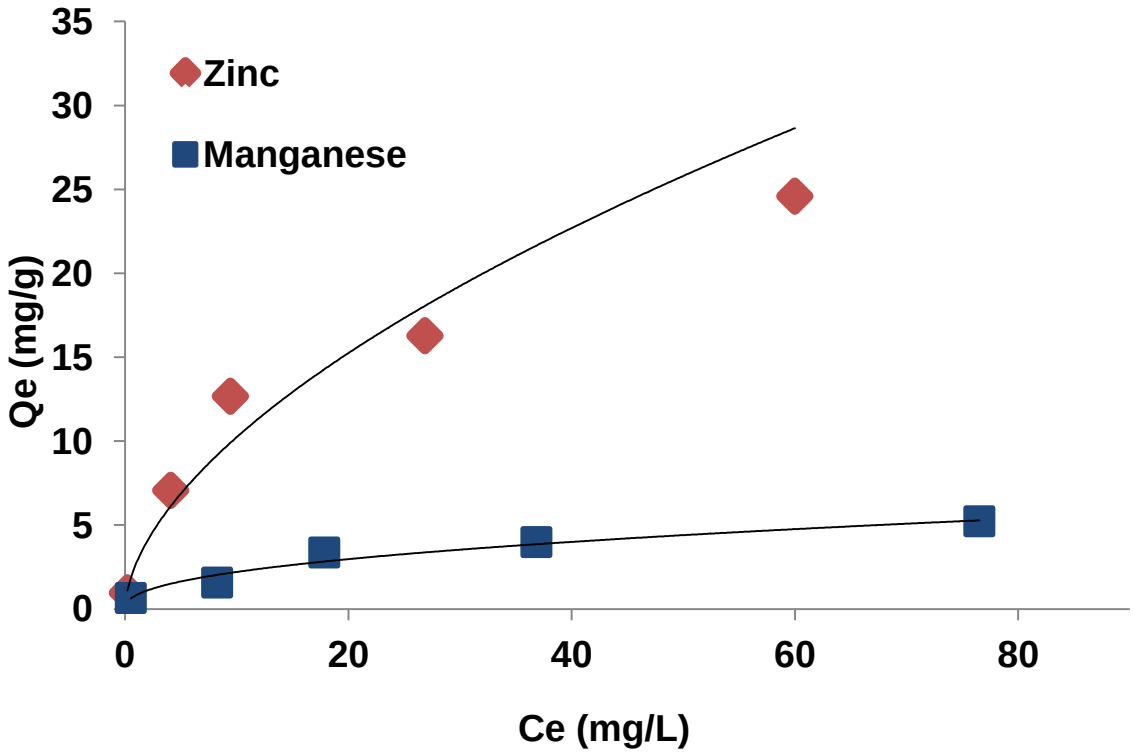


Figure 3.8 Biosorption isotherms of Zn (II) and Mn (II) with Freundlich model fitting [C_0=1, 10, 20, 40 and 80mg/L for both zinc and manganese, pH 6, 120 rpm, 300 min contact time, 30^{o}C] Error bars represent the standard deviation of triplicate measurements.

3.3.6 Kinetic Studies

The kinetics of biosorption process for Zn (II) and Mn (II) were well described by the pseudo-second order model with relatively high correlation coefficients ranging from 0.960 to 0.999 (Table 3.3). The increase in initial biosorption velocity v_o confirms that the initial metal concentration has a significant contribution to driving force to overcome all the mass transfer

resistance of the metal ions between the aqueous and solid phases. The predicted equilibrium removal values all agreed well with the experimental values.

Table 3.3: Kinetic constants for Zn (II) and Mn (II) onto immobilised MWF acclimated bacterial biofilm under the same experimental conditions at different initial metal ion concentrations

Metals	**Parameters**	**C_0 mg/L**				
		1	**10**	**20**	**40**	**80**
Zn (II)	**Q_{exp}* (mg/g)**	0.96	7.07	12.70	16.28	24.60
	Q_{eq} (mg/g)	0.97	7.15	12.64	17.37	25.51
	K_2(g/mg*min)	0.13	$7.52*10^{-3}$	$4.11*10^{-3}$	$3.85*10^{-3}$	$3.14*10^{-3}$
	V_0(mg/g*min)	0.121	0.385	0.658	1.03	1.89
	R^2	0.999	0.984	0.972	0.960	0.984
Mn (II)	**Q_{exp}* (mg/g)**	0.66	1.58	3.37	3.99	5.23
	Q_{eq} (mg/g)	0.68	1.60	3.46	4.16	5.51
	K_2(g/mg*min)	0.082	0.021	$8.35*10^{-3}$	$4.60*10^{-3}$	$3.15*10^{-3}$
	V_0(mg/g*min)	0.038	0.052	0.100	0.080	0.096
	R^2	0.999	0.992	0.993	0.980	0.984

3.3.7 Scanning electron microscopy characterisation

The SEM pictures of MWF acclimated bacterial biofilms are presented in Figure 3.9. Panels A (Figure 3.9) shows SEM image of biofilm with no metal adsorption where a mixture of long-rod shaped and circular plump biofilm cells with smooth surfaces adhered to EPS were observed. After treating with 80

mg/L Zn (II) for 300 min, distinct shape of cells was no longer visible instead the cells appeared to be clumped together (Panels B). There was a higher proportion of long-rod shaped biofilm cells than circular shaped ones on biofilm treated with 80 mg/L Mn (II) for 300 mins compared to unloaded biofilms (Panels C). More morphological differences on the zinc-loaded biofilms (Panels B) can be seen compared to manganese-loaded biofilms (Panel C), which could explain results above where the MWF acclimated bacterial biofilms had a higher Zn (II) removal (mg/g biofilm) than Mn (II) removal. In both cases, the non-metal loaded biofilm appeared smoother than that in metal-loaded samples with some surface shrinking after metal binding also apparent. Also, more EPS, which is known to play a significant role in metal removal by biofilms (Wei et al., 2011), was observed in the metal exposed biofilms compared to control. The microstructures present in some samples may be due to calcium or other crystalloid deposition from the M9 minimal media; these salts were present to stimulate the growth of the biofilm in the reactor (Materials and Methods section 3.2.1). Although the surface appears rougher in the metal loaded biofilms, it is generally quite difficult to discern the morphological changes on biofilms in this study.

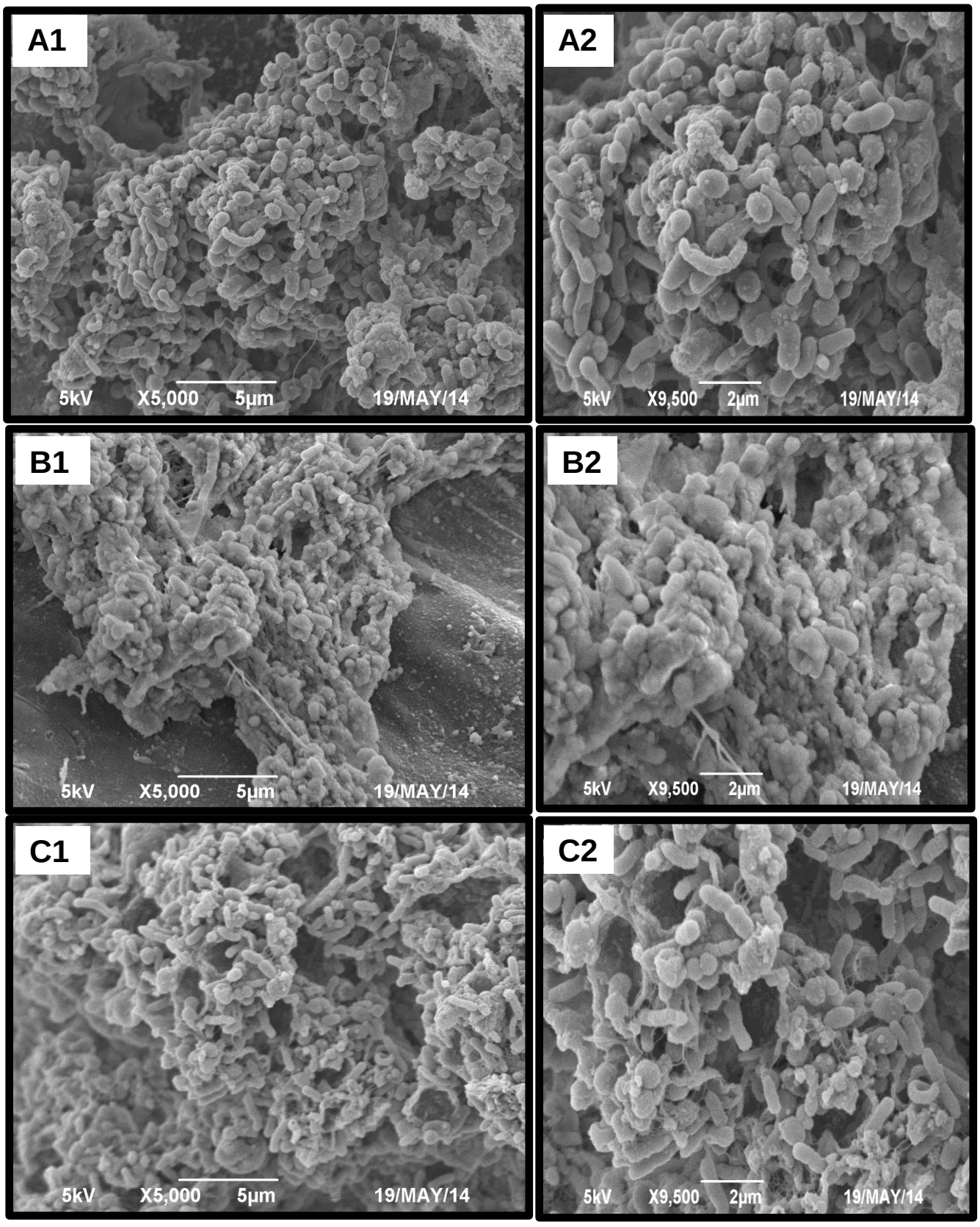


Figure 3.9 SEM images of A) non-loaded biofilm (control) B) Zinc loaded biofilm C) Manganese loaded biofilm (C_0=80 mg/L for Zn and Mn, pH 6, 120 rpm, 300 min, 30^{o}C)

3.3.8 Zinc (II) and manganese (II) competitive biosorption

The equilibrium state in competitive biosorption was rapidly reached, within 60 min (data not shown). Whereas, the single systems needed approximately 300 min to reach the equilibrium state (Figure 3.6). As shown in Figure 3.10, the metal removal of different initial metal ion concentrations of Zn (II) and Mn (II) in single aqueous solutions was compared with the metal removal in binary systems. Equilibrium capacities were lower than the values obtained in single metal ion solutions, suggesting that the biofilms react differently towards a multi-component system where competition takes place additional to other factors. The equilibrium data for binary biosorption of zinc and manganese ions may be analysed in the form of biosorption capacity of one metal ion in the presence of the other metal ion to the biosorption capacity for the same metal ion when it is present alone in the solution. These values for both zinc and manganese ions were found to be less than unity, suggesting that the simultaneous presence of both metal ions reduced biosorption through competition for biosorption sites on MWF acclimated bacterial biofilm. Also Figure 3.10 shows that the zinc biosorption onto MWF acclimated bacterial biofilms was more affected by the simultaneous presence of a competing metal ion compared to manganese biosorption. Zn (II) removal (mg/g biofilm) decreased up to 87% whereas the Mn (II) removal only decreased up to 66% at initial metal concentration of 80 mg/L. This can be explained by the stronger bonding between Mn (II) and biofilm which was observed in single system Freundlich isotherm model, as illustrated in Table 3.3.

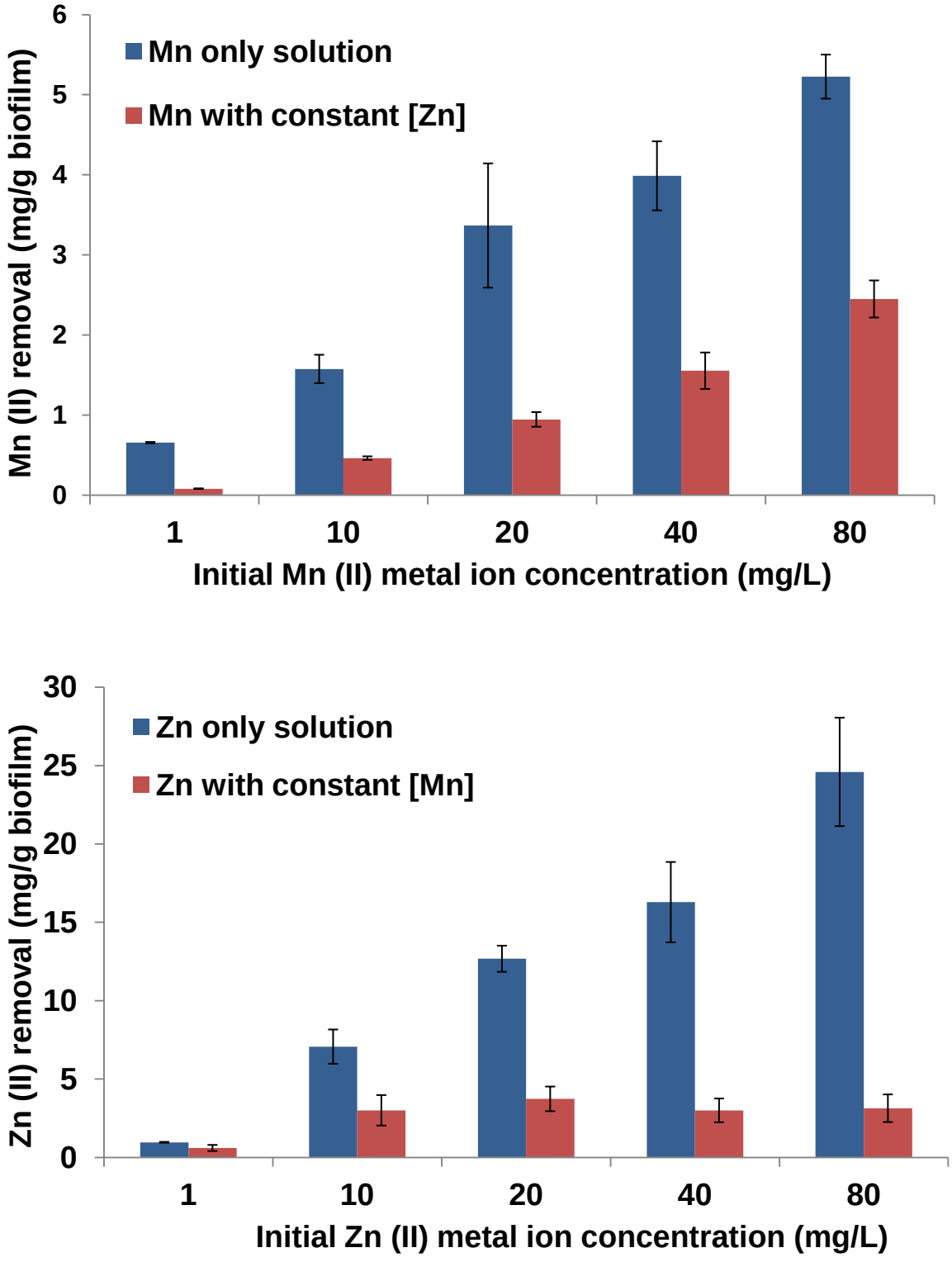


Figure 3.10 Biosorption capacity comparison of Zn (II) and Mn (II) for single and binary systems (constant [metal]= 20 mg/L, pH 6, 120 rpm, contact time 120 min, 30^{o}C). Error bars represent the standard deviation of triplicate measurements.

3.3.9 Toxicity and growth curves

Toxicity was assayed using a bioluminescence based bacterial sensor in a two stage test, involving exposure and post exposure recovery. Post-exposure recovery experiments were used to distinguish any inhibitory and lethal effects, if present. Growth assays were used to determine whether, or to what extent, each metal could be utilised as a sole carbon source by MWF acclimated bacteria. Single and combinatorial toxicity tests for Zn (II) and Mn (II) solutions, individually at 5 different concentrations (1, 10, 20, 40 and 80 mg/L) and also 9 possible combinations of these two metal ions, were tested relative to control samples which consisted of PBS samples. Toxicity results indicated both metals to be acutely toxic individually but lethally toxic in combination (Figure 3.11). However, compared to the control, no significant changes in growth were observed with either of the individual metal concentrations or in combination of the metals.

A consideration for this experimental approach is the use of an undefined bacterial consortium for growth experiments. Bioreactor communities investigated in this study were diverse, dynamic and also contained many uncultivable species making culture based inoculum non representative of an *in situ* community (van der Gast et al., 2004b). The approach used here assumed that the bacterial diversity and functional redundancy from the active bioreactor community was sufficient that biodegradation would not be affected by minor variations in the species composition. The results in Figure 3.11 suggest that the MWF acclimated bacteria were resistant to the metals at the concentrations of zinc and manganese investigated however optical density, which was used

for the degradability growth test, does not determine the viability of the cells. Micro-respiration and post exposure recovery tests would have been better methods for ascertaining toxicity. Since the bioluminescence based bacteria biosensor is more sensitive to toxic metals, the combined metals exhibited a more lethally toxic effect compared to when present individually.

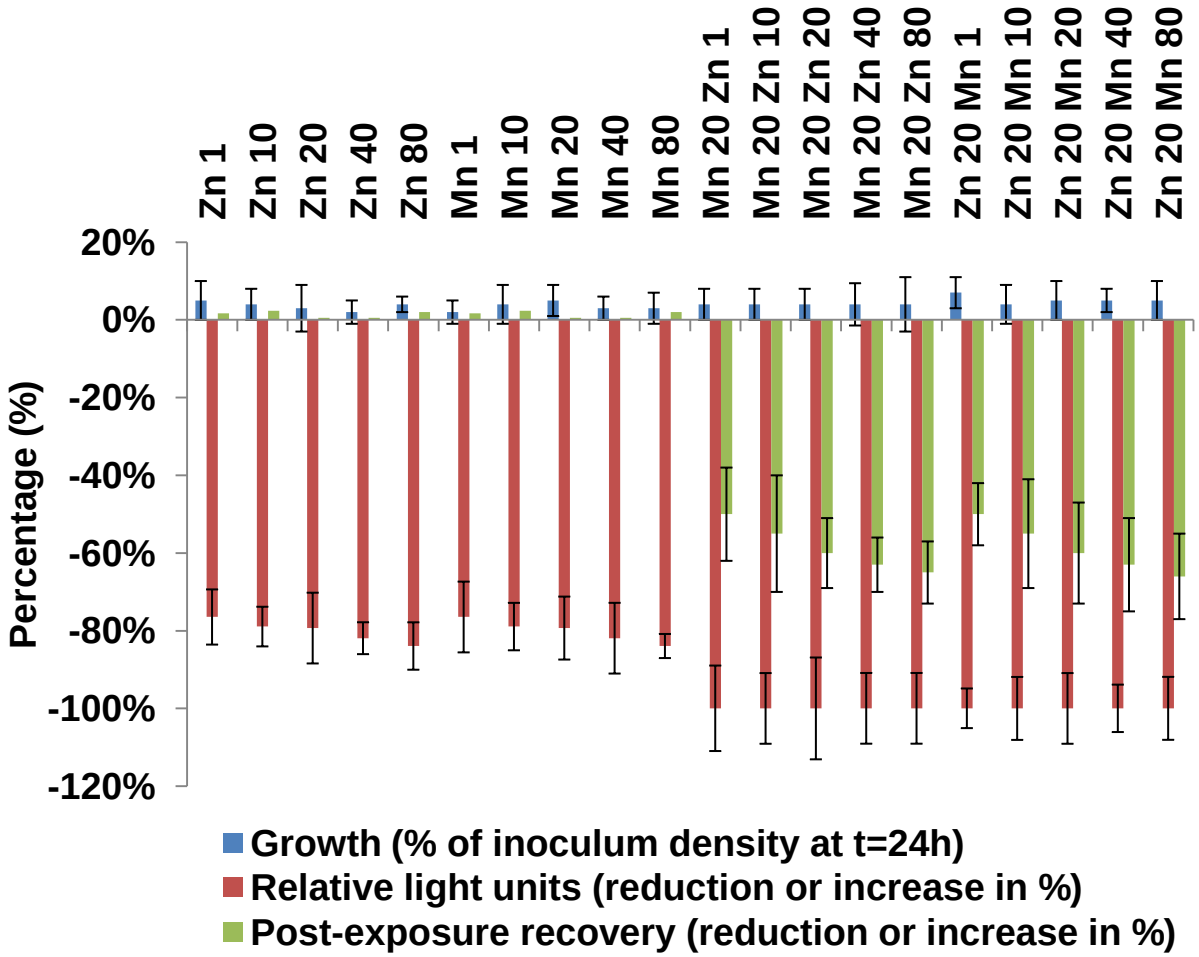


Figure 3.11 Summary of single metals screening of 2 metals at 5 concentrations each and 9 possible combinations of 2 metals combined.

3.3.10 Effect of free-living bacterial cells aging on heavy metal removal

The greatest biomass of free-living bacterial cells was observed after 3 days of growth as can be seen from the optical density (OD) readings listed in Table 3.4. The decrease in OD after Day 3 was due to the decrease in the numbers of viable cells which was confirmed by plate counts determined by Miles and Misra plate count method. On Day 3, the metal removal by the free-living cells reached its maximum with 0.67 mg/g biofilm (± 0.08) and 2.60 mg/g biofilm (± 0.10) at initial Zn (II) concentrations of 10 mg/L and 80 mg/L, respectively (Figure 3.12). Hence, for all further bacterial free-cells experiments, the freeze-dried stock was grown for a period of 3 days.

Table 3.4 Optical density and Miles and Misra plate count measurements of MWF bacterial free-cells for growth assessment.

	Optical Density	**Miles and Misra (cfu/ml)**
2 Days	0.2113± 0.0180	$4.57 \times 10^6 \pm 2.75 \times 10^5$
3 Days	0.3893± 0.0378	$1.27 \times 10^8 \pm 1.46 \times 10^7$
4 Days	0.2817± 0.0472	$1.64 \times 10^6 \pm 1.27 \times 10^5$
5 Days	0.1433± 0.0392	$8.87 \times 10^4 \pm 1.08 \times 10^4$

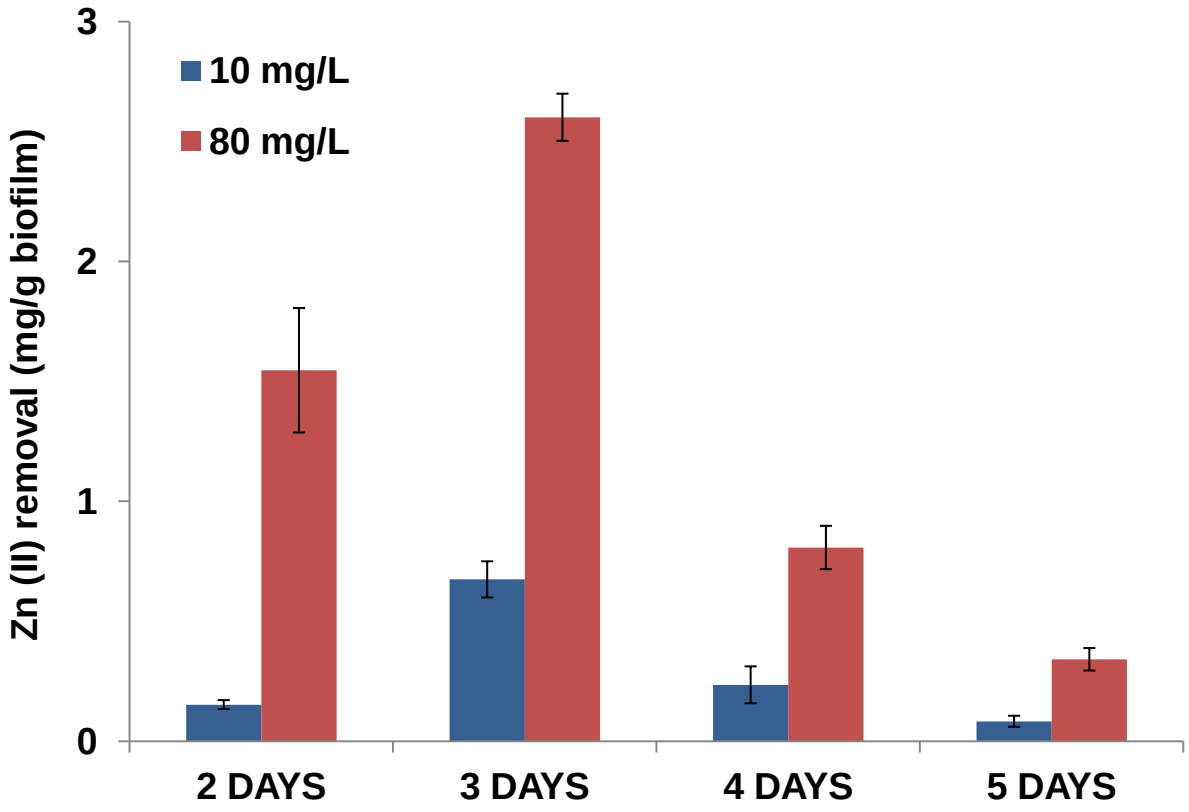


Figure 3.12 The effect of free-living bacterial cells age on Zn (II) removal by MWF acclimated bacterial biofilms (pH 6, 120 rpm, 30^{o}C, 300 min contact time). Error bars represent the standard deviation of triplicate measurements.

3.3.11 Comparison of zinc removal by free-living bacterial cells and biofilms

Increasing initial metal ion concentration affected the Zn (II) removal by free living cells. Figure 3.13 shows Zn (II) removal by free-living cells to be 0.664 mg/L (± 0.07) and 2.60 mg/L (± 0.10) at the initial Zn (II) concentrations of 10 mg/L and 80 mg/L respectively, over a time period of 180 min. However, the system seemed to reach equilibrium within 90 min (Figure 3.13). Zn (II) removal by free-living cells achieved equilibrium quicker compared to biofilms, which took 300 min.

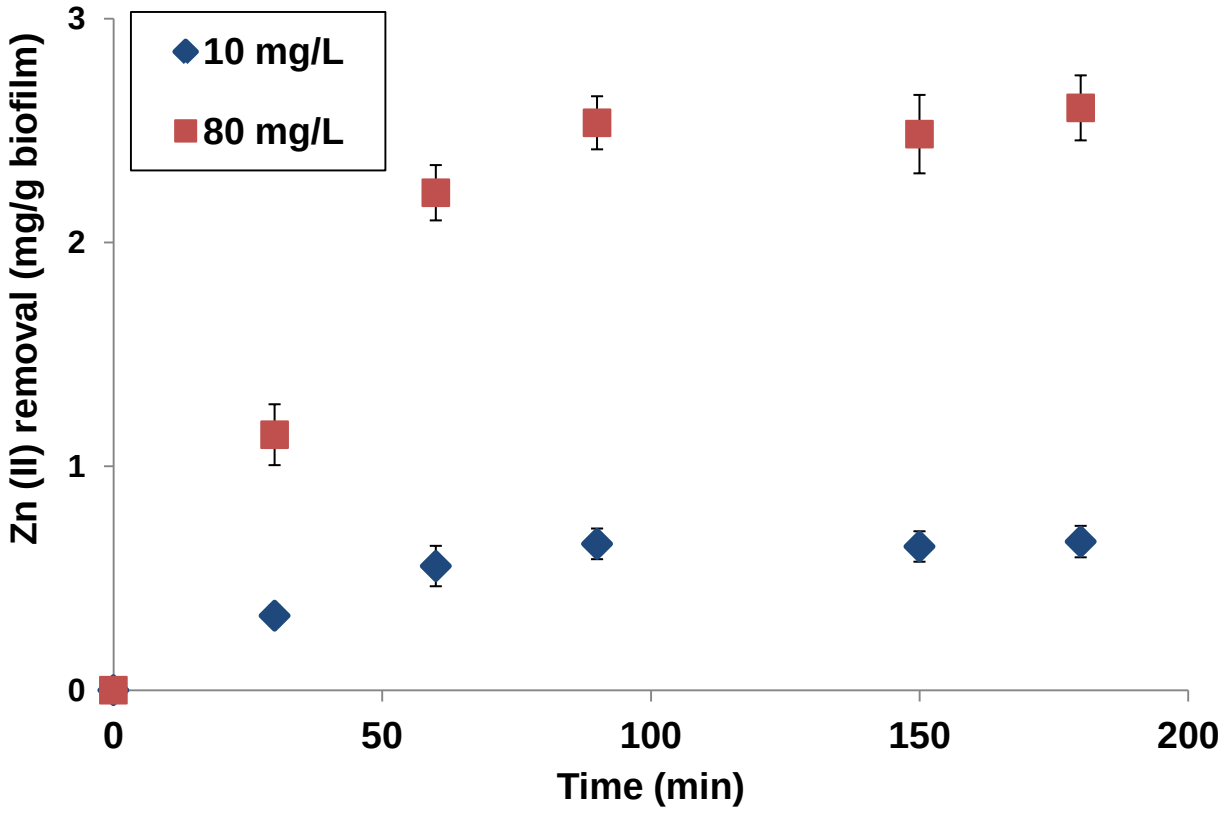


Figure 3.13 Effect of contact time on Zn (II) removal by bacterial free-living cells (pH 6, 120 rpm, 30^{o}C). Error bars represent the standard deviation of triplicate measurements.

In terms of metal removal capacity for zinc, the order of biosorption was biofilms> non-treated free-cells> heat treated and autoclaved free-living cells. Heat-treating and autoclaving free-cells significantly inhibited biosorption, which can be attributed to the disruption of functional groups and denaturing of cells that can lead to loss in extracellular and intracellular removal, respectively (Das and Guha, 2009).

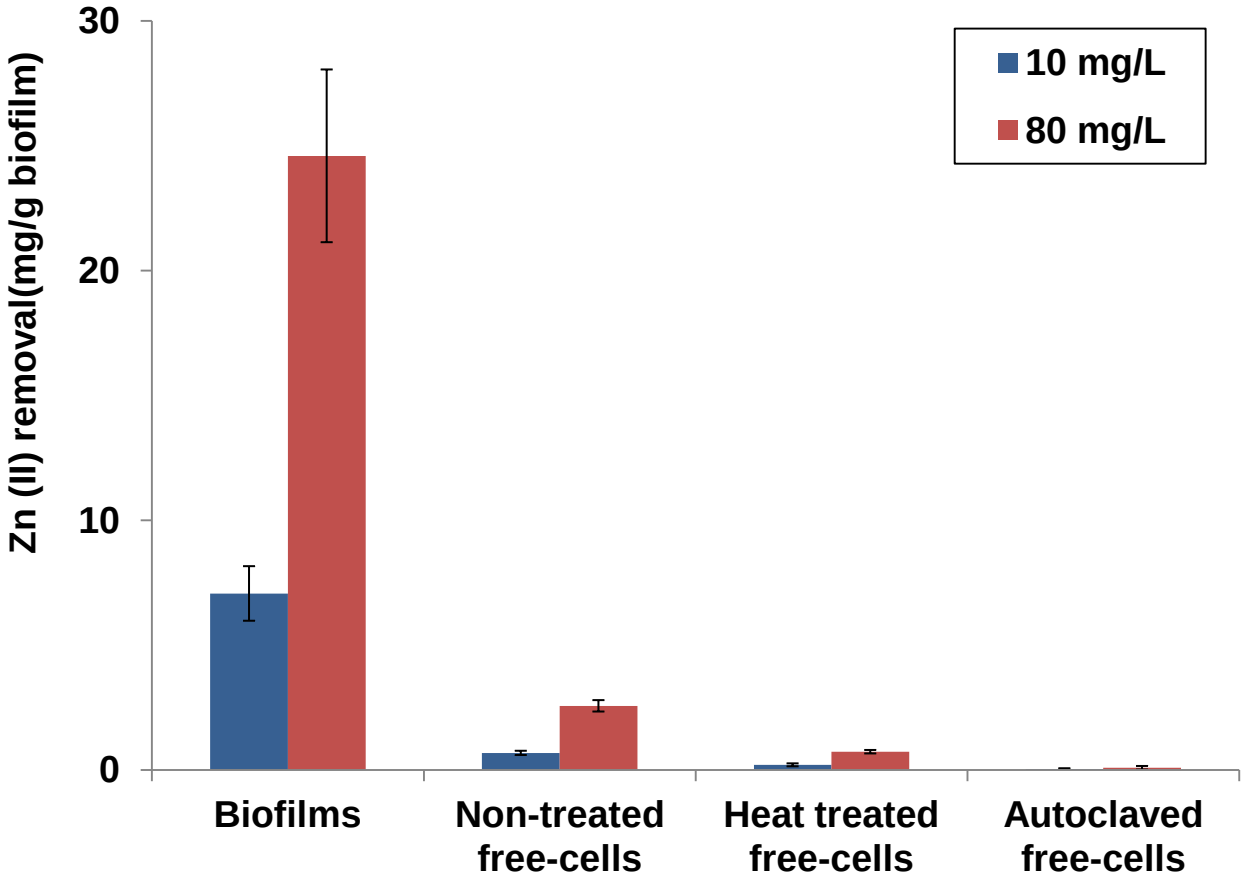


Figure 3.14 Comparison of Zn (II) immobilisation by free-living cells and biofilms. Error bars represent the standard deviation of triplicate measurements.

Biofilms had the greatest amount of Zn (II) removal with 7.07 mg/L and 24.60 mg/L at initial concentrations of 10 mg/L and 80 mg/L respectively, compared to free-living cells which had Zn (II) removal of 0.68 and 2.57 mg/L at the initial Zn (II) concentrations of 10 mg/L and 80 mg/L, respectively (Figure 3.14). This agrees with other researchers who state that bacterial biofilms can biosorb more metals as compared to their free-living counterparts. A control of sterilised biofilm for Zn (II) removal was not used in this study because sterilisation resulted in disintegration of biofilms, which affected the removal amounts.

Toxicity and growth curve tests using MWF acclimated bacterial free-living cells and biofilm cells demonstrated that MWF acclimated bacterial biofilm cells had more resistance to Zn (II) solutions compared to their free-living cells counter parts (data not shown). This was mainly due to the ability of the EPS matrix to delay or buffer toxic substances from reaching target microorganisms within the biofilm through diffusion limitation and/or chemical interactions with EPS; thereby biofilm cells in an attached-growth biological process can tolerate significantly higher toxicant concentrations than free-living bacterial cells (Lee and Ong, 2006, Wuertz et al., 2003). In addition, immobilised biofilms are more tolerant to extreme environmental conditions compared to free-living cells (Davey and O'Toole, 2000), they are able to regenerate the biofilm at the end of process for metal recovery and also there are lowers costs associated with removal of the biosorbent in immobilised biofilm form from industrial reactors as compared to the high costs of removing free-living cells. These reasons encourage us to further investigate biofilms over free-living cells as they are more conducive to an engineering solution.

The maximum Zn (II) and Mn (II) removal of immobilised MWF acclimated bacterial biofilms observed experimentally and predicted by the Langmuir model were compared with that of other bacterial biosorbents reported in literature. Table 3.5 shows that the biofilm in this study exhibited moderate metal removal compared to other bacterial strains for Zn (II) removal and comparatively poor performance for Mn (II) removal. However, the methods and standards for biosorption experiments can vary significantly hence, the reported results are not always comparable. Overall, this part of the project provided insight on the

potential of immobilised MWF acclimated bacterial biofilms to biosorb heavy metals and the nature of competition between metal ions in binary solutions. This serves as foundation research, which is necessary to achieve the overall goal of ascertaining the effect of organics on metal immobilisation efficiency in order to use biofilm-based systems to recover metals from industrial wastewater.

Table 3.5 Results from the literature on metal biosorption by various bacterial species
(E)=experimental removal, (L)=removal predicted by the Langmuir model; NA=not available. [a] Chemically modified [b] Immobilised

Metal	Biosorbent	PH	Temperature (^{0}C)	Removal (mg/g biofilm)	Reference
Zn (II)	MWF acclimated bacterial biofilm[b]	6	30	24.60 (E) 27.17 (L)	This work
	Aphanothece halophytica	6.5	30	133.0(L)	Incharoensakdi, A. & Kitjaharn, P., (2002)
	Pseudomonas putida	7.0	NA	6.9(L)	Pardo, R. et al., 2003.
	Pseudomonas putida CZ1	5.0	30	17.7(L)	Chen, X.C. et al., 2005.
	Streptomyces rimosus	7.5	20	30.0(L)	Mameri, N. et al., 1999.
	Streptoverticillium cinnamoneum[a]	5.5	28	21.3(E)	Puranik, P.R. et al., 1997.
	Thiobacillus ferrooxidans[a]	6.0	25	82.6(L)	Celaya, R.J. et al., 2000.
Mn (II)	MWF acclimated bacterial biofilm[b]	6.0	30	5.23 (E) 6.01(L)	This work
	HAH1	5.5-6.0	37	55.56(L)	Hasan, H.A. et al., 2010.
	Sewage Activated Sludge	5.5-6.0	37	10(L)	Hasan, H.A. et al., 2010.

3.4 Conclusions

In this study, fundamental experiments addressing biosorption of Zn (II) and Mn (II) from single and binary aqueous solution systems by immobilised MWF acclimated bacterial biofilms, grown on polyethylene strips, were performed. Key findings are as follows:

- Immobilised MWF acclimated bacterial biofilms showed great potential for Zn (II) and Mn (II) removal in aqueous systems.
- In single-metal systems, the optimal pH for biosorption of both Zn (II) and Mn (II) was pH 6. The biofilms exhibited rapid metal removal in the first hour and reached equilibrium within 5 hours for both metals. The immobilised MWF acclimated bacterial biofilms biosorbed 4 times more zinc than manganese and this was attributed to the higher electronegativity of the zinc and possibly other factors.
- The isotherm experimental results fitted well for both Langmuir and Freundlich models with correlation coefficients R^2 above 0.9. However, Freundlich model, which assumes the biosorbent to have a heterogeneous surface with biosorption sites that have different biosorption energies, seemed to be the better fitting model for the experimental data. Results revealed that the biofilms were preferential to Zn (II) compared to Mn (II) removal, which is congruent with the experimental data.
- The kinetics of biosorption process for Zn (II) and Mn (II) were well described by the pseudo second order model with relatively high correlation coefficients ranging from 0.960 to 0.999.

- From the SEM images, more differences were detected in the zinc-loaded biofilms than in the manganese-loaded biofilms when compared to control, which could explain the results of MWF acclimated bacterial biofilms having a higher zinc removal than manganese.
- In binary biosorption experiments, strong competition between Zn (II) and Mn (II) in terms of occupying the binding sites of biofilms was observed. Zinc removal (mg/g biofilm) decreased up to 87% whereas the Mn (II) removal (mg/g biofilm) only decreased up to 66% at initial metal concentration of 80 mg/L. This can be explained by the stronger bonding between Mn (II) and the biofilm which was found in single system Freundlich isotherm model.
- Metal removal by free-living cells achieved equilibrium more rapidly (within 90 min) compared to biofilms, which took 300 min. However, biofilms were able to remove 12 times more zinc at initial metal ion concentration of 80 mg/L, confirming that the bacterial biofilms could remove greater amounts of heavy metals as compared to their free-living counterpart cells.

Chapter Four

4. Comparative Study of Chemical and Physical Methods for Distinguishing Between Passive and Metabolically Active Mechanisms of Pollutant Removal by Biofilms.

4.1 Introduction

Microorganisms remove organic pollutants from water through biosorption and active biodegradation while simultaneously accumulating heavy metals intracellularly or immobilising them on the cell surface (Malik, 2004; Aksu, 2005; Vijayaraghavan and Yun, 2008). Researchers have compared removal of pollutants by live and metabolically inactivated biomass, with a view to differentiate between mechanisms of removal and to quantify the extent each contributes to the total pollutant removal from water (Doshi et al., 2007; Johnson et al. 2007; Klein and Kennedy, 1997).

To date, thermal treatments through techniques such as autoclaving have been the most common means of inactivating biomass for physicochemical sorption measurements (Tsezos and Bell, 1989; Wang and Grady, 1994; Das and Guha, 2009). Wang and Grady (1994) demonstrated that the sorption of both viable and metabolically inactivated biomass were identical, after taking into consideration the loss of biomass during autoclaving. Das and Guha (2009)

reported greater chromium (VI) removal in live compared to thermally-inactivated biomass and attributed the difference to the partial loss of binding sites, and/or the intracellular accumulation of chromium by the viable cells. However, the results were difficult to interpret since exposure to high temperatures (> $40^{O}C$) affected cell adsorption capacity and morphology. In contrast, Boyer et al. (1998) investigated the metabolic removal of copper by *Thiobacillus ferrooxidans* at $5^{O}C$. The lower temperature decreased biological metabolism, whilst not disrupting the integral architecture of the biofilms. However, reducing the temperature lowers the total kinetic energy of the system and alters the thermodynamic parameters, which affect the physicochemical removal rates and provides little indication of their potential in real-world applications (Delle Site, 2001).

Chemical inhibitors are an alternative approach which have been widely used for distinguishing pollutant removal mechanisms (Klein and Kennedy, 1997; Johnson et al., 2007; Das and Guha, 2009). They quench the metabolic activity of microbes whilst avoiding cell damage or disruption of the biofilm structure. Formalin and sodium azide are among the most commonly employed. Formalin is known to dehydrate cells by inactivation of proteins, forming covalent crosslinks with several functional groups. Hence, employing formalin to inactivate bacteria alters the cell structure which can lead to misleading results. Sodium azide prevents electron transfer by inhibiting oxidative phosphorylation by binding to the terminal cytochromes of active bacteria (Johnson et al., 2007; Das and Guha, 2009). 2,4-dinitrophenol (DNP) and N,N'-dicyclohexylcarbodiimide (DCCD) are less commonly used as un-couplers of

oxidative phosphorylation preventing ATP synthesis by inactivating ATP synthetase function (Incharoensakdi and Kitjaharn, 2002). Surprisingly, no study to date has investigated the effects of these metabolic inhibitors on cell structure and biomass functional groups, which play a key role in physicochemical sorption of pollutants.

In this study, we compared physical ultraviolet (UV) inhibition with chemical (sodium azide) methods to distinguish and quantify the relative contributions of biosorption and metabolic removal of chemical contaminants in water. UV treatment is a proven, environmentally friendly technology used for effluent disinfection in wastewater systems (Azimi et al., 2014). With this, UV light is absorbed by the nucleic acids (DNA and RNA) of exposed microbial cells, altering DNA structure and thus preventing metabolic activity and any further cell division. Both UV and sodium azide methods were applied to assess the removal of organics (triethanolamine and benzotriazole) and metals (Zn (II), Cu (II) and Cd (II)) individually to: 1) compare UV-treatment and chemical inhibitors method for biofilm inhibition; 2) determine the relative contributions of the different mechanisms (biosorption vs. metabolic uptake) responsible for the individual removal of organics and metals.

4.2 Materials and Methods

4.2.1 Description of bioreactor system

Typically, 4 stainless steel sheets, employed as immobilisation matrix for biofilm formation, of 200 cm^2 (10 cm x 20 cm) were placed in the bioreactor. After biofilm formation, each was cut into 10 small strips (20 cm^2 each) for studies in 250 mL Erlenmeyer flasks. Two mL of phosphate buffered saline (PBS) solution was used to resuspend a freeze-dried inoculum before it was used. The biofilms were grown using two vials of the freeze-dried stocks in a 4 L bioreactor with 800 mL autoclaved M9 minimal medium and 1% v/v fresh MWF (CoolEdge BI concentrate provided by Castrol) at 25^{O}C with continuous air flow over 3 weeks. The bioreactor was decanted and refilled every 3 days with fresh MWF. See Section 3.2.1 for more details about the bacteria consortium and freeze-drying protocol.

4.2.2. Measurement of metabolic inhibition

Respiration activity was measured using a micro-plate based respiration system, known as MicroResp^{TM}, which measures carbon dioxide (CO_2) evolved. This colorimetric method is based on the colour change of a pH indicator dye (cresol red) caused by the presence of CO_2 (Campbell et al., 2003). The CO_2-trap absorbance was measured at 590 nm (Biotek Synergy HT spectrophotometer) initially, and then after incubation (24 hours, 30^{O}C). The conversion of absorbance to CO_2 trapped is a non-linear relationship; the best

fitted curve (regression analysis) is used to obtain the quantities of CO_2 evolved (<5%) by the microbial samples for the incubation period.

Post exposure recovery (PER) tests, using the plate count method (Miles et al., 1938), were performed to enumerate colonies after exposure to the test compounds. After 24 hours exposure in the respiration analysis, 100 µl of the aqueous bacterial solution were diluted using 900 µl phosphate buffered saline (PBS) and then, regrown on LB agar plates. The plates were then incubated (24 hours, $35^{O}C$) and resulting colonies were counted. LB agar plates were made using LB Miller agar (40 g/L, Fisher Scientific) in deionised (DI) water with the additional D-glucose (5 g/L).

The following chemicals, commonly reported to be used as metabolic inhibitors, were tested against UV: formalin (0.1%, 0.5%, 1%, 2% and 5%), sodium azide (1 mM, 5mM and 10 mM), DNP (0.5 mM, 1 mM, 5 mM and 10 mM) and DCCD (0.1 mM, 0.2 mM and 0.4 mM). All four were supplied from Sigma-Aldrich. The degree of inhibition was assessed by plotting colony-forming units (CFU / mL expressed as % of control) from post exposure recovery method versus respiration activity (CO_2 % of control). This method was also used to determine the minimum UV-exposure time required for complete biofilm inhibition and to assess the toxicity effects of individual organics and metals. Biofilms in our study required a UV dose of approximately 300 mJ/cm^2 for complete inhibition.

4.2.3 Analytical methods

All chemicals were analytical reagent grade supplied from Sigma-Aldrich. Stock solutions of 10% v/v triethanolamine (TEA) and 2% w/v benzotriazole (BTA) were prepared by suspending appropriate amounts of triethanolamine (GC) (≥ 99.0%) and 1-H Benzotriazole (≥ 98.0%) in deionised (DI) water. Zn (II), Cu (II) and Cd (II) stock solutions (each at 1 g/L concentration) were prepared by suspending $ZnSO_4.7H_2O$ (≥ 99.0%), $CuSO_4.5H_2O$ (≥ 98.0%) and $CdSO_4.8/3H_2O$ (≥ 99.0%) in DI water. These were further diluted to obtain concentrations for practical use. Samples collected in all experiments were centrifuged at 12,000 rpm for 15 min to minimise background biofilm content. The total organic carbon (TOC) concentration of the supernatant was measured by a Shimadzu TOC V-CPH. The system was calibrated with standards prepared from potassium hydrogen phthalate in the range of 0-1000 mg/L.

Atomic absorption spectroscopy (Agilent 240FS AA) was used to measure metal concentrations, calibrated in the linear ranges of 0.2-1.2 mg/L, 0.5-4 mg/L and 0.4-1.8 mg/L for zinc, copper and cadmium, respectively. Before measurement, the heavy metal solutions were appropriately diluted with DI water to ensure that the heavy metal concentration in the sample was linearly dependent to the absorbance detected.

4.2.4. Comparing UV and chemical inhibitor for pollutant removal

The organic/metal removal Qe was calculated as follows (Gabr et al., 2008):

$$Q_e = V\left(\frac{C_o - C_e}{M}\right) \quad \textbf{(1)}$$

where Q_e is the organic/metal removal (mg/ g biofilm); C_O and C_e are the initial and equilibrium organic/metal concentrations in solution (mg/L) respectively; V is the solution volume (L); and M is the biofilm dry weight (g).

To determine dry-weight, biofilms grown on stainless steel strips were first rinsed with DI water, and then were dislodged into a 50 mL test tube by vigorous shaking. The dry weight of the biofilm was calculated as the sum of the dry weight of the centrifuged biofilm cells collected at the bottom of the test tube and the dry weight of the biofilm cake floating on the top of the solution after centrifugation. The dry weight of the biofilms cells was measured after drying them at 70^{O}C for 1 hour to constant weight. The dry weight of the biofilm cake was measured after vacuum filtering and then drying in the furnace at 50^{O}C for 4 hours. Total suspended solids were not determined following the Standard Methods for the Examination of Water and Wastewater (No. 2540) (APHA, 2001) because the biofilm cake disintegrated at higher temperatures and parts of the solids were lost in the measurement.

4.2.4.1 TEA and BTA removal

TEA and BTA were studied in the concentration ranges of 0.1% v/v-10% v/v and 0.1% w/v-2% w/v, respectively. TEA is commonly used as pH buffer and BTA is a corrosion inhibitor that is used in MWFs. These widespread contaminants of waste- and surface-water pose major risks to public health and the environment and hence are extensively studied in literature (Wu et al., 1998; Cheng et al., 2005). These were chosen to account for the differences in composition of all the different types of metalworking fluids (MWFs) that exist. It is important to note that operationally exhausted MWFs are 95% water. Based on toxicity analysis, biofilms exposed to 10 mM sodium azide for 24 hours were selected to test against UV (Section 4.3.1). Hence two types of inhibited biofilms were used for all further experiments: UV treated and sodium azide treated.

All organic (TEA and BTA) removal experiments were undertaken by exposing the biofilm strips (20 cm^2 area covered on both sides) to 100 mL of the organic solution at a range of concentrations. Biofilms on the stainless steel metal strips were placed in 250 mL Erlenmeyer flasks (120 rpm, 30^OC) for the removal experiments. Samples were centrifuged at each time point and the supernatant liquid was analysed for TOC content. Negligible removal of organics and metals were detected when using blank stainless steel strips (without biofilms).

4.2.4.2 Metals removal

The experimental procedure to quantify the removal of metals was similar to that described for organics (Section 4.2.4.1) except that these experiments were conducted in 200 mL metal solution. Samples were centrifuged at each time point and the supernatant liquid was analysed for metal concentration.

4.2.5. Measuring the effect of oil interference on biosorption

Mineral oil, from the oil-based MWF used to grow the biofilms, was found to have accumulated on the biofilm surface, significantly decreasing the biosorption of TEA and zinc of both UV and sodium azide treated biofilms. Triton X-100, an oil-emulsifying surfactant, did not alter the surface morphology of the biofilm cells and therefore was used in investigations to remove the oils. Biofilms were washed 3 times with 0.2% Triton X-100 to remove adsorbed oil, and then five times using DI water to remove residual surfactant (this removal was ensured by TOC removal measurements, results not shown).

4.2.6. Measurement of biofilm carbon released due to inhibitors

This test was carried out to explore the effect of each of the inhibitors (i.e. chemical treatments and UV) on the structural integrity of the cells. Any treatment resulting in destruction of the cell membrane will lead to the release of intracellular components into solution. Since many of these components are organic in nature (e.g. DNA), there will be an increase in the carbon of the

solution in which the treatment occurred. It is thus possible to estimate the percentage of cell destruction due to the specific treatment (chemical or physical) by calculating the percentage of biofilm carbon released in the bulk solution:

$$\textbf{Percentage of biofilm carbon released in the bulk solution} = \left(\frac{C_2 - C_1}{C_{avg}}\right) X100 \quad \textbf{(2)}$$

C_2: Average carbon content of the supernatant of inhibitor solution plus biofilm after undergoing centrifugation

C_1: Average carbon content of inhibitor solution with no biofilm

C_{avg}: Average carbon content of cells on one strip of biofilm

C_2 represents the average carbon content, after centrifugation, of the supernatant of inhibitor solution that was exposed to the biofilm. C_1 represents the average carbon content of the inhibitor solution without biofilm. Hence, any positive difference in carbon content of these two values corresponded to the biofilm carbon released due to cellular destruction. Thus, the relative amount of biofilm carbon in the bulk solution can be represented by Equation (2).

The average carbon content on one strip of biofilm was calculated from the average of 20 stainless steel strips (20 cm^2 each). Each biofilm strip was exposed to 0.2% Triton X-100 (an oil-emulsifying surfactant) in a 50 mL centrifuge tube and placed on a rotating shaker at 50 rpm for 10 min. This was repeated three times until all the oil was washed away from the surface of the biofilm, then was washed with DI water 5 times to remove all traces of Triton. The oil-free biofilm was dislodged from the stainless steel strip by vigorous

shaking and then was centrifuged in DI water at 4000 rpm for 30 min, after which all the cells were collected. This pellet was lysed using 30 ml of 0.1% sodium hypochlorite. The total carbon released due to lysis of the biofilm cells was then measured using a Shimadzu TOC V-CPH. Thus, the average carbon content of the biofilm cells on one strip was found to be 28.03 mg (± 0.25).

4.2.7. Scanning electron microscopy

Same procedure as Section 3.2.5.

4.2.8 UV intensity measurement

A Spectroline UV lamp (ENF-260C, low pressure lamp 254 nm) was used for inhibiting biofilms (thickness: 2-3 mm) in all the UV related experiments. The UV dose for complete biofilm inactivation was determined to be approximately 300 mJ/cm^2 (Bolton and Linden, 2003). The UV intensity at 254 nm was determined by microprocessor-controlled radiometer (Cole-Parmer). Inactivated biofilms did not show photo-reactivation in the time scale of the experiments (tested with microrespiration and plate counts, results not shown here). It should be noted that the UV dose required for biofilm inactivation is dependent on the packing density of the biofilm, composition of wastewater used, and the compositional variations of the biofilm. Therefore, it is recommended that the UV dose required for complete inactivation of other biofilms to be determined experimentally.

4.2.9 Statistical analysis

All experiments were conducted in at least three replicates and the data represent the mean values ± standard error. T-test and ANOVA followed by Tukey's test were applied when appropriate, and P values less than 0.05 and 0.01 were considered statistically significant.

4.3 Results and Discussion

4.3.1. Selection of a metabolic inhibitor to test against UV

4.3.1.1 Micro-respiration and post exposure recovery

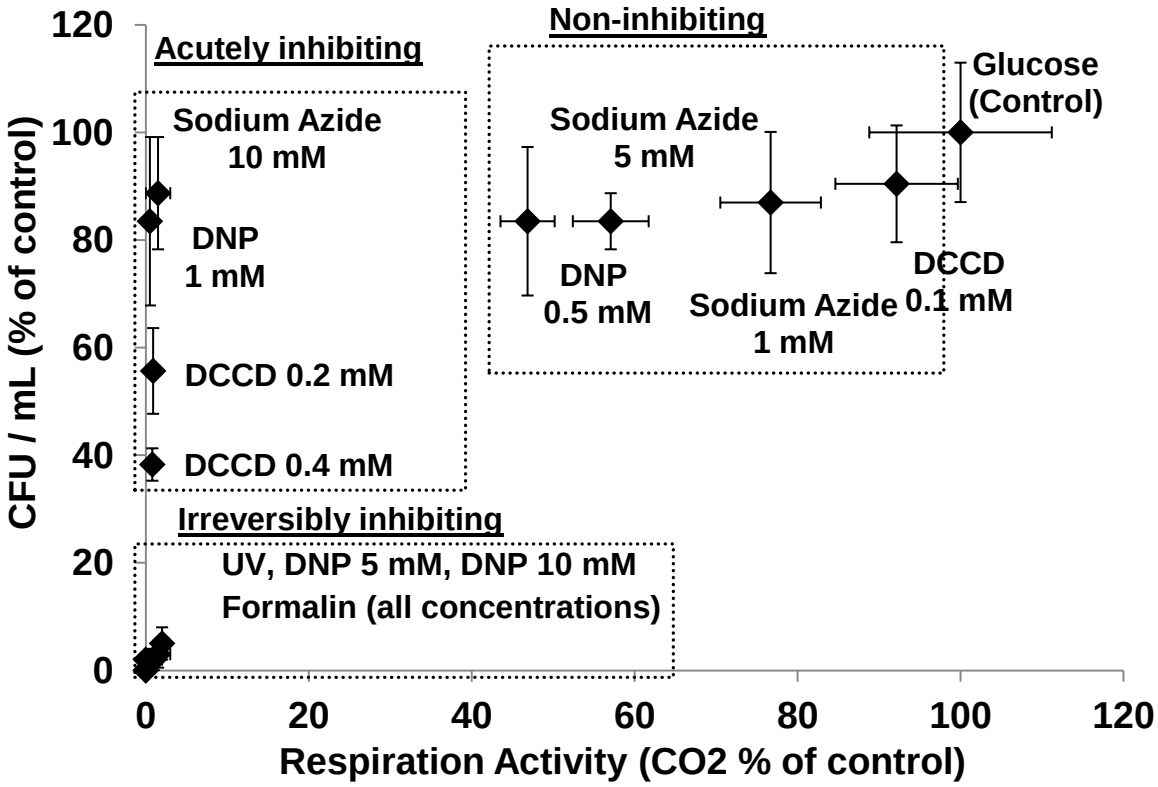


Figure 4.1 Respiration activity and post-exposure growth of select metabolic inhibitors after 24 hour exposure. Error bars represent the standard deviation of triplicate measurements.

The results of respiration and post exposure recovery (PER) tests conducted for a 24-hour time period are illustrated in Figure 4.1. The behaviours of the chosen metabolic inhibitors can be classified into three groups: 1) non-inhibiting i.e. those that do not completely eliminate both respiration activity and PER, 2) acutely inhibiting i.e. those that eliminate respiration activity but not PER, and 3) irreversibly inhibiting or completely toxic i.e. those that completely eliminate

both respiration activity and PER. These results are further confirmed by growth curve studies conducted for 24 hour period (data not shown). In these studies, we took respiration activity to be a measure of carbon dioxide given off by the microbial biofilm community as a result of exposure to the test compound and this is given as a percentage of control. A cell exposure to toxic compound induces low respiration activity and low CFU/ ml (expressed as a percentage of control). An acutely toxic compound demonstrates low respiration activity but high CFU/ml (expressed as a percentage of control) as the microbial community will most likely be revived during the post exposure recovery test in the absence of the toxic stress from the compound. A nontoxic compound has high respiration activity and high CFU/ml and will be present close to the data point of the control, in this case glucose. This test was repeated for a 5-hour time period, as illustrated in Figure 4.2, for the compounds that were either acutely inhibiting or irreversibly inhibiting in Figure 4.1.

Micro-respiration and post exposure recovery tests for the metabolic inhibitor conditions that did not completely inhibit respiration activity at 24 hours (Figure 4.1) were repeated for 5 hour exposure time (Figure 4.2). This exposure period was found to be the minimum amount of time required for the UV treatment to completely inhibit the metabolic activity of biofilm (thickness: 2-3 mm) used in this study (data not shown), and therefore this time interval was chosen to select an equally inhibiting chemical to test against UV.

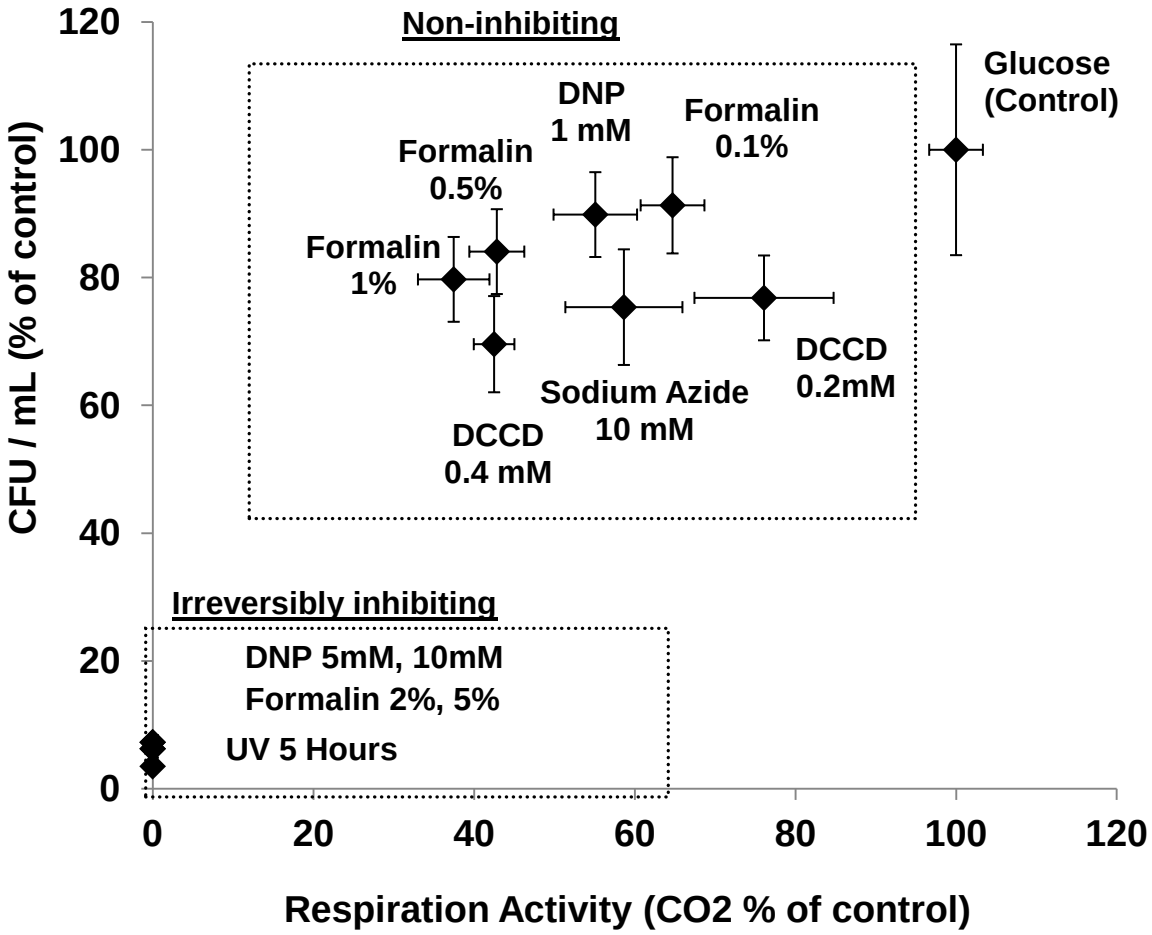


Figure 4.2 Respiration activity and post-exposure growth of select metabolic inhibitors after 5 hours exposure. Error bars represent the standard deviation of triplicate measurements.

As seen in Figure 4.2, only DNP at concentrations of 5 mM and 10 mM, formalin at concentrations 2% and 5% and UV treatment inhibited respiration activity of the biofilm cells. All of these treatments resulted in a low level of PER, and thus it was unclear if the cellular structure has been destroyed, or if it has remained intact. Any treatment resulting in destruction of the cell membrane will lead to the release of intracellular components into solution. Since many of these components are organic in nature (e.g. DNA), there will be an increase in the carbon of the solution in which the treatment occurred. It is thus possible to

estimate the percentage of cell destruction due to the specific treatment (chemical or physical) by calculating the percentage of biofilm carbon released in the bulk solution using Equation (2) from Section 4.2.6.

4.3.1.2 Biofilm carbon released due to UV and select metabolic inhibitors

Significantly less biofilm carbon was released in the bulk solution after exposure to UV, sodium azide and DI water (control) compared to those treated using chemical inhibitors formalin and DNP (Figure 4.3). Six percent (± 2%) of biofilm carbon was released into the bulk solution after exposure to UV compared to 33-41% (± 8%) with exposure to 5 mM and 10 mM DNP and 69-80% (± 5%) for 2% w/v and 5% w/v formalin, respectively. Many studies such as Johnson et al. (2007) for example, used 2.5% w/v formalin because it was unlikely to have any damaging effects on the cell wall. The results in combination with micro-respiration and post exposure recovery infer that only UV-treatment completely inhibited the metabolic activity of biofilms without significant amounts of biofilm carbon released into solution for 5 hour exposure. Hence, in order to select an equally inhibiting chemical, 24 hr respiration activity and post-exposure recovery data were used (Figure 4.1). Sodium azide (10 mM) and DNP (1 mM) significantly stopped respiration activity with similar growth in post-exposure recovery tests; hence biofilms exposed to both treatment methods were used to perform SEM analysis to observe for any structural changes on biofilm morphology.

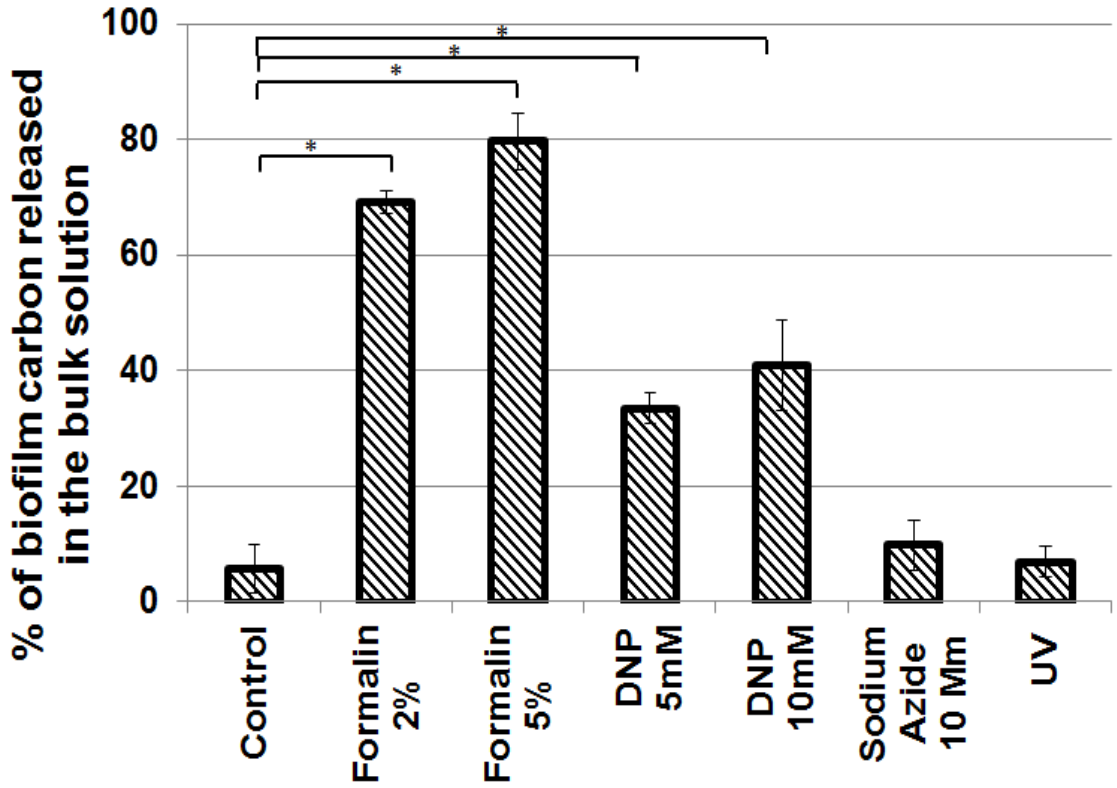


Figure 4.3 Percentage of biofilm carbon released in the bulk solution after exposure to select metabolic inhibitors and UV (300 mJ/cm^2) after 24 hours exposure. Error bars represent the standard deviation of triplicate measurements. Asterisks (*) denote significant difference ($P \leq 0.05$).

4.3.1.3 SEM analysis

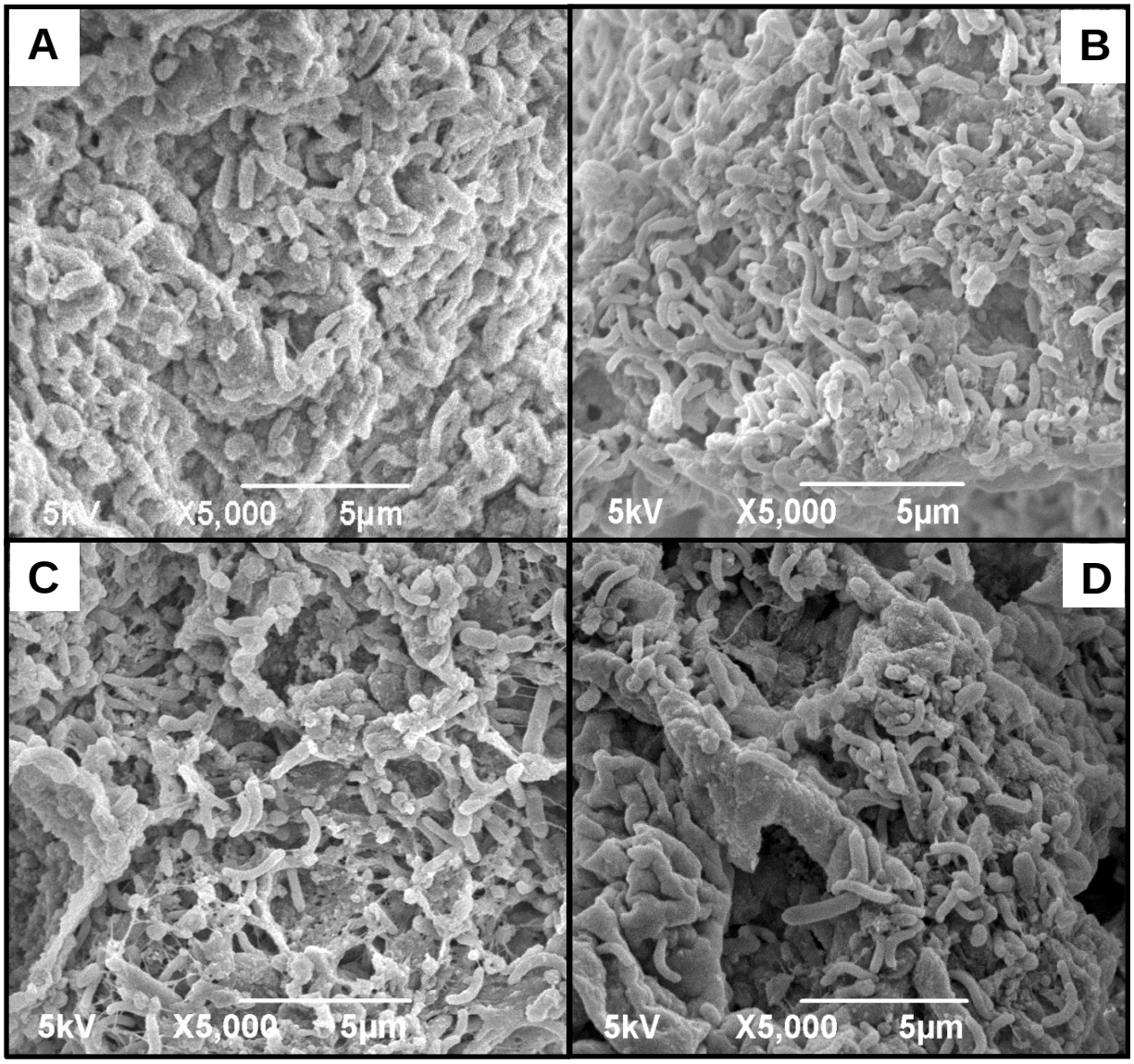


Figure 4.4: Scanning electron micrographs of biofilms at 5000X magnification: A) Uninhibited (control), B) Inhibited by UV with 5 hour exposure, C) Inhibited by 10 mM sodium azide with 24 hour exposure, D) Inhibited by 1 mM 2,4-dinitrophenol with 24 hour exposure

Scanning electron micrographs of uninhibited (control) and inhibited biofilms using UV (300 mJ/cm^2), sodium azide (10 mM) and 2,4-dinitrophenol (1 Mm) are given in Figure 4.4: A-D. Control and inhibited biofilms by UV and sodium

azide revealed no detectable impact on the cells nor the extracellular polymeric substance (EPS). In contrast, distinct morphological differences were observed between the control biofilms and those treated with 1 mM DNP. These cells were clumped into each other with few intact rod-like shaped cells present on the surface of the biofilm. The community in Figure 4.4 looks very simple because the bacterial communities in operationally exhausted MWFs have low diversity and are similar in species composition from different locations and uses. Moreover, the seed consortium was composed of only six different bacterial species as described by van der Gast and Thompson (van der Gast and Thompson, 2004a).

4.3.2 Comparison of UV and chemical inhibition for organic and metal removal

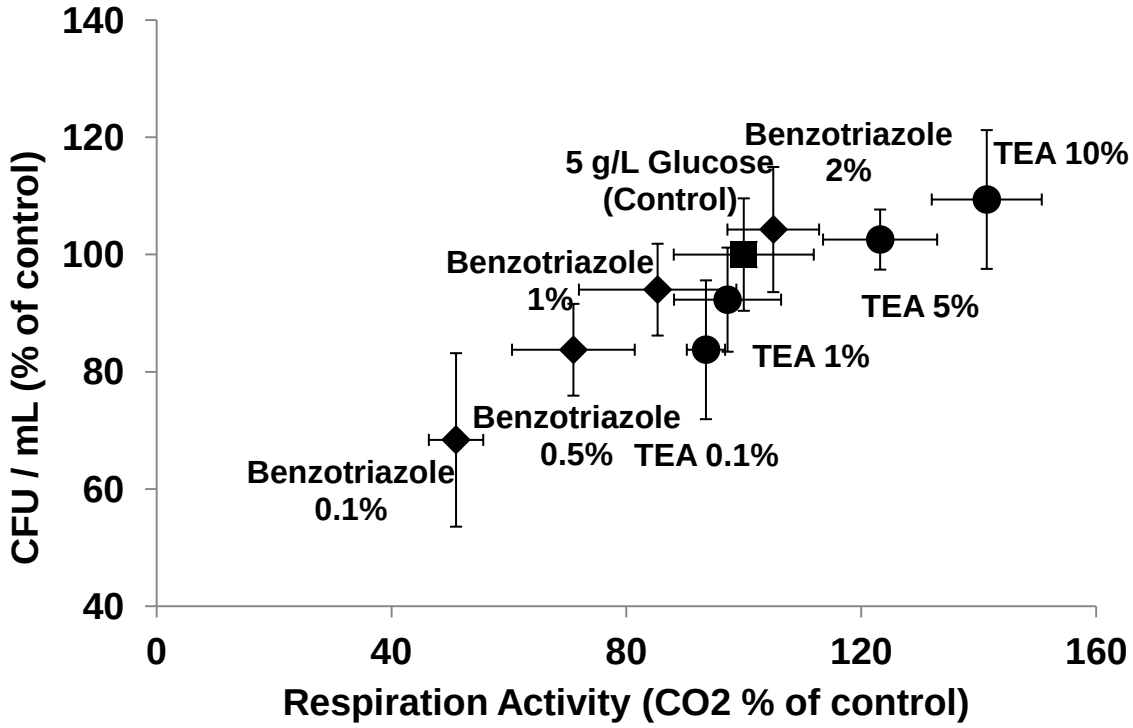


Figure 4.5 Respiration activity and post-exposure growth after 24 hour exposure demonstrating the toxicity of benzotriazole and triethanolamine individually. Error bars represent the standard deviation of triplicate measurements.

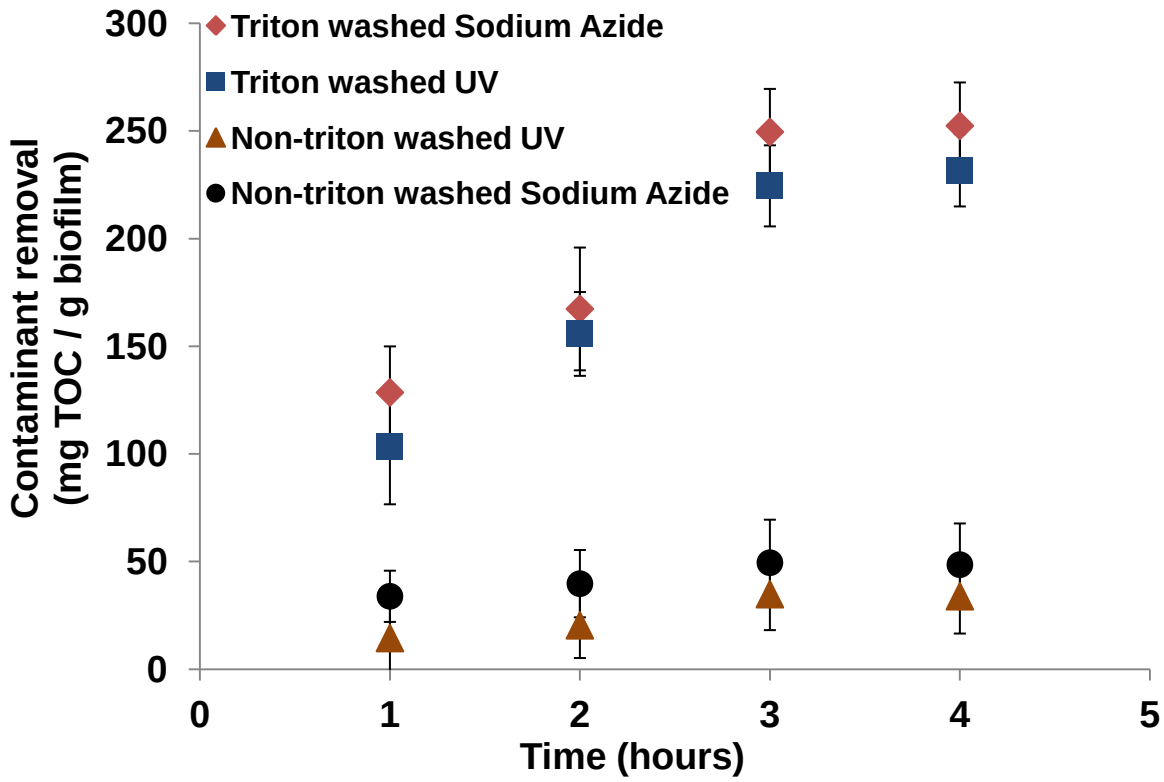


Figure 4.6 Effect of oil interference on the removal of 1% triethanolamine (mg TOC/g biofilm) by biofilms that have been inhibited by UV and sodium azide. Error bars represent the standard deviation of triplicate measurements.

TEA and BTA, within the concentration ranges of 0.1% v/v- 10% v/v and 0.1% w/v- 2% w/v, respectively, had no detectable toxic effect to the biofilm cells (Figure 4.5). The respiration rate and the level of post exposure recovery growth increased linearly with increasing concentrations for both compounds. The implication is that the cellular metabolic activity observed during the biodegradation was not the result of a stress response, but was due entirely to the utilisation of the compounds as carbon sources.

Figure 4.6 demonstrates the effect of oil interference on the removal of TEA by biofilms. In the case of non-triton-washed biofilms, no significant TEA

removal was detected by biofilms inhibited with sodium azide and UV ($P \geq 0.05$). However, washing with Triton X-100, an oil-emulsifying surfactant, led to significant carbon removal (Figure 4.6). Similar trends were observed for zinc removal by non-triton-washed and triton-washed biofilms (data not shown).

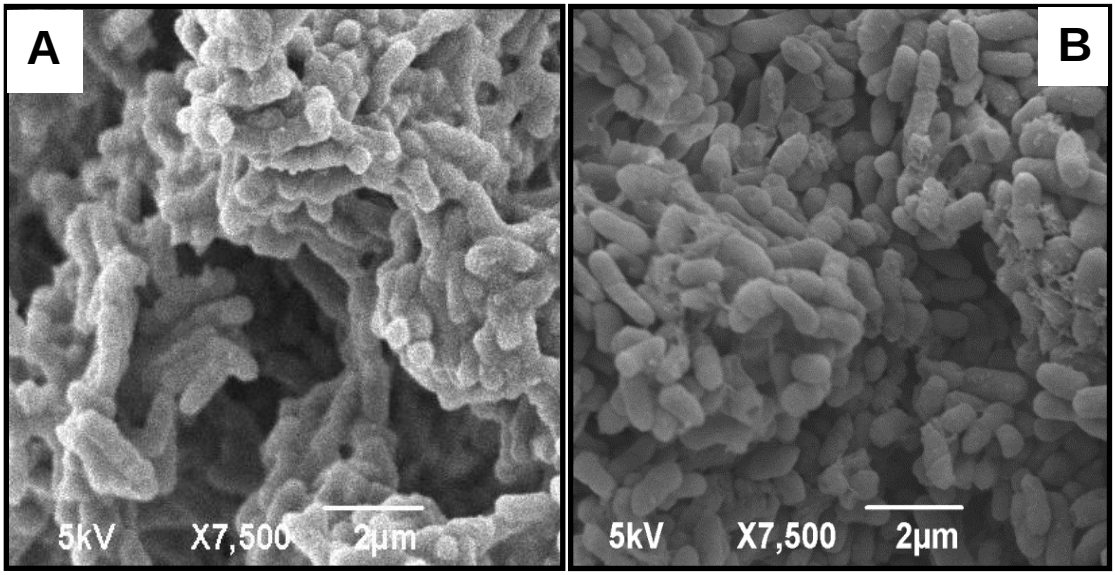


Figure 4.7 Scanning electron micrographs of biofilms at X7500 magnification reveal that Triton X-100 washes away the oils from the biofilm surface without altering the biofilm cell structure: A) non-triton-washed, B) triton-washed.

Scanning electron micrographs of non-triton-washed and triton-washed biofilm cells are presented in Figure 4.7. Images reveal that the biofilm cells were coated with oil (indicated by the smoother surface in washed microorganisms) that was present in the metalworking fluid, which was initially used to propagate the biofilms. This lowered the biosorption of organics onto the surface of the biofilms for both UV and sodium azide treatment. Triton did not alter the surface morphology of the biofilm cells and therefore could be used in our investigations to remove the oils. This was required since the oil

interfered with biosorption. Since this study aimed to show the trends in the organics removals, it was important to remove the oils for all further experiments.

4.3.2.1 Comparing UV and chemical inhibitor on organic removal by biofilms

TEA and BTA removal by inhibited biofilms treated with UV and sodium azide were identical ($P \geq 0.05$). However, the kinetics and the total TEA/BTA removal were significantly different ($P < 0.01$) for inhibited (both UV and chemical inhibitor) and control biofilms (Figure 4.8). Table 4.1 summarises the amount of TEA and BTA removed by control (uninhibited) and inhibited biofilms by both UV and sodium azide. In both cases (TEA and BTA), control biofilms removed significantly more organics compared to inhibited biofilms ($P < 0.01$), confirming the importance of microbial activity (leading to biodegradation) in the removal of the selected organics. BTA removal by both the inhibited and control biofilms followed similar trends to that observed for TEA, although the extent of BTA removal was much lower (Figure 4.8).

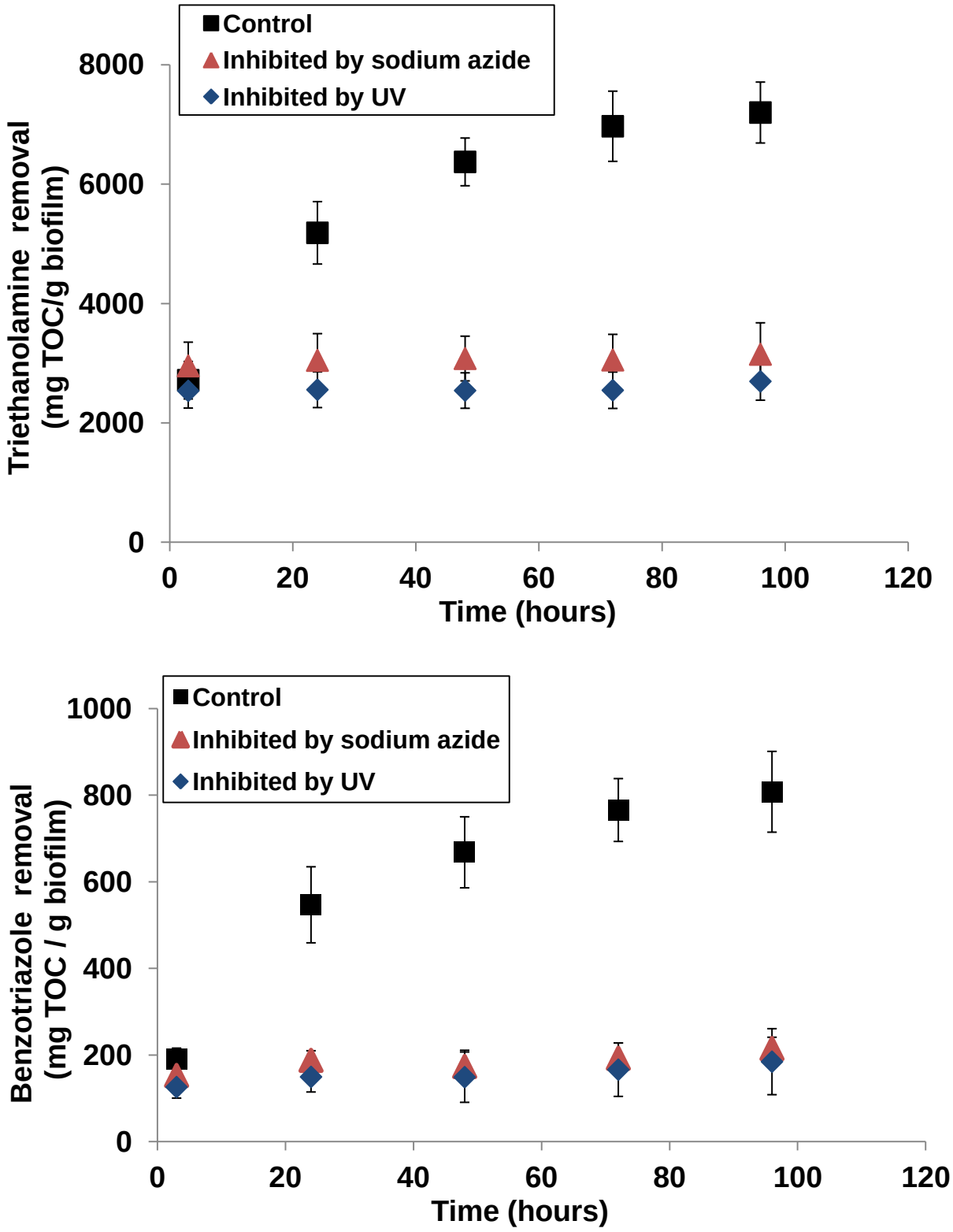


Figure 4.8 Triethanolamine [Co = 10%] (top) and benzotriazole [Co = 2%] (bottom) removal (mg TOC/ g biofilm) by biofilms that were uninhibited (control) and those inhibited by UV and sodium azide. Error bars represent the standard deviation of triplicate measurements.

Table 4.1 Triethanolamine (TEA) and benzotriazole (BTA) removal (mg TOC/ g biofilm) by biofilms that were uninhibited and those inhibited by UV and sodium azide (± SD of three replicates)

	Triethanolamine removal (mg TOC/ g biofilm)			Benzotriazole removal (mg TOC/ g biofilm)		
Concentration	1%	5%	10%	0.5%	1%	2%
Molarity (mol/L)	0.067	0.335	0.670	0.042	0.084	0.168
Uninhibited (Control)	1040 (±59)	4240 (±206)	6970 (±510)	293 (±30)	519 (±62)	808 (±93)
Inhibited by UV	256 (±34)	1360 (±142)	2690 (±316)	78 (±19)	135 (±18)	184 (±62)
Inhibited by sodium azide	308 (±21)	1600 (±259)	3150 (±527)	94 (±10)	145 (±30)	215 (±26)

Even though the molarity of benzotriazole at concentrations ≥ 1% was higher than the molarity of TEA at 1%, both the biosorption and biodegradation were lower for benzotriazole compared to those for TEA. Hence, a possible explanation for the lower removal rates of benzotriazole over TEA could be the inherent structural differences between the molecules of the two compounds. Benzotriazole is a more complex molecule than TEA since it is a fused aromatic nitrogen heterocycle of the benzene ring with triazole (Wu, 1998). This structural difference could have altered the biodegradation kinetics of benzotriazole compared to TEA by the biofilm cells.

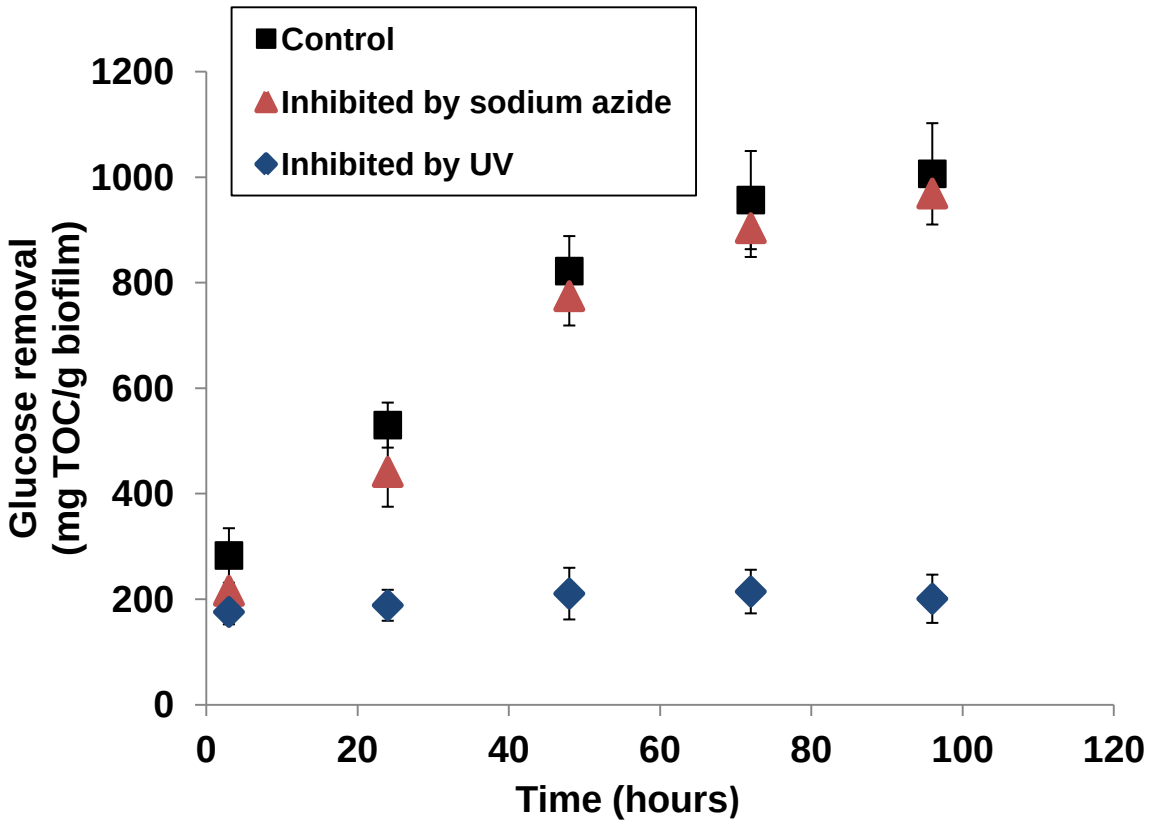


Figure 4.9 Glucose [Co = 5 g/L] removal (mg TOC/ g biofilm) by biofilms that were uninhibited (control) and those inhibited by UV and sodium azide. Error bars represent the standard deviation of triplicate measurements.

The glucose removal by sodium azide inhibited biofilms were similar to that for the control biofilms ($P \geq 0.05$), suggesting that cells treated with sodium azide recovered when tested for glucose removal (Figure 4.9). However, the UV inhibited biofilms remained irreversibly inhibited for the whole duration of the experiment (4 days). This result agrees with the respiration and post-exposure recovery results for the 24 hour time period (Figure 4.1), where the respiration activity in the presence of sodium azide (10 mM) was significantly lower compared to control but the post exposure recovery growth was similar to that of the control sample. This confirmed that sodium azide was not a long-term

inhibitor, and that the biofilm cells were able to resume metabolic functionality in the presence of glucose.

4.3.2.2 Comparing UV and chemical inhibitor on heavy metals removal by biofilms

Metal removal tests were conducted at concentrations that demonstrated to be non-inhibitory. Zinc and copper were toxic at ≥ 40 mg/L for their respective salts whereas, cadmium was toxic at ≥ 10 mg/L (Figure 4.10).

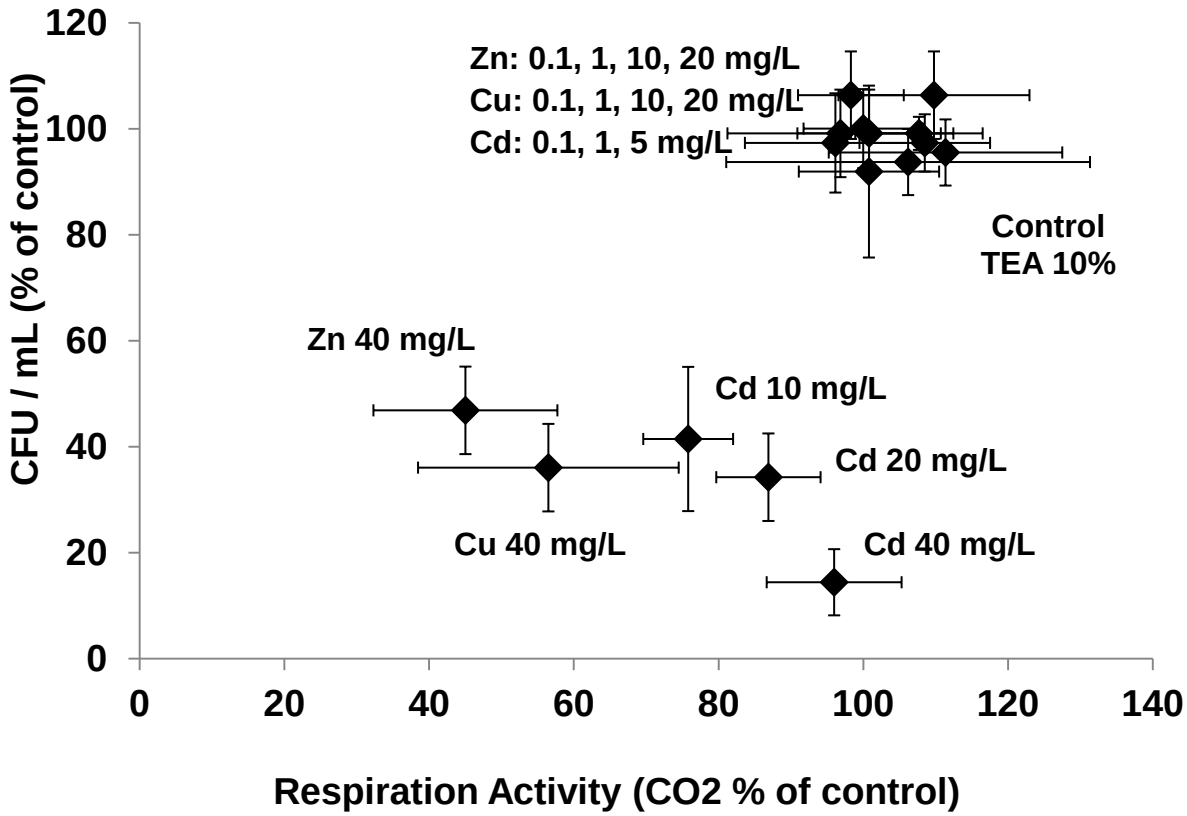


Figure 4.10 Respiration activity and post-exposure growth after 24 hours exposure demonstrating the toxicity of individual metals in 10% TEA: zinc, copper and cadmium. Error bars represent the standard deviation of triplicate measurements.

Biofilms treated with UV and sodium azide removed similar quantities of zinc at the initial concentration of 20 mg/L ($P \geq 0.05$): 6.52 mg/g biofilm and 7.10 mg/g biofilm, respectively (Figure 4.11). The removal of metal ions by inhibited biofilms appeared to take place in two steps: a very rapid initial sorption over the first few hours, followed by a long period of slower uptake eventually reaching equilibrium within 5 hours. Live uninhibited biofilms had similar physicochemical removal rate compared to inhibited biofilms within the first 5 hours; however, zinc removal was greater at the end of 5 days compared to inhibited biofilms. For control biofilms, zinc and copper removal at the concentration of 20 mg/L were 8.76 mg/g biofilm (±0.90) and 6.62 mg/g biofilm (±0.70), respectively (Table 4.2). As for UV inhibited biofilms, the removal capacities were 6.32 mg/g biofilm (±0.33) and 3.80 mg/g biofilm (±0.49) for zinc and copper, respectively. In contrast to the above, no changes in the cadmium removal kinetics were detected between the inhibited and control biofilms over 4 day period (Figure 4.12). Both the inhibited and control biofilms showed a rapid initial metal removal rate, reaching equilibrium within 5 hours. At this time point, both UV-inhibited and control biofilms removed 0.65 mg/g biofilm and 0.71 mg/g, respectively ($P \geq 0.05$).

Table 4.2 shows the metal removal of zinc, copper and cadmium by the control biofilms and those that were inhibited by sodium azide and UV. The physicochemical metal removal increased as the concentration of metal increased in all three metal systems. Even though Zn (II) and Cd (II) have very similar chemical property, their removal amounts were different mainly because the biofilms used in this study were initially acclimated to zinc metal, which is a

common heavy metal present in MWFs, but not to cadmium metal. Moreover, zinc and copper are required by the micro-organism unlike cadmium and thus, higher Zn (II) removal was observed by live biofilms compared to inhibited ones whereas Cd (II) removal was similar between the inhibited and live biofilms.

Table 4.2 Zinc, copper and cadmium removal (mg/g biofilm) by biofilms that were uninhibited and those inhibited by UV and sodium azide (5 days) (± SD of three replicates)

	Zinc removal (mg/g biofilm)		Copper removal (mg/g biofilm)		Cadmium removal (mg/g biofilm)	
[Inhibitor]	1 mg/L	20 mg/L	1 mg/L	20 mg/L	0.1 mg/L	5 mg/L
Uninhibited (control)	0.27 (±0.02)	8.76 (±0.90)	0.23 (±0.02)	6.62 (±0.70)	0.09 (±0.01)	0.71 (±0.01)
Inhibited by UV	0.22 (±0.02)	6.32 (±0.33)	0.16 (±0.01)	3.80 (±0.49)	0.10 (±0.01)	0.65 (±0.06)
Inhibited by sodium azide	0.23 (±0.02)	6.90 (±0.29)	0.19 (±0.01)	4.89 (±0.73)	0.11 (±0.01)	0.61 (±0.01)

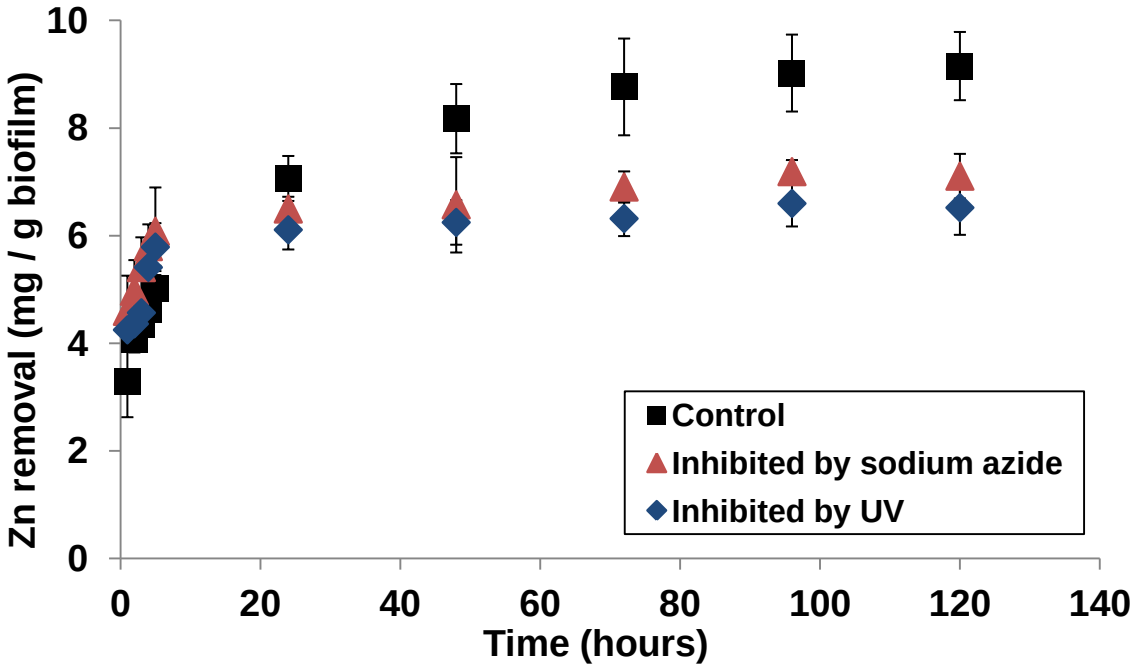


Figure 4.11 Zinc [Co = 20 mg/L] removal (mg/g biofilm) in 10% TEA by biofilms that were uninhibited (control) and those inhibited by UV and sodium azide. Error bars represent the standard deviation of triplicate measurements.

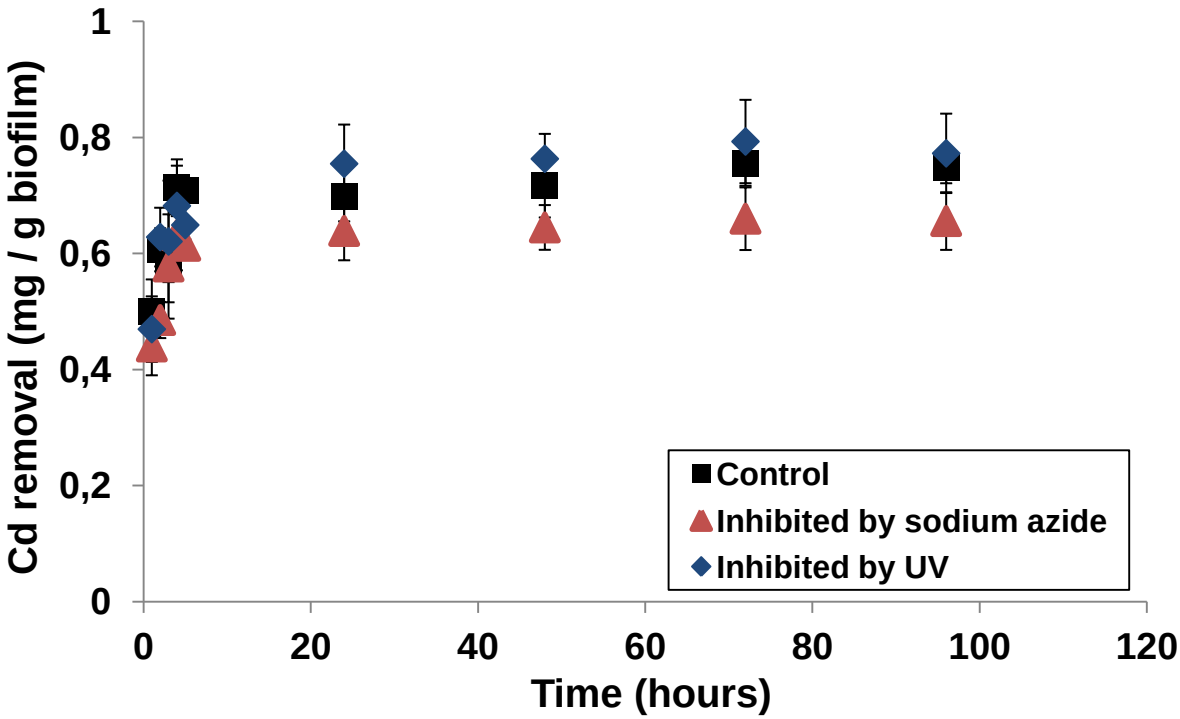


Figure 4.12 Cadmium [Co = 5 mg/L] removal (mg/g biofilm) in 10% TEA by biofilms that were uninhibited (control) and those inhibited by UV and sodium azide. Error bars represent the standard deviation of triplicate measurements.

4.4 Discussion

There is a growing pressure to make water treatment methods more sustainable (Jagadevan et al., 2012) and hence, there is increasing interest in exploiting biofilms for both biodegradation of organics and also immobilisation and recovery of metals (Singh and Cameotra, 2004; Singh et al., 2006; Edwards and Kjellerup, 2013). In order to optimise pollutant removal by biofilms, it is essential to understand how biofilms function and the relative contributions of metabolic activity and physicochemical processes. To achieve this objective, it is essential we have appropriate analytical techniques and determine their effectiveness in providing reliable insights of biofilm functioning.

The results of this study confirm that UV treatment and sodium azide inhibit the metabolic activity of the biofilms whilst not compromising its structural integrity. Five and 24 hour exposure were found to be the minimum amount of time required for UV (300 mJ/cm^2) and sodium azide (10 mM) treatment, respectively, to completely inhibit the metabolic activity of the biofilm (thickness: 2-3 mm) for the duration of the experiments in this study.

Biofilms treated with UV and sodium azide (i.e. inhibited biofilms) removed similar amounts of triethanolamine (TEA) and benzotriazole (BTA), but removed significantly less organics compared to live uninhibited biofilms ($P < 0.01$). Since the control biofilms removed TEA by a combination of biosorption and biodegradation, it was not possible to quantify the amount of TEA removal by biodegradation alone. But it can be concluded that metabolic removal via biodegradation was the dominant mechanism.

Increasing the initial concentration of TEA and BTA improved their removal in both the control and inhibited biofilms. For the latter, this implies that the biofilm's sorption capacity increased with increasing organic concentration, which can be explained by the shift in the adsorption/desorption equilibrium kinetics (Delle Site, 2001; Aksu, 2005). For the control biofilms, the greater organic concentration induced cellular growth and production of EPS in the biofilm. Both of which led to an increase in the removal by biodegradation and biosorption as well as an increase in absolute carbon utilization.

The results of this study suggest that biofilms treated with sodium azide were reversibly inhibited, and their cells were able to resume metabolic functionality in the presence of glucose. Chemical inhibitors were not effective for distinguishing the removal mechanisms of simple sugars (e.g. glucose), however UV-inhibited biofilms continued to remain in an inhibited state in the presence of simple sugars. This was expected since UV irradiation results in irreversible DNA damage within the cells (Zimmer and Slawson, 2002), thereby causing irrevocable inhibition.

The dominant mechanism responsible for zinc removal was biosorption (the removal fraction of inhibited to uninhibited biofilm was 76% ± 5%). In contrast, no significant difference in cadmium removal was observed between the inhibited and the uninhibited biofilms, suggesting its immobilisation was a passive process. A similar trend was reported by Guo et al. (2012), who demonstrated similar adsorption capacity of Cd (II) with live and heat treated *P. plecoglossicida* cells. In contrast, Tangaromsuk et al. (2002) demonstrated that the cadmium removal capacity of living cells was significantly greater than that

of autoclaved cells *S. paucimobilis.* They attributed this to the partial destruction of metal binding sites as a consequence of autoclaving in the heat-treated cells. However, Srinath et al. (2002) reported that the heat treatment of chromate resistant bacterial cells breaks the cell wall, so yielding more binding sites. Zinc is essential for enzyme systems that influence cell division (MacDonald, 2000), and copper serves as a cofactor in respiration, thus is required for aerobic metabolism (Santo et al., 2011). This could be the origin of differences observed in the removal of zinc and copper compared to cadmium, the latter of which does not play any specific role in cell growth and development.

This is the first study, as far as we are aware, to report that UV has potential as an analytical tool for determining mechanisms of pollutant removal by biofilms. UV has the key advantage over that of commonly employed chemical inhibitors; resulting in irreversible cell inhibition whilst maintaining cellular integrity. Autoclaving is an effective inhibition tool; however it causes significant structural changes to the biofilm. Reducing temperature (i.e. to 5ºC) is also effective in reducing metabolic activity, but it also alters the pollutant absorption kinetics. Sodium azide (10 mM) provides similar inhibition to UV however, in the presence of simple sugars, the cells exposed to sodium azide recovered and resumed metabolic activity. Hence, this chemical method is a preferred option if the revival of inhibited cells is required for subsequent experimentation or applications. However, chemicals must be chosen carefully for cell inactivation as some lead to structural disintegration, whilst others cause reversible inhibition in the presence of certain organics. When using UV as an inhibition tool, attention must be paid to choose the UV dose required to completely inhibit

the cells whether suspended or in the form of biofilms. The UV dose employed will also be dictated by the age and thickness of the biofilm since thicker and older forms are likely to be more resistant (Azimi et al., 2012). For metal removal studies, understanding the type of mechanism responsible for the removal is crucial for choosing appropriate desorption methods to maximise recovery with minimal biofilm damage, thus making the whole biological treatment process more sustainable and efficient.

4.5 Conclusions

In this study, UV was applied for the first time and its potential as a physical inhibitor to study the mechanisms of biofilm function in terms of water contaminant interactions was investigated. The following conclusions are drawn from this study:

- Chemical inhibitors such as formalin and dinitrophenol caused biofilm structural damage and cell lysis, whereas UV did not cause cell membrane rupture or leakage of intracellular components that could affect the parameters of interest.
- UV tool led to irreversible metabolic inhibition of biofilms, whereas the impact of sodium azide was reversible. Biofilm cells inhibited with sodium azide revived upon exposure to glucose, which depending on the circumstances (e.g. subsequent cell analysis) could prove to have advantages in terms of developing tools for understanding biofilms.
- The dominant mechanisms of organic and metal removal were found to be biodegradation (active) and biosorption (passive) processes, respectively.
- UV, as an analytical tool, could be widely applied to study pollutant removal in biofilms/microbial aggregates across various processes (e.g. industrial and municipal water and wastewater treatment processes) and provide novel insights.

Chapter Five

5. Investigation of the Interaction of Metals and Organics in Biofilms Established to Treat Chemically Mixed Industrial Wastewaters.

5.1 Introduction

A high percentage of industrial effluents are co-contaminated with both heavy metals and organic compounds and these sites can pose a major threat to the public health and the environment. The US Environmental Protection Agency estimates that 40% of hazardous waste sites in the United States are co-contaminated with organic and metal pollutants (Cheng, 2003; Sandrin and Maier, 2002 & 2003). Conventional treatment methods are costly, energy demanding and can be sub-optimal when dealing with heterogeneous wastewaters (Barakat, 2011). In contrast microbial communities, particularly in the form of biofilms, have a recognised potential for sustainable remediation of both organics and metals, individually and when mixed, whilst having low energy demands (Burke and Gaines, 2006; Gadd, 2009; Lesmana et al., 2009).

Operationally exhausted metalworking fluids (MWFs) are representative of chemically mixed effluents which are generated in significant quantities by engineering industries during machining and other processes and are also challenging to treat sustainably (Teli et al., 2014). Due to the tightening environmental regulations around the world, the typical means of disposing spent MWFs through incineration is no longer viable (van der Gast et al., 2004;

Jagadevan et al., 2013). The disposal costs of MWFs can be over ten times the cost of their purchase and specifically estimated to be £16 million within the United Kingdom alone (Cheng et al., 2005 & 2006).

Treatment of chemically mixed waste is challenging since both the metal and the organic present can interact synergistically and have a toxic impact. This is particularly the case if the treatment method proposed is biological, since the presence of metal ions can slow the degradation of organic contaminants and the efficiency of metal recovery can be reduced by the presence of organic species. The negative impact of metals on the biodegradation of organic pollutants has been extensively studied and appropriate strategies to deal with these challenges have been reported (Said and Lewis, 1991; Sandrin and Maier, 2002; Gadd, 2010; Olaniran et al., 2013). Approaches for increasing organic biodegradation in the presence of metals involve the reduction of metal bioavailability, and the application of metal-resistant bacteria, clay minerals, divalent cations and adjustment of pH (Sandrin and Maier, 2002 & 2003). However, the effects of the organic compounds present in mixed effluents on metal recovery have received little attention.

The aim of this study was to determine the effects of the individual organic components that form an integral part of MWFs, and their impact on metal removal in waste streams. Specifically, the presence of non-interacting organics on metal removal and the influence of other organics on metal chelation, toxic response of the biofilm and surface tension were investigated. Approaches to increase the metal removal in the presence of these organic compounds are discussed in the light of the results.

5.2 Materials and Methods

5.2.1 Metalworking fluids (MWFs)- the model wastewater

Waste MWFs were selected since they are representative of the many challenges faced by industries to develop sustainable end-of-pipe treatment methods. They are 95% water in composition and the rest composed of constituents summarised in Table 5.1. Specifically, semi-synthetic MWF formulations compromise of water, base oil, surfactants and special additives (Childers, 2006).

Table 5.1: Typical organic components present in metalworking fluid formulations.

Components	Purpose in MWFs	References
Surfactants Nonionic: Octylphenol Ethoxylate (Triton X-100) Ionic: Sodium Dodecyl Benzene Sulphonate (SDBS)	Critical for producing a stable oil-in-water emulsion	Skerlos et al. 2008 Negm et al., 2015
Corrosion inhibitor Benzotriazole (BTA)	Prevent rust formation and corrosion on work pieces, machine parts and tools made of metals	Jagadevan et al., 2013
Biocide Benzisothiazolinone (BIT)	Inhibit microbial growth	Rizvi, 2003
Propylene Glycol	Coupler additives	Rizvi, 2003

5.2.2 Description of bioreactor system

Biofilms were grown on stainless steel matrix as described in Section 4.2.1. For metal removal experiments, all biofilms were washed 3 times using 0.2% Triton X-100 (an oil-emulsifying surfactant) and then five times using DI water to remove residual surfactant. The oils, which inhibit the removal of metal and organics by biofilms, were thus washed away from the surface of the biofilms by dissolving them in the wash water.

Biosorption, the passive non-metabolically mediated process of metal ion binding, was investigated employing UV-inhibited biofilms. UV is an effective method for accurately quantifying passive biosorption and active metabolic metal removal (Adapa et al., 2016). Same procedure as described in Section 4.2.8 was used in this chapter to inhibit biofilms using UV.

5.2.3 Measurement of metabolic inhibition

As described in Section 4.2.2, respiration activity was measured using a micro-plate based respiration system, known as MicroResp^{TM}, which measures carbon dioxide (CO_2) evolved. This colorimetric method is based on the colour change of a pH indicator dye (cresol red) caused by the presence of CO_2 (Campbell et al., 2003). Post exposure recovery (PER) tests, using the plate count method (Miles et al., 1938), were performed to enumerate colonies after exposure to the test compounds. The degree of inhibition was assessed by plotting colony-forming units (CFU / mL expressed as % of control) from post exposure recovery method versus respiration activity (CO_2 % of control). This

method was also used to determine the minimum UV-exposure time required for complete biofilm inhibition and to assess the toxicity effects of individual organics and metals.

5.2.4 Analytical methods

All chemicals were analytical reagent grade and supplied from Sigma-Aldrich. Stock solutions of 10 g/L propylene glycol, 1% w/v benzisothiazolinone (BIT), 2% w/v benzotriazole (BTA) and 1 g/L Zn (II) were prepared by dissolving appropriate amounts of 1,2-Propanediol (≥ 99.5%), 1,2-Benzisothiazol-3(2H)-one (≥ 97.0%), 1-H Benzotriazole (≥ 98.0%) and $ZnSO_4.7H_2O$ (≥ 99.0%) in DI water, respectively. For fully chelated zinc, EDTA-Na_2Zn (≥ 98.0%) was used and its stock solution of 1 g/L Zn (II) was prepared.

Samples collected in all experiments were centrifuged at 12,000 rpm for 15 min to minimise background biofilm content. The total organic carbon (TOC) concentration of the supernatant was measured by a Shimadzu TOC V-CPH. The system was calibrated with standards prepared from potassium hydrogen phthalate in the range of 0-1000 mg/L. Atomic absorption spectroscopy (Agilent 240FS AA) (AAS) was used to measure zinc metal concentrations and was calibrated in the linear ranges of 0.2-1.2 mg/L for zinc.

5.2.5 Zinc removal

Zinc removal measurements and the dry weight of the biofilms were measured as described in Section 4.2.4. All zinc metal removal experiments were undertaken in batch mode operation by exposing the biofilm strips to 200 mL metal solution at various concentrations in 250 mL Erlenmeyer flasks (120 rpm, 30^{O}C). Samples were centrifuged at each time point and the supernatant liquid was analysed for metal concentration. Negligible metal removal was detected when using blank stainless steel strips (in the absence of biofilms).

5.2.6 Chelation test

In a mixture of a chelating organic and a metal, it is difficult to differentiate the amount of chelated and non-chelated metal because the AAS used in this study to measure the amount of metal gives the total amount (chelated + non-chelated) of metal in a given sample. Therefore, the following procedure was developed to measure the amount of chelated metal using pH adjustment. The chelating organic and metal were first allowed to mix at 30^{O}C and then the sample was adjusted to pH 10 using 1 M NaOH and incubated at 30^{O}C for 1 hour. One mL of the sample was then centrifuged at 10,000 rpm for 20 min and the supernatant was measured using AAS. The zinc concentration of the supernatant represented the amount of chelated metal because the non-chelated metal precipitated at pH 10 and thus, was separated from supernatant during the centrifugation step. The organic-metal mixture was tested again after 5 days to confirm that chelation did not change over time.

5.2.7 HPLC analysis of propylene glycol, BTA and BIT

To study the effect of toxicity, metal removal experiments in the presence of both propylene glycol (carbon source) and BIT (biocide) were conducted. The concentration analyses of the organics were performed by high performance liquid chromatography (Agilent 1100). This consisted of a Water Associates model 2695, equipped with a C18 column, and photodiode array detector at a wavelength of 254 nm.

For the detection of propylene glycol, the derivatisation method was adapted using a previously described method (Holcapek et al., 1999). One hundred µL of real sample along with 50 µL of ethylene glycol (internal standard), 250 µL of 30% NaOH and 10 µL of benzoyl chloride were all vortexed for 10 min. Immediately after, 100 µL of glycerol was added to the sample to prevent any further reaction from occurring and vortexed for 1 min. Then, 400 µL of hexane was added to the reaction mixture, then vortexed for 5 min, and centrifuged at 14,500 rpm for 5 min. Two hundred µL of supernatant was collected, and special care was taken not to collect any trace of the aqueous phase. The collected hexane was evaporated at ambient temperature under a stream of nitrogen. The residue was re-dissolved in 200 µL methanol for the UV detection and 25 µL injected into the liquid chromatography. The calibration curves were plotted as the ratios of the peak areas of each glycol to the peak area of the internal standard. For the analysis of BTA, no derivatisation step was required. The mobile phase for both compounds was a 70:30 (v/v) mixture of methanol and DI water, used at a flow rate of 0.75 mL/min^{-1} under isocratic conditions. All

solvents, standards and sample solutions were filtered (0.45 um membrane filters) before injection.

For the analysis of BIT, the mobile phase was 30:70 (v/v) methanol to DI water until 15 min and then changing to 70:30 (v/v) methanol to DI water until 16 min and then leaving the composition up to 29 min and finally increasing it to 100% methanol until 30 min.

5.2.8 Measuring the effect of oil interference on biosorption

To distinguish between the metal removal mechanisms (extracellular vs intracellular), 0.1 M HCl was used to desorb the zinc ions from the functional groups and cell surfaces of the biofilm. Many researchers have shown that 0.1 M HCl is effective as an elutant enabling 95-99% metal recovery and allowing the reuse of the biomass in sorption-desorption cycles thrice without loss of biosorption capacity (Aryal et al., 2010; Chen et al., 2005). Recovery experiments were conducted in 250 mL Erlenmeyer flasks with 100 ml of 0.1 M HCl for a period of 2 hours.

5.2.9 Statistical analysis

All experiments were conducted in at least three replicates and the data represent the mean values ± standard error. T-test and ANOVA followed by Tukey's test were applied when appropriate, and P values less than 0.05 and 0.01 were considered statistically significant.

5.3 Results and Discussion

5.3.1 Extent of metal chelation with various organics used in MWF

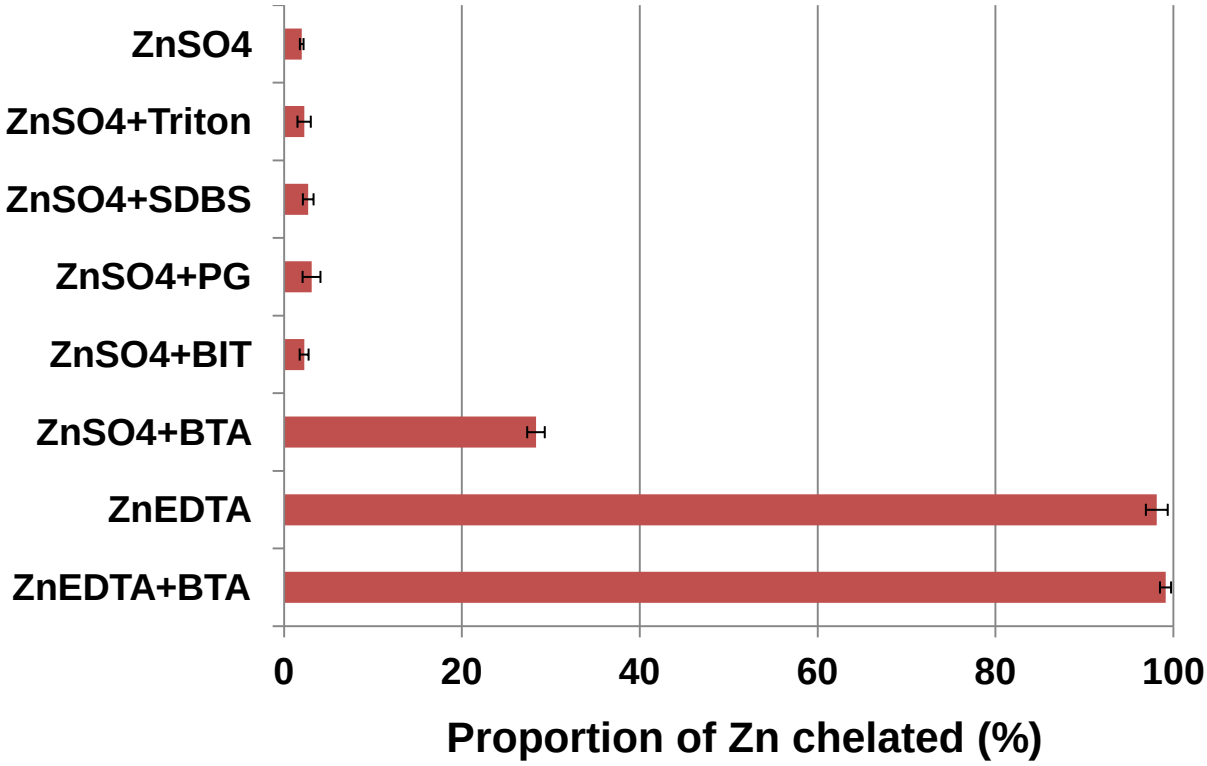


Figure 5.1: Chelated zinc (mg/L) in different mixtures with initial concentration of 20 mg/L Zn: a) ZnSO4 in DI water, b) ZnSO4 in 0.15% v/v Triton X-100, c) ZnSO4 in 0.15% w/v SDBS, d) $ZnSO_4$ in 1 g/L propylene glycol, e) $ZnSO_4$ in 0.1% BIT, f) $ZnSO_4$ in 0.015% w/v BTA, g) Zn-EDTA complex in DI water and h) Zn-EDTA complex in 0.015% w/v BTA. Error bars represent the standard deviation of triplicate measurements.

To investigate the degree to which chelation affected metal uptake, the extent of zinc chelation by 0.15% octylphenol ethoxylate (Triton X-100), 0.15% sodium dodecylbenzene sulphonate (SDBS), 1 g/L propylene glycol, 0.1% benzisothiazolinone (BIT) and 0.015% benzotriazole (BTA), respectively, were investigated using the pH adjustment method (Figure 5.1). In solutions containing 20 mg/L of zinc and 0.015% BTA, 28% (± 1%) of the zinc was

chelated, while other systems showed negligible chelation. In order to validate the experimental method, a positive control experiment using Zn-EDTA complex salts in DI water and in 0.015% BTA was used, which resulted in 100% of zinc being chelated. This confirmed that the reduction in metal concentration in the Zn-BTA system was not due to BTA interference in the AAS.

5.3.2 Effect of chelation on metal removal

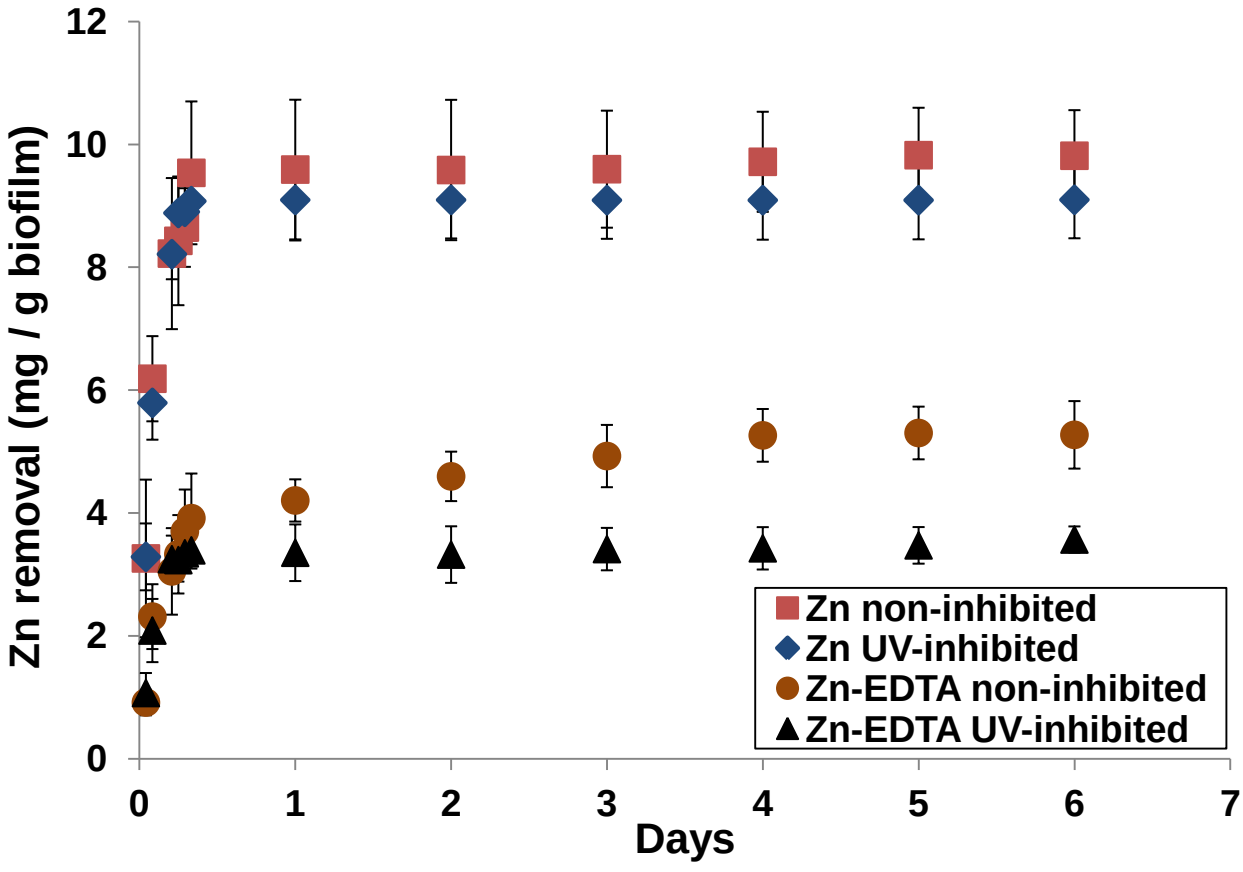


Figure 5.2 Effect of chelation on zinc [Co = 20 mg/L] removal (mg/g biofilm) by live biofilms and those inhibited by UV using non-chelated Zn^{2+} salt and fully chelated Zn-EDTA complex, both in 0.85% NaCl. Error bars represent the standard deviation of triplicate measurements.

Micro-respiration and post exposure recovery tests revealed that 0.015% BTA with Zn-EDTA complexes or $ZnSO_4$ salts, at initial concentration of 20 mg/L Zn (II), were not toxic to the microbes used in this study (data not shown). Therefore, BTA and Zn-EDTA complexes were used in all further experiments to study the effect of partial and full chelation, respectively, on zinc removal.

Figure 5.2 illustrates the effect of zinc chelation on Zn (II) removal by live biofilms and those inhibited by UV by using two compounds: 1) non-chelated Zn^{2+} salt and 2) fully chelated Zn-EDTA complex, both in 0.85% NaCl. Zn (II) removal by both live biofilms and those inhibited by UV was higher in non-chelated Zn^{2+} salt solution compared to fully chelated Zn-EDTA complex, clearly confirming that metal chelation inhibits metal removal. At an initial concentration of 20 mg/L, chelation significantly reduced ($P < 0.01$) zinc removal by both live and UV inhibited biofilms (Figure 5.2). Biofilms inhibited by UV removed 9.10 mg/g biofilm (± 0.63) of zinc in the form of non-chelated Zn^{2+} ions compared to 3.57 mg/g biofilm (± 0.22) in the form of fully chelated Zn-EDTA complexes. Both systems reached equilibrium within the first 3 hours, reflecting the process occurred by biosorption. Moreover, the metal removal in non-chelated Zn^{2+} salt solution was identical for live biofilms and those inhibited by UV, however in the case of fully chelated Zn-EDTA complex, higher metal removal was found by live biofilms compared to those inhibited by UV, mainly due to the carbon source being present in the second solution.

Several studies have demonstrated that in addition to the biosorption process, metals can be removed by intracellular accumulation by viable cells in biofilms (Malik, 2004) and through passive diffusion or metabolic accumulation

(Das and Guha, 2009). The Zn^{2+} system with 0.85% NaCl contained no carbon source, therefore as expected both the live and the UV-inhibited biofilms removed similar quantities of zinc ($P > 0.05$). In contrast, the live and UV-inhibited biofilms removed different amounts of Zn-EDTA complexes (initial concentration of Zn (II): 20 mg/L): 5.27 mg/g biofilm (± 0.55) by live biofilms compared to 3.57 mg/g biofilm (± 0.22) by the UV-inhibited biofilms ($P < 0.01$) (Figure 5.2). Live biofilms revealed a similar biosorption removal trend as UV-inhibited biofilms; however after the initial fast biosorption kinetics, zinc removal continued in the live system and the overall removal was greater at the end of 4 days compared to inhibited biofilms. An interesting observation was that the live biofilms in the fully chelated system reached a stable value after 4 days, at which point the EDTA removal was also found to reach equilibrium at a value of 19% (± 2%). A possible explanation could be that as the live biofilms degraded EDTA from the Zn-EDTA complex, the bioavailability of the zinc ions increased, thereby increasing zinc removal by the biofilm. There is also the possibility that the EDTA on the surface of the biofilm was degraded, which resulted in more available active sites and hence, this increased zinc removal.

In the chelated systems, about 98% zinc was recovered from the live biofilm in the Zn-EDTA complex system with 0.1 M HCl, suggesting that the zinc removal was predominantly extracellular uptake. Degradation of the Zn-EDTA, resulted in more zinc to be taken up.

Similar zinc removal patterns as fully chelated Zn-EDTA complex system were observed in partially chelated systems (zinc sulphate in 0.015% w/v BTA) by both live and inhibited biofilms: live biofilms removed 6.52 mg/g ± (0.41) as

compared to 5.01 mg/g ± (0.34) by inhibited biofilms after 3 days (Figure 5.3). The non-chelated zinc was preferentially removed to the chelated form by the biofilms (Figure 5.4). Moreover, 30% lower BTA removal (data not shown) was observed by biofilms (live and inhibited by UV) in a system containing Zn (II) compared to one without Zn (II). The latter suggests that the free Zn (II) and BTA present in one single system compete for adsorption spaces on biofilms.

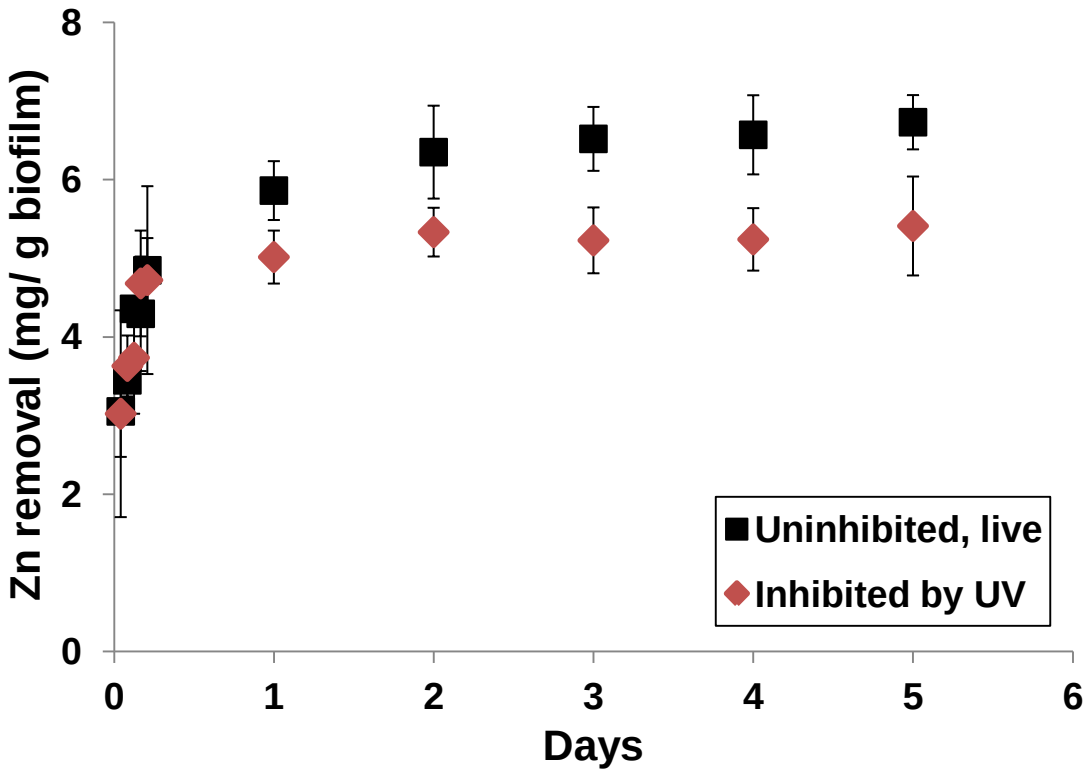


Figure 5.3 Zinc [Co = 20 mg/L] removal (mg/g biofilm) in 0.015% w/v benzotriazole (BTA) by live biofilms and those inhibited by UV. Error bars represent the standard deviation of triplicate measurements.

Chemically mixed wastes in industries such as MWFs contain various metals and organics with chelating properties. Therefore, a high concentration of chelated metal is usually present in such waste streams. Chelating agents, such as BTA, are not very biodegradable, and therefore it is important to

understand their effect on metal removal. Practitioners dealing with such wastes should ideally separate chelating agents from the metal-chelate complex to make metals more bio-available before the bioprocess and then, should consider utilising biofilms for the removal of the non-chelated portion of the metals. A two-stage sequential process is recommended as a preferable alternative to simultaneously degrading the chelating agent and removing the metals from the chelated system. Not least here we demonstrated that chelation inhibited biosorption of metals and that biofilms preferentially uptake the non-chelated metals in systems containing organics which have limited biodegradability.

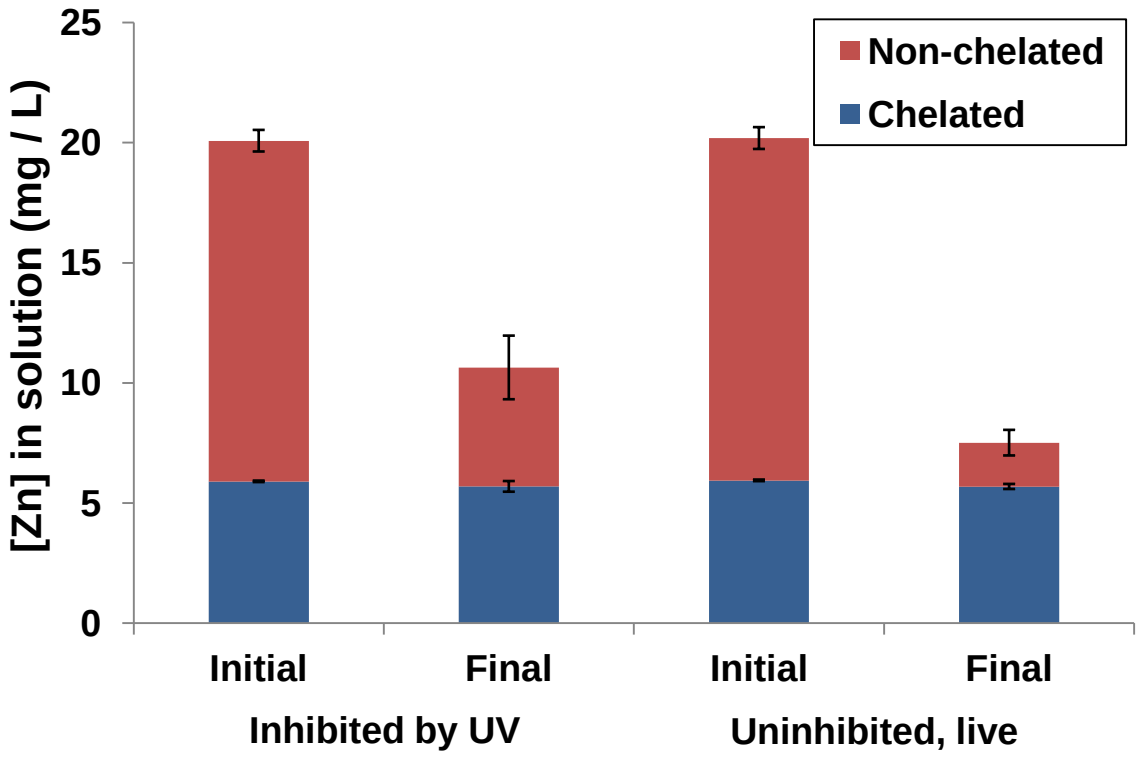


Figure 5.4 Initial and final concentrations of chelated and non-chelated zinc (mg/L) present in solution by live biofilms and those that were inhibited by UV (initial [Zn]= 20 mg/L, [BTA]=0.015%, final measurements taken at the end of 5 days). Error bars represent the standard deviation of triplicate measurements.

5.3.3 Effect of toxicity on metal removal

Since MWF formulations are susceptible to microbial attack, microbial biocides are included to inhibit microbial growth (Passman, 2006). This leads to a negative impact on the subsequent end-of-life biotreatment. Because of this, the effects of biocide toxicity on metal removal were investigated by comparing the biosorption and the metabolic uptake of zinc in the presence and the absence of benzisothiazolinone (BIT) (biocide commonly used in MWFs). Propylene glycol is added to MWFs to assist emulsifiers in stabilising water-based MWFs (Childers, 2006), hence it was also added to the solutions as the carbon source for the biofilms.

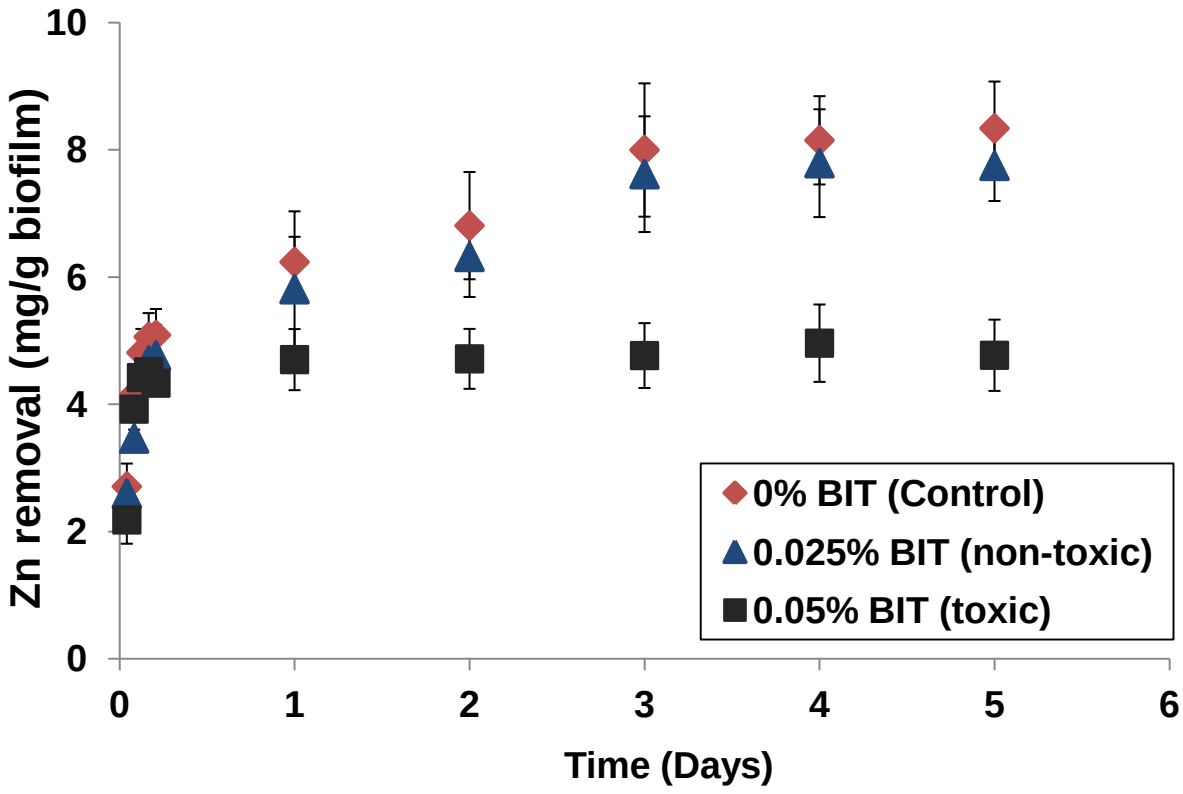


Figure 5.5 Zinc [Co = 20 mg/L] removal (mg/g biofilm) by live biofilms in 1 g/L propylene glycol and at 0% (control), 0.025% (non-toxic) and 0.05% (toxic) concentrations of benzisothiazolinone (BIT) biocide. Error bars represent the standard deviations of triplicate measurements.

BIT was found to be toxic at concentrations ≥ 0.05% in the presence of 1 g/L propylene glycol (data not shown), and therefore a concentration of 0.025% and a concentration of 0.05% was chosen to represent a non-toxic and toxic concentration, respectively.

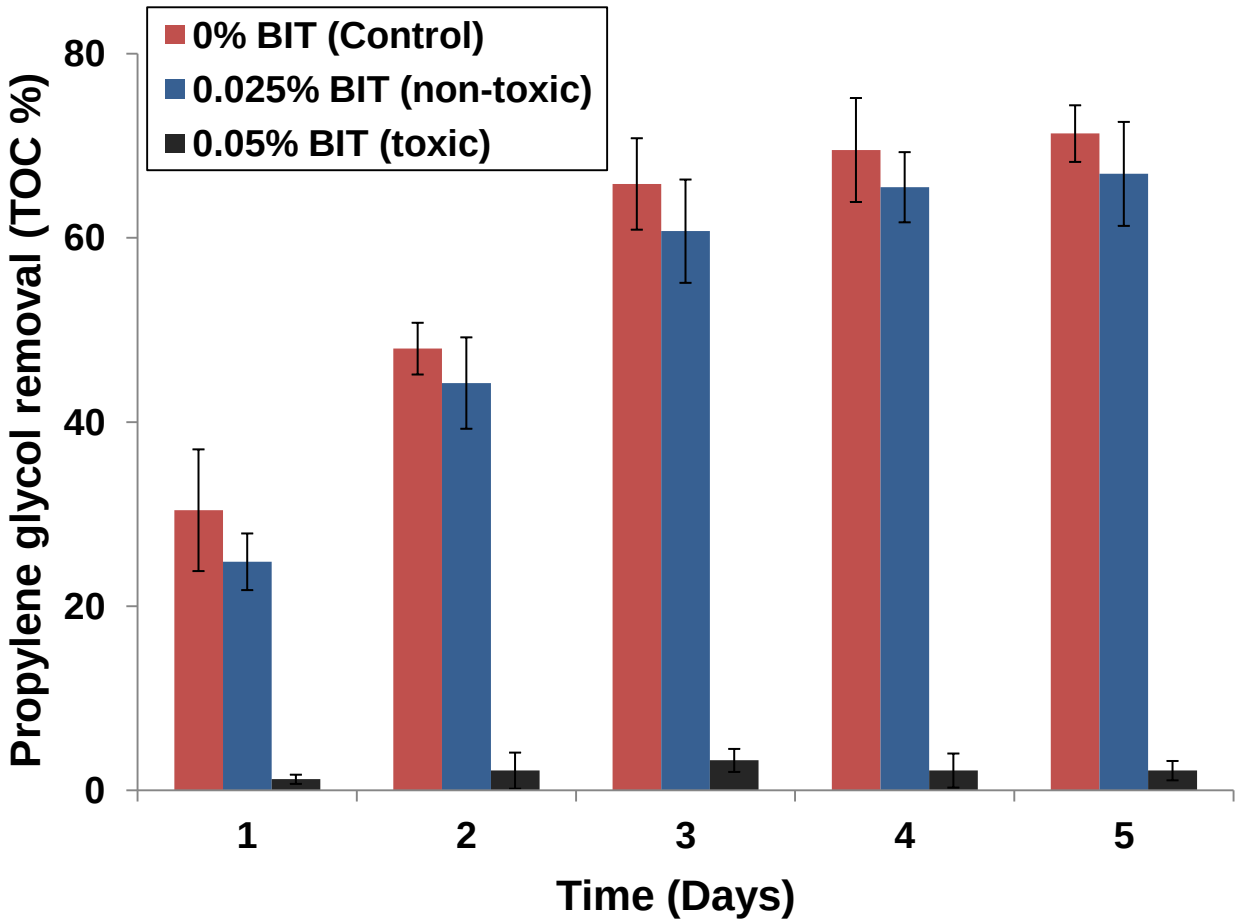


Figure 5.6 Propylene glycol [Co = 1 g/L] removal (TOC %) by live biofilms in 1 g/L propylene glycol and at 0% (control), 0.025% (non-toxic) and 0.05% (toxic) concentrations of benzisothiazolinone (BIT) biocide. Error bars represent the standard deviation for three replicates.

Figure 5.5 and 5.6 depicts the zinc (mg/g biofilm) and propylene glycol removal (% TOC), respectively, by live biofilms exposed to 0%, 0.025% (non-toxic) and 0.05% (toxic) concentrations of BIT, respectively, over a 5-day

period. Toxicity did not affect the biosorption process of Zn (II). UV-inhibited biofilms at 0%, 0.025% (non-toxic) and 0.05% (toxic) of BIT concentrations removed similar quantities of Zn (II) at the initial concentration of 20 mg/L ($P > 0.05$): 5.09 mg/g biofilm (± 0.41), 4.45 mg/g biofilm (± 0.37) and 4.33 mg/g biofilm (± 0.12), respectively (Figure 5.5). The removal in all three cases reached equilibrium quickly and consistently within 2 hours. This trend is characteristic of the physicochemical non-metabolic mechanism of removal, also known as biosorption. For metabolic removal, similar quantities of Zn (II) were removed by biofilms exposed to both 0% and to 0.025% BIT (non-toxic) ($P > 0.05$) (Figure 5.5). However, in the presence of 0.05% BIT which was the toxic concentration, metabolic zinc removal was significantly lower (4.77 mg/g biofilm ± 0.56) than the non-toxic concentration of BIT (7.75 mg/g biofilm ± 0.51).

Figure 5.6 shows that the propylene glycol removal was inhibited in the presence of 0.05% BIT (toxic). At 0% and 0.025% BIT (non-toxic), propylene glycol removal increased to 66 % (± 5%) and 61 % (± 6%), respectively, within 3 days when it reached a stable value. In parallel, as shown in Figure 5.5, zinc removal by live biofilms exposed to 0% and 0.025% BIT (non-toxic) also reached a plateau within 3 days. This suggests that toxicity inhibited degradation of propylene glycol, which then reduced the Zn (II) removal by the metabolically-active live biofilms. However, in the presence of a lower concentration of BIT which was not toxic (0.025%), the live biofilms behaved similarly to control biofilms. Both biofilms recovered >95% Zn (II) using 0.1 M HCl, confirming that the increase in Zn (II) removal was not due to intracellular

metabolic uptake. Hence, it is likely that they degraded propylene glycol present on the surface of the biofilm, hence freeing-up more active sites and allowing for more Zn (II) to be extracellularly removed.

Some researchers have found that metabolically active biomass is less effective at metal removal compared to dead biomass, whilst others have shown the opposite (Malik, 2004; Gabr et al., 2008; Li et al., 2010). Biosorption, the fast metabolically independent process, was shown to be unaffected by the presence of toxic BIT, however this might not be true for all toxic biocides.

5.3.4 Effect of non-interacting organics on metal removal

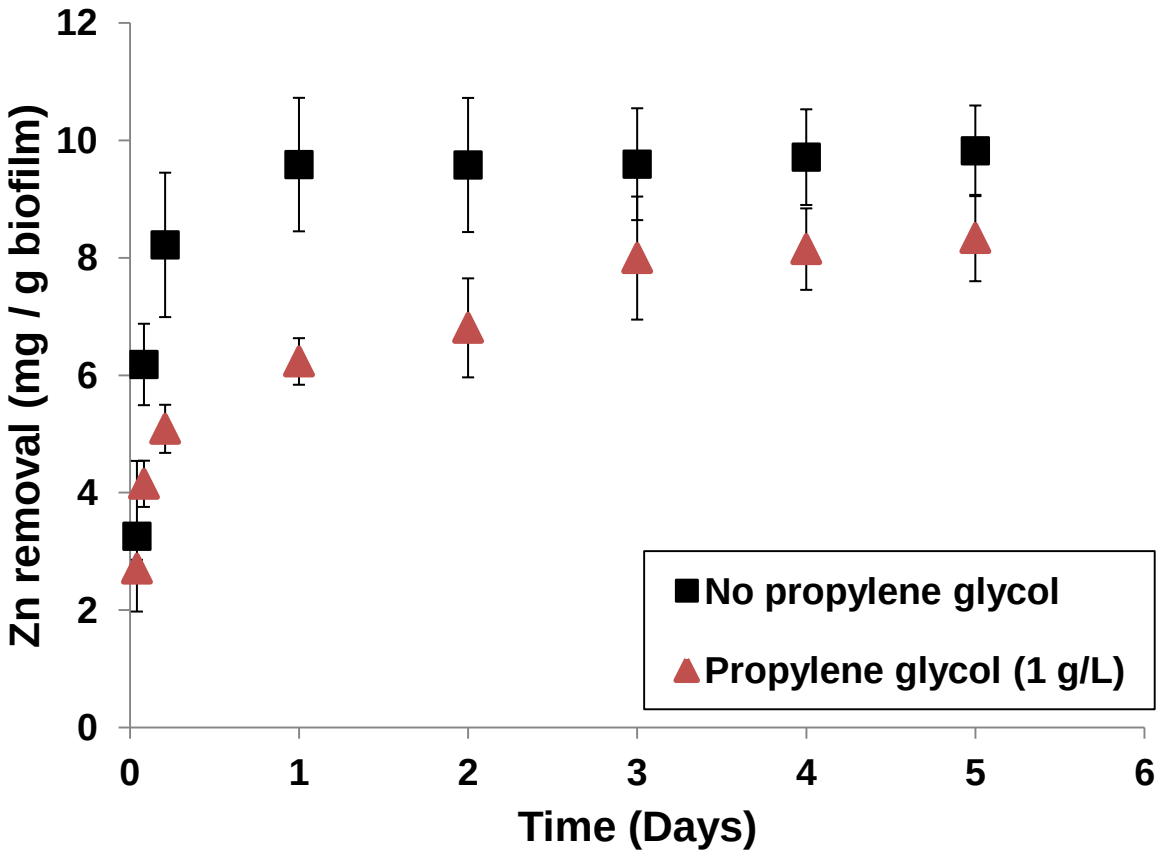


Figure 5.7 Effect of non-interacting organic on zinc [Co = 20 mg/L] removal (mg/g biofilm) by live biofilms with 1 g/L propylene glycol (soft organic), in

0.85% NaCl. Error bars represent the standard deviation of triplicate measurements.

The influence of a non-interacting organic on metal removal was studied using 1 g/L propylene glycol and 20 mg/L of Zn (II) (Figure 5.7). Zinc removal by live biofilms was slower in the presence of propylene glycol, reaching equilibrium within 3 days compared to 3 hours in its absence. This suggests competition between the metal and organic present. However, equilibrium values were identical to control biofilms where propylene glycol was absent ($P > 0.05$). In both systems, biofilms recovered >95% zinc using 0.1 M HCl, confirming that the increase in Zn (II) removal in both systems was predominantly extracellular uptake. Moreover, live biofilms removed about 65% of propylene glycol in the presence of Zn (II) at the initial concentration of 20 mg/L, via biodegradation, within 3 days (Figure 5.6). This confirms that the propylene glycol which competes for active sites on the surface of the biofilm was eventually degraded, allowing for more active sites on the biofilm. In the propylene glycol system, this resulted in an increase in metal removal until day 3, at which point the system reached equilibrium and removed similar amount of zinc as the system without propylene glycol.

5.3.5 Effect of surfactants on metal removal

Surfactants are significant and integral components of MWF formulations as they facilitate the formation of oil-in-water emulsions, thereby giving the MWF

both lubrication and cooling properties (Skerlos et al., 2008; Negm et al., 2015). Anionic and nonionic emulsifiers are often used together in MWFs (Rizvi, 2003). Consequently, it was important to determine the effects of an anionic and a nonionic surfactant on the metal removal. Sodium dodecylbenzene sulphonate (SDBS) and octylphenol ethoxylate (Triton X-100) were used as a representative anionic and nonionic surfactant, respectively.

Triton X-100 accelerated the biosorption process of zinc removal without altering the removal capacity, whereas SDBS inhibited the zinc removal process (Figure 5.8). Since the biofilms were unable to degrade either of these surfactants, only the fast metabolically independent process was considered to determine the effects of the surfactants on zinc removal. In the presence of Triton X-100, biofilm inhibited by UV removed 8.74 mg/g biofilm (± 0.32) of zinc ions within 180 min compared to 8.88 mg/g biofilm (± 0.58) within 360 min in the absence of Triton X-100 (Figure 5.8). However, in the presence of the anionic SDBS, the UV-inhibited biofilm only removed 2.08 mg/g biofilm (± 0.42) in 300 min.

Triton X-100 is known to be the most widely used nonionic surfactant employed to permeabilise the living cell membrane, making the cells porous (Koley and Bard, 2010). However, porosity was not the reason for the speed-up of the biosorption process in this case: no significant change in zinc removal was observed between the biofilm pre-treated overnight with Triton X-100 and the control (data not shown).

A possible explanation for the acceleration of the biosorption process with Triton X-100 could be the reduction of surface tension. It is well established that

biofilms are hydrophobic and negatively charged in water and that metal ions exist in a hydrated form in solution. Thus, there exists an attractive electrostatic force between the metal cation and the negatively charged biofilm surface, and a retardation force is caused due to hydration shell being more attracted to the bulk liquid than the biofilm surface. Since the electrostatic force is orders of magnitude greater than the retardation force, there is a net attractive force, resulting in biosorption. The addition of surfactant within the system could result in a further decrease in the strength of the retardation force since the surfactant molecules reduce the induced surface tension that occurs between the biofilm and the bulk liquid. This is analogous to the reduction in the surface tension that would occur on the air-water interface.

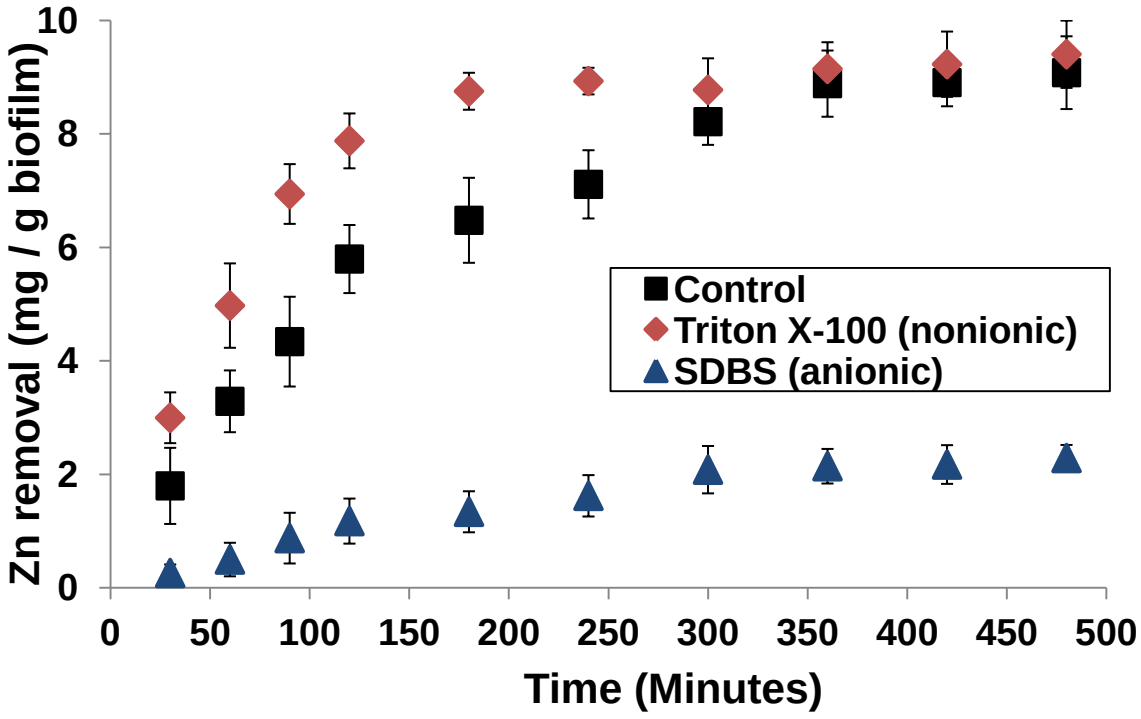


Figure 5.8 Zinc [Co = 20 mg/L] removal (mg/g biofilm) by UV-inhibited biofilms in 0.15% octylphenol ethoxylate (Triton X-100) (nonionic surfactant) and 0.15% sodium dodecylbenzene sulphonate (SDBS) (anionic surfactant). Error bars represent the standard deviation of triplicate measurements.

The results from this study suggest that Triton X-100, which is a nonionic surfactant, had a positive effect on metal removal: it sped up the rate of metal removal compared to the control. In contrast, SDBS, which is an anionic surfactant, had a negative effect on metal removal: it inhibited the biosorption process. This may be largely due to the fact that SDBS is negatively charged. Hence, the anionic surfactant facilitates the metal desorption from the biofilm rather than the biosorption of metal onto the biofilm. Semi synthetic MWFs contain both nonionic and anionic surfactants whereas soluble oils use ionic surfactants in their formulations (Childers, 2006). Therefore, industries focusing on metal recovery should concentrate on removing the anionic and cationic surfactants, but not nonionic surfactants, from MWFs before employing bacteria or biofilms to immobilise the metals on them. Since these surfactants are present at concentrations well above the CMC and as long as the nonionic surfactants do not exhibit toxicity or chelation effects on the metals present in the effluents, they could prove to be beneficial in the metal removal process. Some of the conventional ways of removing anionic surfactants from water are coagulation-flocculation (Kaleta and Elektorowicz, 2013), adsorption (Adak et al., 2005), ultrafiltration and ion exchange (Kowalska, 2008).

5.4 Conclusions

In this study, the impacts of organic-metal interaction on metal immobilisation by bacterial biofilms were investigated and the following conclusions were drawn:

- Metal removal was inhibited when organics were present which indicated competition for biofilm adsorption sites.
- Organics that chelate metals should be completely removed before biotreatment because, in addition to the competition between the organics and metals, they inhibit metal removal. Biofilms preferentially uptake non-chelated metals relative to chelated metals.
- Biosorption was unaffected by the presence of toxic organics but this may not have been the case for all organics, especially those that are known to change the structural integrity of biofilms. Metabolic removal of organics was hindered by toxic compounds, which resulted in less active sites being available for metal removal as these were taken-up by the organics.
- Nonionic surfactants were the only organics that increased the rate of metal removal, but did not affect the biosorption capacity. Hence, they are likely to stop the metal-organic competition or at least assist the metals over the organics. Anionic surfactants are good at desorbing metals, hence they have negative effects on metal removal and should be removed from MWFs or similar waste streams before treatment using biofilms.

Chapter Six

6. A novel method of recovering biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate.

6.1 Introduction

Biosorption has emerged as a sustainable and cost-effective alternative for wastewater treatment, especially for decontaminating heavy metals (Gadd, 2009). It is a passive low energy process whereby metal ions in solution bind to the extracellular structure of certain biomass predominantly in the form of bacteria. Biofilms in particular have recognised potential for remediating both organics and metals in wastewater (Edwards and Kjellerup, 2013). As a result, their potential in industrial applications has received increasing attention since conventional treatment methods for dealing with hazardous effluent score poorly in terms of sustainability (Veglio and Beolchini, 1997; Aksu, 2005). However, biosorption process can only be economical and feasible if a suitable protocol is used to recover metals from the biosorbent (Volesky, 2001).

An effective recovery process requires desorption of metals whilst regenerating the biomass that can be re-used in subsequent biosorption and recovery cycles. Therefore, the recovery agent must be: (i) non-damaging to the biomass, (ii) cheap and/or reusable, (iii) environmentally sustainable and (iv) effective (Veglio and Beolchini, 1997; Gadd, 2009). Several researchers have

conducted exhaustive screening experiments to identify appropriate recovery agents for this process, including dilute mineral acids (HCl, H_2SO_4, HNO_3), organic acids (citric, acetic, lactic) and complexing agents (EDTA, thiosulphate) (Vijayaraghavan, 2013). Kuyucak and Volesky (1989) examined several chemical agents for desorbing Co (II) and demonstrated that acid, alkaline and potassium thiocyanate treatment resulted in alterations in the cellular structure. Organic acids such as citric acid are less commonly used in metal removal by bacteria due to its lower desorption capability and its damaging effect to the biomass. Shetty and Rajkumar (2010) reported 44% copper desorption using citric acid from metal resistant *Pseudomonas sp.* during the first cycle, which significantly decreased during subsequent cycles, a decline attributed to deterioration of the biofilm by the acid. In contrast, several have reported that 80% zinc desorption using EDTA with the reuse of biomass in sorption-desorption cycles over three cycles without accompanying loss of biosorption capacity (Kucayak, N., Volesky, B., 1989; Gaur and Dhankar, 2009). However, due to EDTA's poor biodegradability, EDTA and its related ligands are found to be persistent and present in nature for up to 15 years following disposal. Consequently, EDTA is now banned in parts of the USA, severely restricted in some countries and carefully controlled in others by setting maximum levels for discharge (Chaudhary et al., 2000; Oviedo and Rodriguez, 2003). Hence, there is an urgent need for alternative desorbing/recovery agents facilitating metal recovery in a more sustainable manner.

Ion flotation holds significant potential for heavy metal removal from wastewaters (Fu and Wang, 2011). With this, metal species in wastewaters are

immobilised using surface active agents, such as surfactants, with subsequent recovery using hydrophobic species via air bubbles (Polat and Erdogan, 2007). Surfactants are also used for soil washing or flushing because of their excellent surface active properties, anionic nature and low toxicity (Mulligan et al., 2001; Wuana et al., 2011). The surfactants absorb onto the soil surface, form complex with the metal, then detach the metal from the soil into the pore-water and into the surfactant micelles, so they effectively remove the metals adsorbed on the soil particles (Peng et al., 2009). Surfactants remove organics from soils employing two mechanisms: 1) at lower concentrations, they increase mobility of hydrophobic organic compounds (Duffield et al., 2003); 2) at higher concentrations, they enhance the solubilisation of hydrophobic organic compounds by increasing micellar solubilisation (Huang et al., 1997). For example, anionic (SDS, SOS) and nonionic (Triton X-100, TX100) surfactants enhance the desorption rates of cadmium, lead and zinc, whereas the addition of cationic surfactants (cetyltrimethylammonium bromide) decrease the desorption efficiency of heavy metals (Doong et al., 1998). Surprisingly, no study to date has investigated the use of surfactants for recovering metals from bacteria, after biosorption. Possible explanation could be that most surfactants, such as sodium dodecyl sulphate (SDS), are known to prevent the formation of biofilm and in some cases kill bacteria (Ridgway et al., 1996; Li et al., 2013).

In this study, we propose for the first time employing sodium dodecylbenzene sulphonate (SDBS) as a desorbing/recovery agent to recover biosorbed Zn (II) from metalworking fluid (MWF) acclimated biofilms. Spent MWF was selected as a model wastewater since they are representative of

many waste streams co-contaminated by both metals and organics. Surfactants were introduced for the first time as metal recovery agents from bacterial biofilms treating chemically mixed contaminants. SDS and SDBS are both inexpensive surfactants (Ridgway et al., 1996) however, SDBS has a lower critical micelle concentration (CMC) than SDS. SDBS is used as an emulsifier in MWFs and therefore the assumption was that the biofilms established to recover the metals from the waste stream would have been acclimated to it. Moreover, SDBS was found to be less damaging to the biofilm at its CMC compared to that of SDS. The recovery of Zn (II) was studied in batch mode with respect to the initial pH, temperature, surfactant concentration, contact time and salt presence.

6.2 Materials and Methods

6.2.1 Description of bioreactor system

Biofilms were grown on stainless steel matrix as described in Section 4.2.1.

6.2.2 Measurement of metabolic inhibition

Same as described in Section 4.2.2.

6.2.3 Analytical methods

All chemicals were analytical reagent grade and supplied from Sigma-Aldrich. Stock solutions of 0.5 M EDTA at pH 8, 1 M citric acid, 10 g/L propylene glycol and 1 g/L Zn (II) were prepared by dissolving appropriate amounts of Ethylenediaminetetraacetic acid (≥ 98.0%), Citric acid (≥ 99.5%), 1,2-Propanediol (≥ 99.5%) and $ZnSO_4.7H_2O$ (≥ 99.0%) in DI water, respectively. Stock solution of 5 mM SDBS was prepared from technical grade Sodium dodecylbenzene sulphonate. Samples collected in all experiments were centrifuged at 12,000 rpm for 15 min to minimise background biofilm content. The total organic carbon (TOC) concentration of the supernatant was measured by a Shimadzu TOC V-CPH. The system was calibrated with standards prepared from potassium hydrogen phthalate (KHP) in the range of 0-1000 mg/L. Atomic absorption spectroscopy (Agilent 240FS AA) was used to measure metal concentrations and was calibrated in the linear ranges of 0.2-1.2 mg/L for zinc. Before measurement, the heavy metal solutions were appropriately diluted.

6.2.4 Determination of CMC of surfactant

The conductivity-concentration data were obtained from SDBS surfactant solution with ZnSO4 at initial Zn (II) concentration of 20 mg/L. The conductivity of the various solutions was measured at constant temperature (30 ± 0.5^{O}C) on a hot plate. The conductivity values were determined by a YSI professional plus multimeter with conductivity probe, with an accuracy of ± 0.1 µs/cm in the range below 500 µs/cm and ± 1 µs/cm in the range above, after thorough mixing and reaching temperature equilibrium at each dilution. The conductivity meter was calibrated with standards prior to the experiment. A 500 mL solution of ZnSO4 solution at Zn (II) concentration of 20 mg/L was prepared from stock solution and 1 mL of 0.1 M surfactant SDBS was then added stepwise into the solution. Readings were taken when the conductivity reading was established. A correction factor for the dilution was considered. In some relatively unstable zones, the readings could vary by 2 µs/cm in a few seconds. In this case, only the highest conductivity reading was taken to achieve consistent results.

6.2.5 Biosorption and recovery experiments

For metal removal experiments, all biofilms were washed 3 times using 0.2% Triton X-100 (an oil-emulsifying surfactant) and then five times using DI water to remove residual surfactant. The oils, which inhibited the removal of metal and organics by biofilms, were thus washed away from the surface of the biofilms. Adsorption of zinc onto the walls of conical flasks and on blank stainless steel strips (immobilisation matrix in the absence of biofilm) was found to be

negligible. The dry-weight of the biofilms growing on stainless steel strips were measured using the protocol described in Section 4.2.4.

All zinc metal removal experiments were undertaken in batch mode operation by exposing the biofilm strips to 200 mL metal solution at 20 mg/L Zn (II) in 250 mL Erlenmeyer flasks (120 rpm, 30^{O}C) for a period of 5 hours. Samples were centrifuged at each time point and the supernatant liquid was analysed for metal concentration. After biosorption measurements were taken, the biofilm was exposed to 200 mL of recovery agent (SDBS or EDTA or citric acid) in new 250 mL Erlenmeyer flasks (120 rpm, 30^{O}C). Samples were again centrifuged at each time point for the desorption experiments and the supernatant liquid was analysed for metal concentration.

Metal recovery was calculated using the following formula:

$$\text{Metal Recovery (\%)} = \frac{\text{recovered metal (mg)}}{\text{biosorbed metal for that cycle (mg)}} \text{ x } 100$$

6.2.6 Using HPLC for the analysis of propylene glycol

Same procedure as described in Section 5.2.7.

6.2.7 Statistical analysis

All experiments were conducted in at least three replicates and the data represent the mean values ± standard error. T-test and ANOVA followed by Tukey's test were applied when appropriate, and P values less than 0.05 and 0.01 were considered statistically significant.

6.3 Results and Discussion

6.3.1 Toxicity test

Micro-respiration and post exposure recovery tests demonstrated that SDBS was toxic to the biofilm cells at concentrations ≥ 5 mM (Figure 6.1).

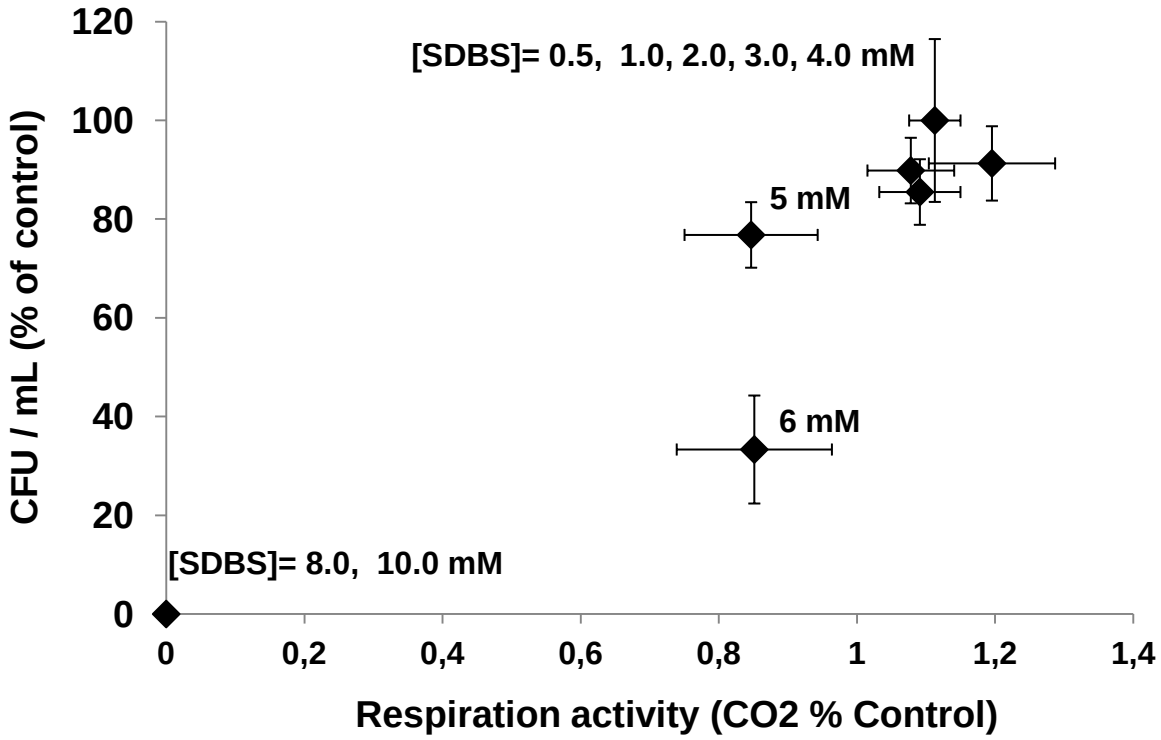


Figure 6.1 Respiration activity and post-exposure growth after 24 hours exposure demonstrating the toxicity of sodium dodecylbenzene sulphonate (SDBS). Error bars represent the standard deviation of triplicate measurements.

6.3.2 CMC determination

The differential conductivity was plotted against surfactant concentration, which resulted in two straight lines with different slopes when the conductivity of solutions with increasing concentration of surfactant was measured. The first one corresponded to the concentration below the CMC, where only monomers of surfactant existed in solution. At concentration above the CMC, micelles are

known to form and a change of slope appears because the conductivity increases in a different manner (Fuguet et al., 2005). The intersection of these two straight lines is taken as the CMC value of the surfactant (Carpena et al., 2002), hence the CMC of SDBS is found experimentally to be 2.6 mM (Figure 6.2).

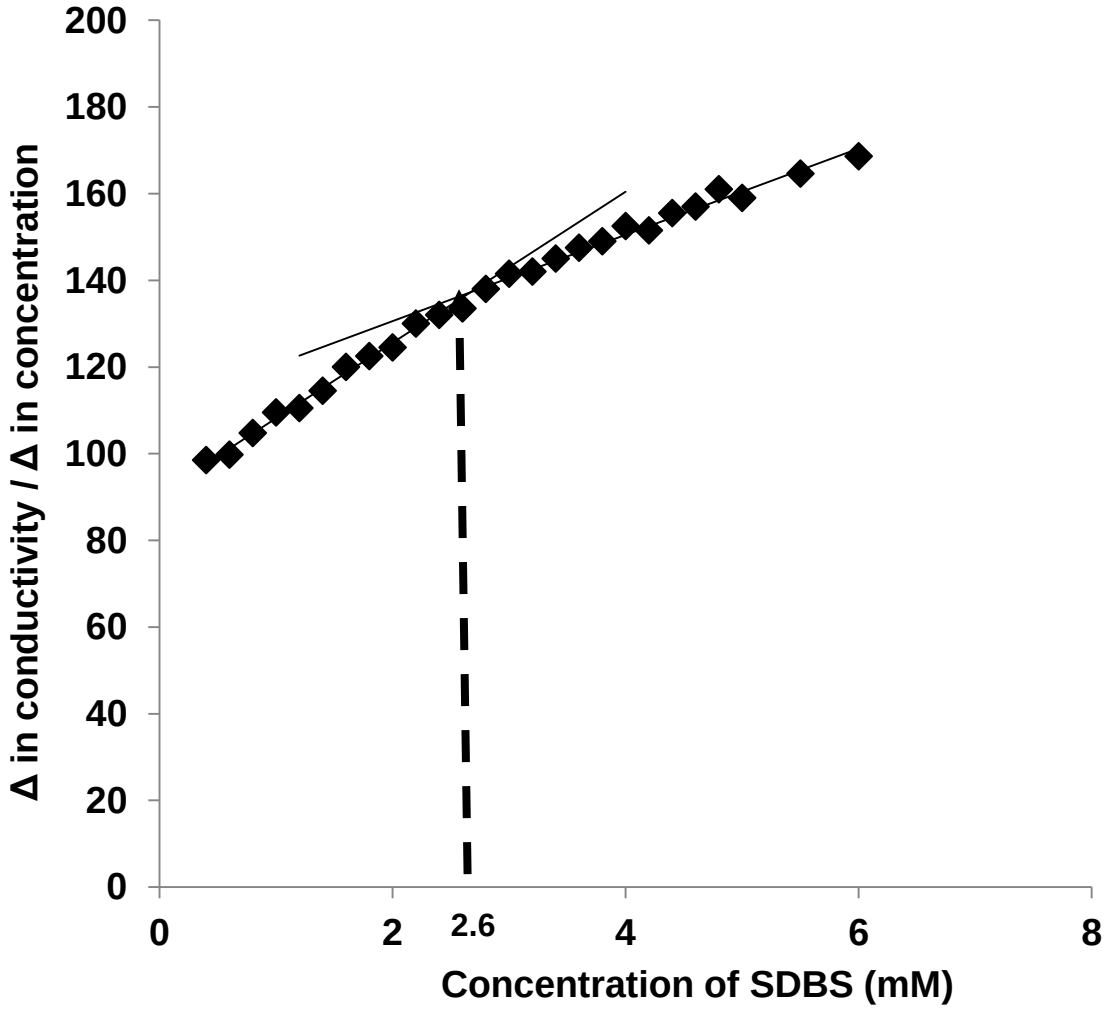


Figure 6.2 Experimental data of differential conductivity versus concentration of sodium dodecylbenzene sulphonate (SDBS) (temperature: 30^{O}C). The dashed lines correspond to the critical micelle concentration (CMC) of SDBS.

6.3.3 Effect of initial pH

The effect of initial pH on Zn (II) recovery from the biofilms was evaluated within the pH range of 2-6. Figure 6.3 shows that Zn (II) recovery percentage increased with the increasing initial pH of the surfactant solution. A possible explanation for this trend could be that at lower pH values, the surfactant charge is neutralised and hence, its ability to attract the metal ions from the biofilm diminishes. Experiments were not conducted above pH 6 to avoid possible metal hydroxide precipitation.

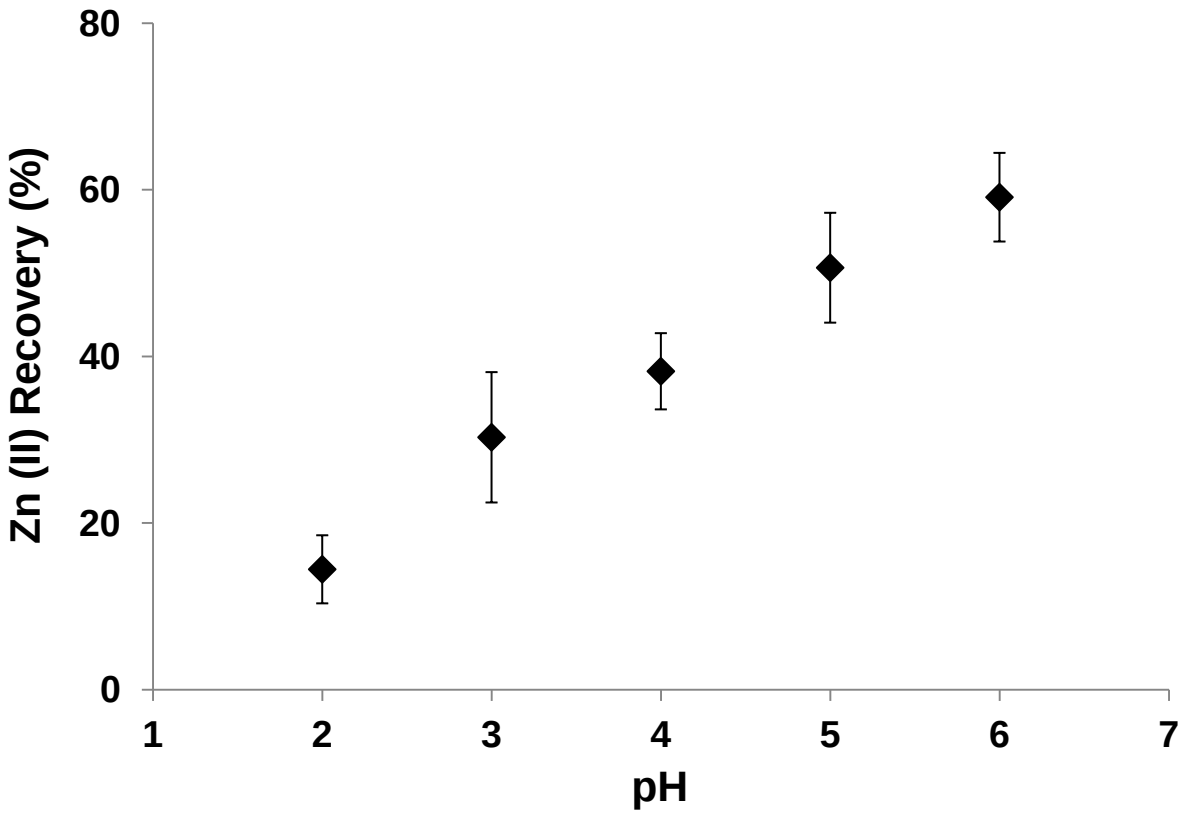


Figure 6.3 Effect of initial pH on Zn (II) recovery (%) from metalworking fluid acclimated biofilms (biosorbed zinc: 1.08 ± 0.08 mg; SDBS concentration: 4 mM; temperature: 30^{O}C). Error bars represent the standard deviation of triplicate measurements.

6.3.4 Effect of initial temperature

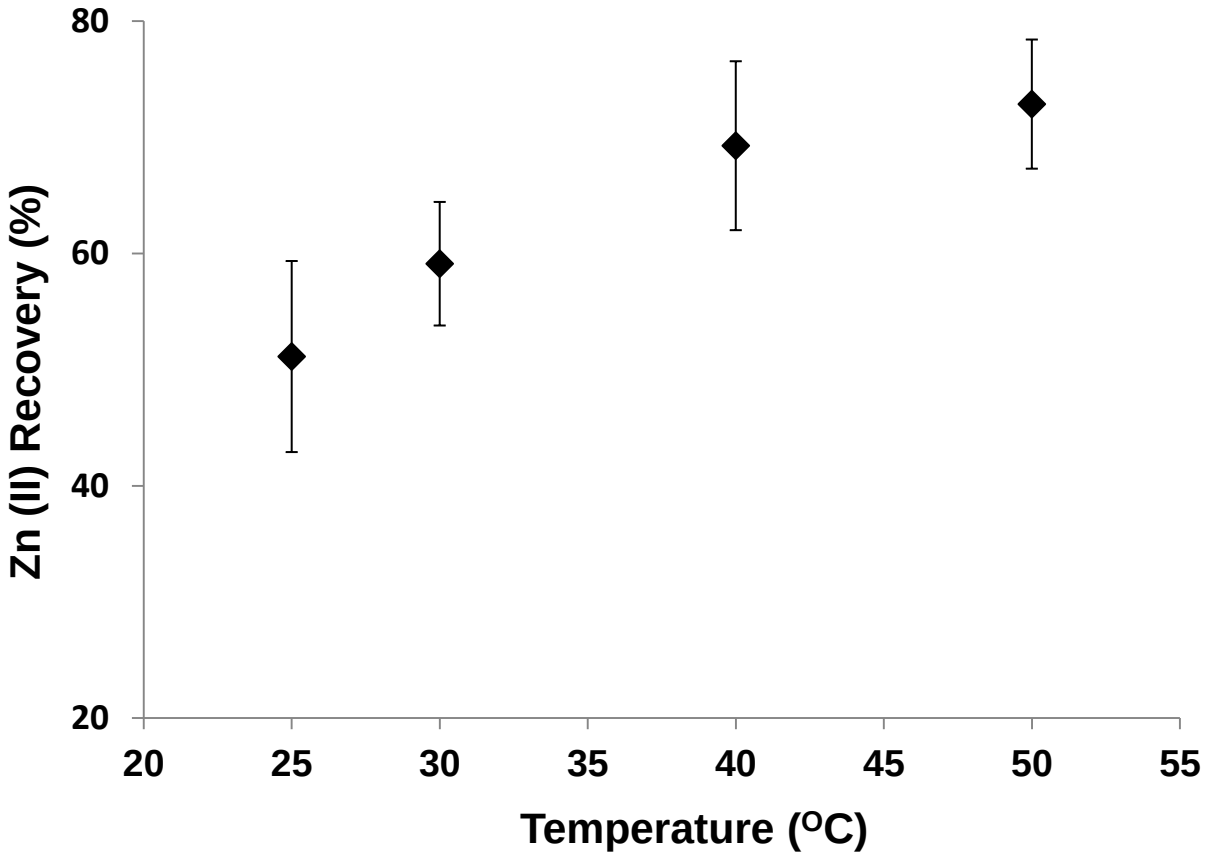


Figure 6.4 Effect of temperature on Zn (II) recovery (%) from metalworking fluid acclimated biofilms (biosorbed zinc: 1.08 ± 0.08; SDBS concentration: 4 mM; pH: 6). Error bars represent the standard deviation of triplicate measurements.

Figure 6.4 represents the effect of temperature on Zn (II) recovery (%). Increasing the temperature increased Zn (II) recovery from 51% (± 8 %) at 25^{o}C to 73% (± 6%) at 40 ^{o}C, after which a plateau was reached. However, higher temperatures may damage the biofilm structure and kill cells, making it unsuitable for subsequent biosorption and recovery cycles. Consequently, all subsequent recovery experiments were performed at 30^{o}C, the optimal temperature for metal recovery whilst avoiding biofilm damage. It is very likely that higher temperatures might have destroyed the biofilm thereby, releasing the biosorbed zinc into the solution. Similar Zn (II) recovery trend can be

observed in Figure 6.4 as compared to Zn (II) removal in Figure 3.3. Zn (II) removal and Zn (II) recovery appear to be at their best around 25-30 because the original consortium is known to grow and thrive best around these temperatures. Moreover, higher temperatures can destroy the biofilm, thereby releasing the biosorbed zinc into the solutions and decreasing the amount of metal removal at higher temperatures and increasing the amount of metal recovery at higher temperatures, as observed in Figure 3.3 and Figure 6.4.

6.3.5 Effect of SDBS concentration

Figure 6.5 illustrates the Zn (II) recovery percentage at varying concentrations of SDBS, ranging from 0 to 5 mM. In the absence of SDBS, only 5% (± 6%) of Zn (II) ions were recovered from the biofilm. Increasing the amount of SDBS increased the Zn (II) recovery percentage up to 3 mM SDBS. At which point, the maximum recovery of 58% (± 5%) was obtained. The CMC of SDBS was 2.6 mM (Figure 6.2) and hence, a possible explanation could be that the system had reached the CMC: so not enabling any further recovery of zinc ions (Gecol, 2006). Above the CMC, the concentration of monomers was constant, while the counterion and the micellar concentrations increase linearly. This should have resulted in a noticeable increase of metal recovery at or near CMC concentrations. However, the results in this study revealed no significant variations in Zn (II) recovery above 3 mM. This could have been due to metal precipitation at higher surfactant concentrations. There is a possibility that the

precipitate metal did not appear in the supernatant which was analysed for metal concentration.

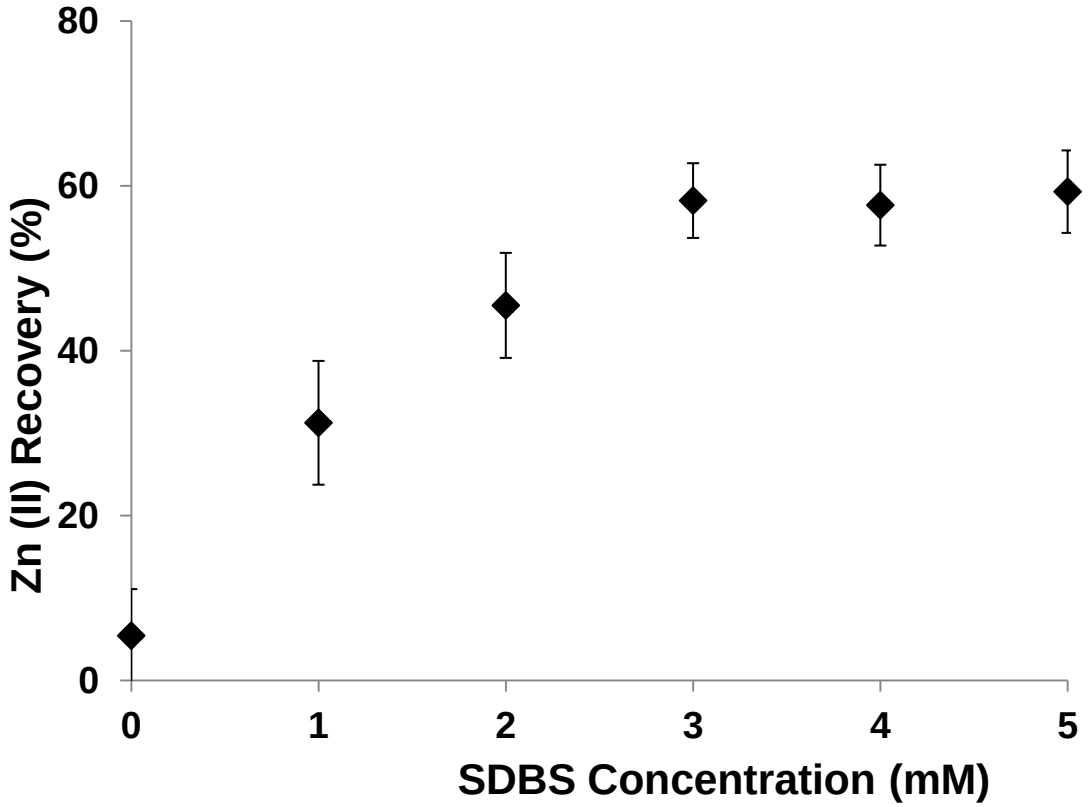
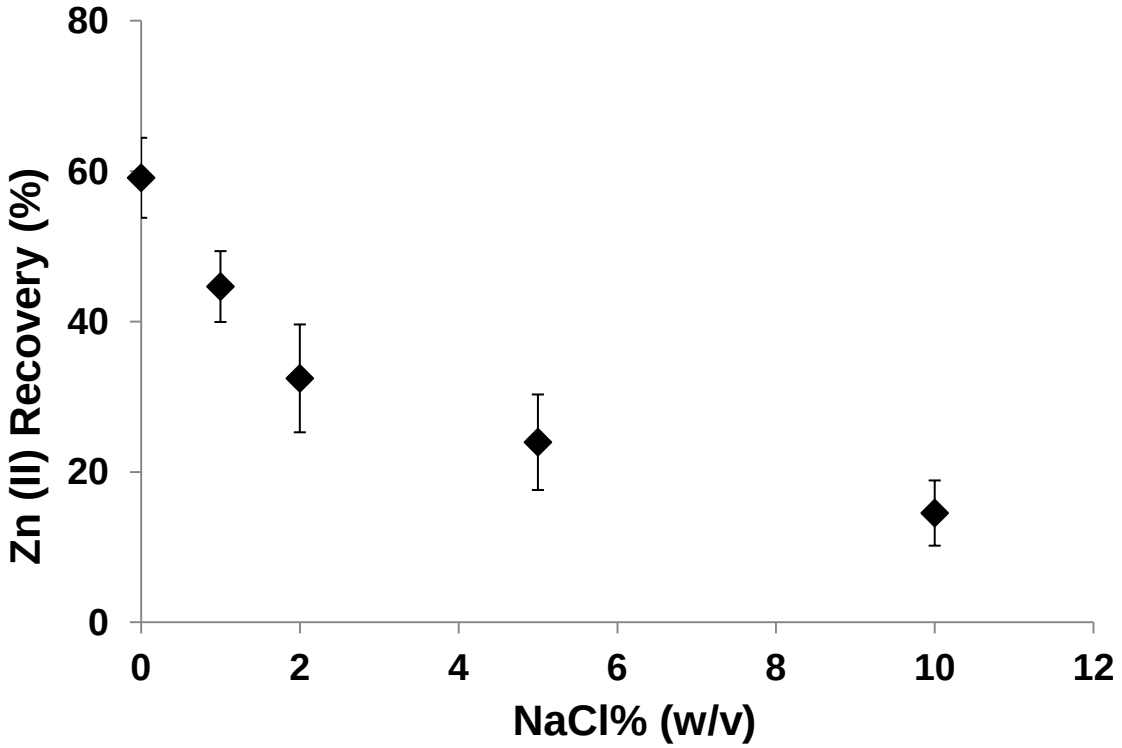


Figure 6.5 Effect of sodium dodecylbenzene sulphonate (SDBS) concentration on Zn (II) recovery percentage from metalworking fluid acclimated biofilms (biosorbed zinc: 1.08 ± 0.08 mg; temperature: 30^{O}C; pH: 6). Error bars represent the standard deviation of triplicate measurements.

6.3.6 Effect of salt presence

The effect of salt presence on Zn (II) recovery was investigated by the addition of NaCl in the range of 0-10% (w/v). As the NaCl concentration increased, the Zn (II) recovery significantly decreased from 59% (± 5%) to 15% (± 4%) in the presence of 10% NaCl (w/v) ($P < 0.05$) (Figure 6.6). The addition of NaCl decreased the thickness of the electrical double layer as the sodium ions shielded the negative charge of the micelles (Wasan et al., 1998). This

reduced the electrostatic attraction between the Zn (II) ions and the micelles resulting in the observed decline in Zn (II) recovery.



Effect 6.6 Effect of salt presence on Zn (II) recovery percentage from metalworking fluid acclimated biofilms (biosorbed zinc: 1.08 ± 0.08 mg; SDBS concentration: 4 mM; temperature: 30^{O}C; pH: 6). Error bars represent the standard deviation of triplicate measurements.

6.3.7 Effect of SDBS on propylene glycol and Zn (II) removal over 4 cycles

Since this removal technique could potentially be used for treating real-world mixed wastes containing metals and organics, it was therefore important to assess the durability of the microbial biofilm and its potential for reuse. This was tested by assessing the effect of SDBS on the ability of the biofilm to remove propylene glycol over four successive biosorption-recovery cycles. In each cycle, propylene glycol removal (TOC %) and zinc removal (%) during the biosorption part of the cycle was investigated for five days. The propylene glycol

removal reached a stable value in each cycle within 3 days at which point, the zinc system reached equilibrium in every cycle (Figure 6.7). Seventy-one percent (± 3%) of propylene glycol was removed in Cycle 1 compared to 87% (± 6%) in Cycle 4: the percentage removal increased from 1st to 4th cycle.

The equilibrium Zn (II) removal efficiency over the four cycles showed little variation with 53% (± 6%) and 43% (± 5%) for Cycle 1 and Cycle 4, respectively ($P > 0.05$). The residence of metal ions immobilised on the biofilm at the end of each cycle decreased the number of active adsorption sites available for subsequent recovery. However, the increase in biomass, as a result of propylene glycol degradation, was sufficient to provide new active sites. Also, Table 6.1 suggests that the Zn (II) recovery decreased from 51% (± 4%) at the end of Cycle 1 to 38% (± 3%) at Cycle 4. This could possibly have been due to a decrease in the washing efficiency due to the formation of thicker biofilms over the cycles. System stability is important when the intention is to employ the same biomass for multiple metal removal and recovery cycles. Therefore, the reusability of biofilm was examined based on removal/recovery ability. Overall, SDBS was an effective metal recovery agent which did not inhibit organic degradation during the biosorption process; however metal recovery decreased slightly with increasing growth of biofilm. Eventually once the system reaches a point when the biofilm stops growing, then a steady removal and recovery efficiency can be obtained with several wash cycles. Hence, further investigation on the possibility of recovering more zinc by developing effective washing strategy unique to each cycle is required if this technology is to be exploited.

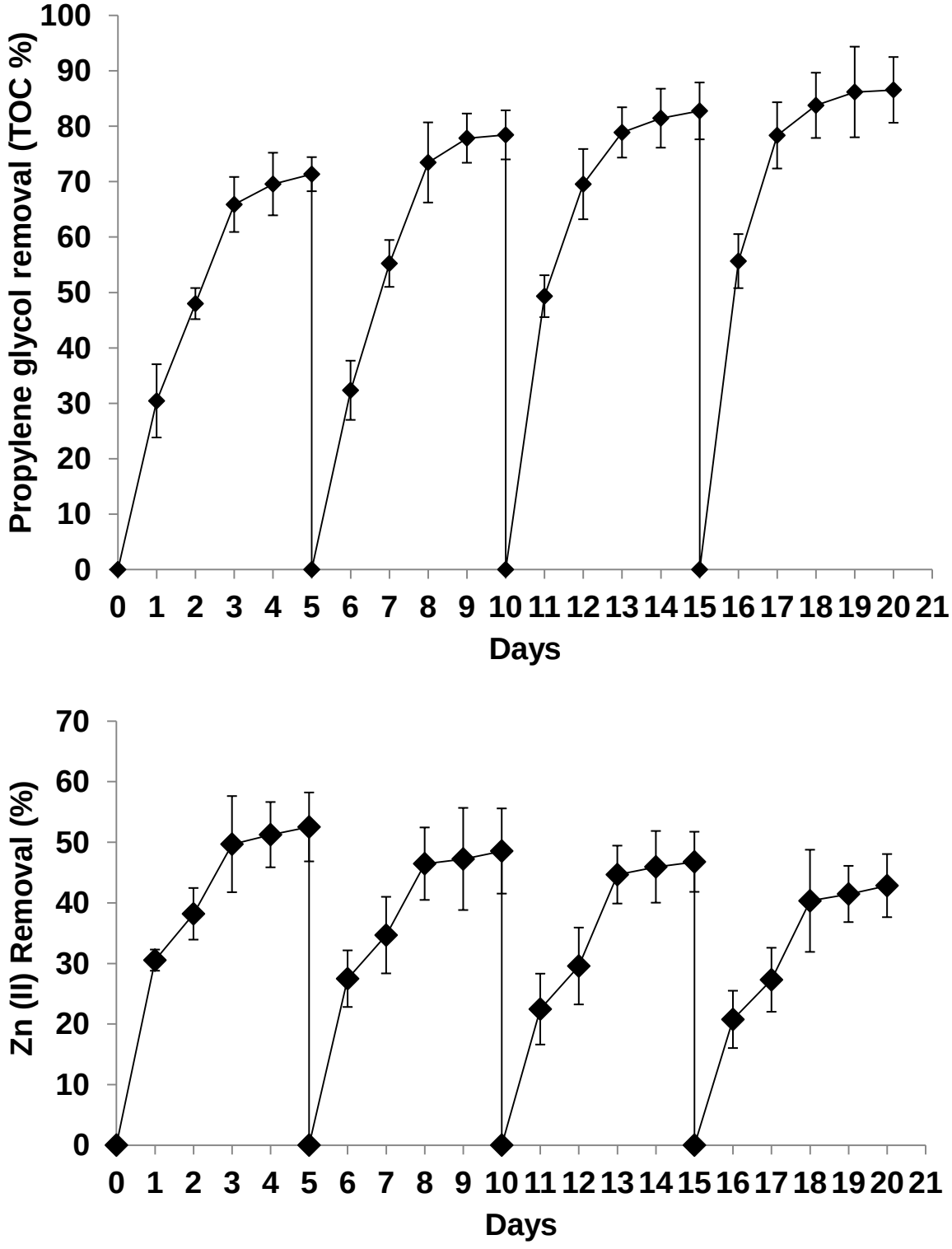


Figure 6.7: Propylene glycol (above) and Zn (II) removal (below) over four cycles employing sodium dodecylbenzene sulphonate (SDBS) for recovery from biofilms. Initial propylene glycol concentration: 1 g/L; initial zinc concentration: 20 mg/L; SDBS concentration for recovery at the end of each cycle: 4 mM; pH: 6; temperature: 30^{o}C. Error bars represent the standard deviation of triplicate measurements.

Table 6.1: Zn (II) recovery at the end of each Zn (II) removal cycle. SDBS concentration: 4 mM; pH: 6; temperature: 30^{o}C (± S.D. of triplicate measurements).

Cycle number	1	2	3	4
Zn (II) Recovery (%)	51% (± 4%)	46% (± 6%)	41% (± 4%)	38% (± 3%)

6.3.8 Comparison of SDBS removal technique to commonly used methods

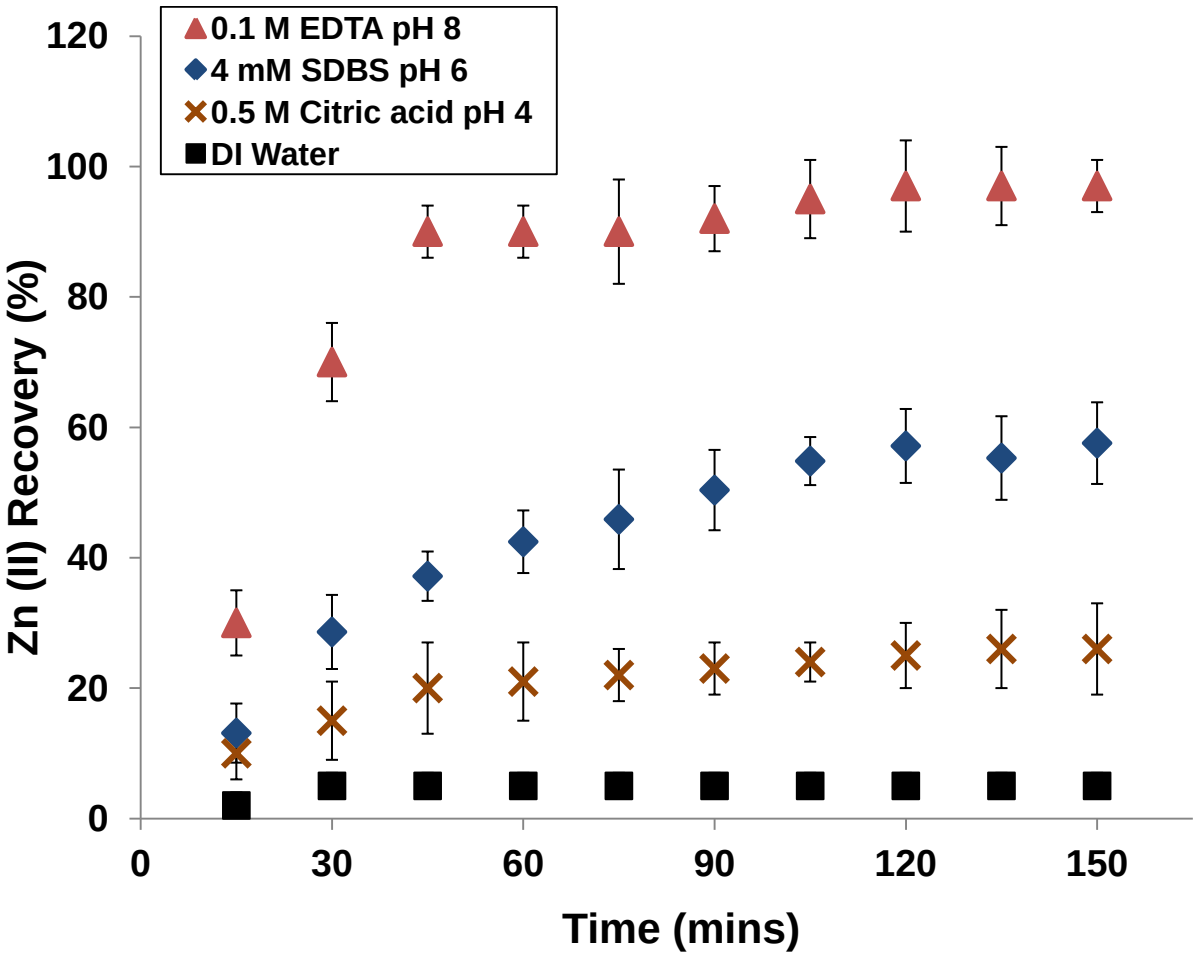


Figure 6.8 Zn (II) recovery kinetics for the first metal recovery cycle: comparison of sodium dodecylbenzene sulphonate (SDBS) removal technique to commonly used methods (biosorbed zinc: 1.08 ± 0.08 mg; temperature: 30^{o}C). Error bars represent the standard deviation of triplicate measurements.

Zn (II) recovery kinetics for a single cycle for three different recovery agents (optimised for pH and concentration of recovery agent: data not shown) is illustrated in Figure 6.8. Only 5% (± 3%) of Zn (II) was recovered using DI water (control), confirming the relatively strong affinity of Zn (II) to biofilms. Ninety percent (± 8%) of Zn (II) was recovered from the biofilm flushed with 0.1 M EDTA, reflecting its high efficiency for dislodging the metal. SDBS at 4 mM (concentration well above the CMC) performed relatively well with 57% (± 6%) recovery compared to 26% (± 7%) with 0.5 M citric acid. Recovery using SDBS was quick, reaching equilibrium within 120 min.

The metal recovery process had the purpose of recovering metals while regenerating the biomass to be used in subsequent biosorption-recovery cycles. Hence it was important to compare the SDBS removal technique with the two commonly used methods over 4 cycles, with zinc biosorption at initial Zn (II) concentration of 20 mg/L for each cycle (Figure 6.9). Table 6.2 shows the amount of Zn (II) biosorbed, recovered and resident for each cycle, along with the total amount of Zn (II) biosorbed and recovered and the change in dry weight of the biofilm at the end of 4 cycles. In terms of recovery, SDBS and EDTA performed similarly with 2.03 mg (± 0.01) and 2.16 mg (± 0.08) of Zn (II) recovered over the 4 cycles however, citric acid recovered only 0.85 mg (± 0.05). The loss in the dry weight of the biofilm was greatest for EDTA (72.1 ± 9.3 %) when compared to SDBS (11.6 ± 0.7 %) and citric acid (8.4 ± 0.5 %). Hence, these results suggest that recovery using EDTA is unsuitable for cases where it is desirable to reuse the biofilm, which is essential for longer term

sustainability. Moreover, regenerating EDTA is more difficult than regenerating surfactants due to EDTA's metal chelating ability.

Figure 6.9 illustrates Zn (II) biosorption and recovery percentages using the three recovery agents for each of the four cycles. For each recovery agent, the recovery percentages for all four cycles were identical ($P > 0.05$), which was expected. This is however not true for the biosorption percentages. In the first cycle, Zn (II) biosorption was 53% (± 7.9%), then this decreased to 28% (± 6.5%), 26% (± 5.4%) and 23% (± 5.1%) in cycles 2, 3 and 4, respectively, with SDBS. The resident Zn (II) ions on the biofilm caused this decrease between the first and second cycle and since the amount of resident ions did not vary over the cycles, the biosorption removal remained constant from cycle 2 onwards. Similar trends were observed for citric acid. In the case of EDTA, the Zn (II) biosorption decreased over the 4 cycles because as observed earlier, EDTA lead to detachment and loss of biofilm, which reduced the biosorption capacity of the biofilm after each cycle. Hence, Zn (II) recovery using SDBS was better compared to EDTA and citric acid because it allowed the reuse of the biofilm for at least four biosorption-recovery cycles whilst avoiding destruction of the biofilm (as in the case of EDTA) and provided a better recovery amount when compared to citric acid.

Table 6.2 The amount of Zn (II) biosorbed, recovered and resident on the biofilm using sodium dodecylbenzene sulphonate (SDBS), ethylenediaminetetraacetic acid (EDTA) and citric acid recovery agents including change in dry weight of biofilm at the end of fourth cycle (temperature: 30^{o}C). Error bars represent the standard deviation of triplicate measurements.

	Amount of Zn (II) (mg)	4 mM SDBS pH 6	0.1 M EDTA pH 8	0.5 M Citric acid pH 4
Cycle 1	Biosorbed	1.08 ± 0.08	1.08 ± 0.08	1.08 ± 0.08
	Recovered	0.61 ± 0.05	1.06 ± 0.04	0.28 ± 0.02
	Resident	0.46 ± 0.06	0.04 ± 0.00	0.80 ± 0.05
Cycle 2	Biosorbed	0.56 ± 0.06	0.59 ± 0.06	0.24 ± 0.03
	Recovered	0.54 ± 0.05	0.59 ± 0.06	0.23 ± 0.02
	Resident	0.48 ± 0.05	0.04 ± 0.00	0.82 ± 0.06
Cycle 3	Biosorbed	0.52 ± 0.06	0.30 ± 0.01	0.21 ± 0.02
	Recovered	0.46 ± 0.04	0.30 ± 0.02	0.20 ± 0.03
	Resident	0.54 ± 0.04	0.04 ± 0.00	0.83 ± 0.04
Cycle 4	Biosorbed	0.46 ± 0.06	0.20 ± 0.04	0.18 ± 0.01
	Recovered	0.43 ± 0.05	0.20 ± 0.03	0.15 ± 0.01
	Resident	0.57 ± 0.03	0.03 ± 0.00	0.86 ± 0.05
Total Zn (II) biosorbed (mg)		2.61 ± 0.02	2.19 ± 0.02	1.71 ± 0.03
Total Zn (II) recovered (mg)		2.03 ± 0.01	2.16 ± 0.08	0.85 ± 0.05
Change in dry weight of biofilm after 4 cycles		-11.6 ± 0.7 %	-72.1 ± 9.3 %	-8.4 ± 0.5 %

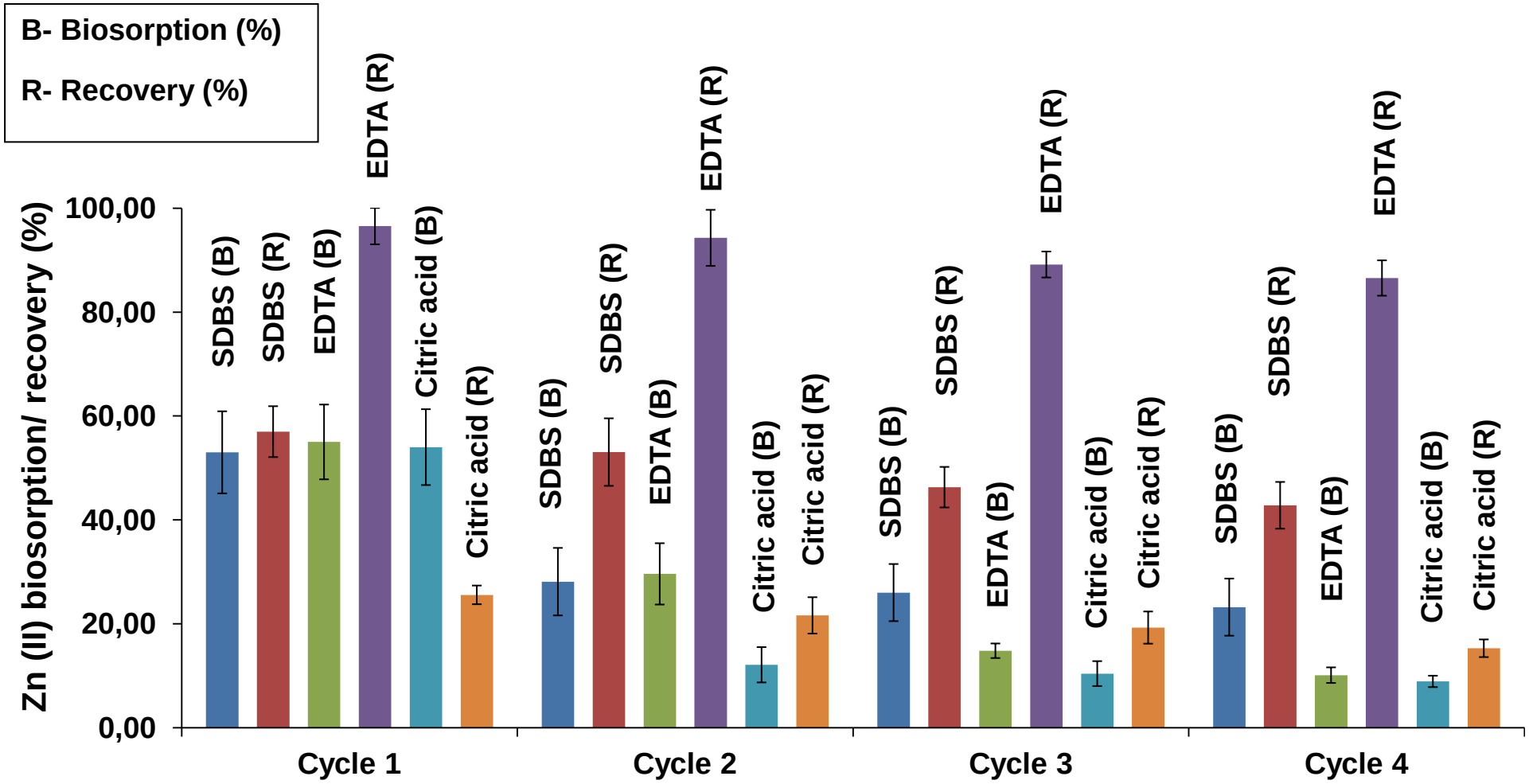


Figure 6.9 Zn (II) biosorption and recovery employing 4 mM sodium dodecylbenzene sulphonate (SDBS) at pH 6, 0.1 M ethylenediaminetetraacetic acid (EDTA) at pH 8 and 0.5 M citric acid at pH 4 recovery agents for four cycles (Initial Zn (II) concentration in 0.85% NaCl for biosorption experiments in each cycle: 20 mg/L; temperature: 30^{o}C). Error bars represent the standard deviation of triplicate measurements.

6.3.9 Cumulative Zn (II) recovery with successive wash cycles

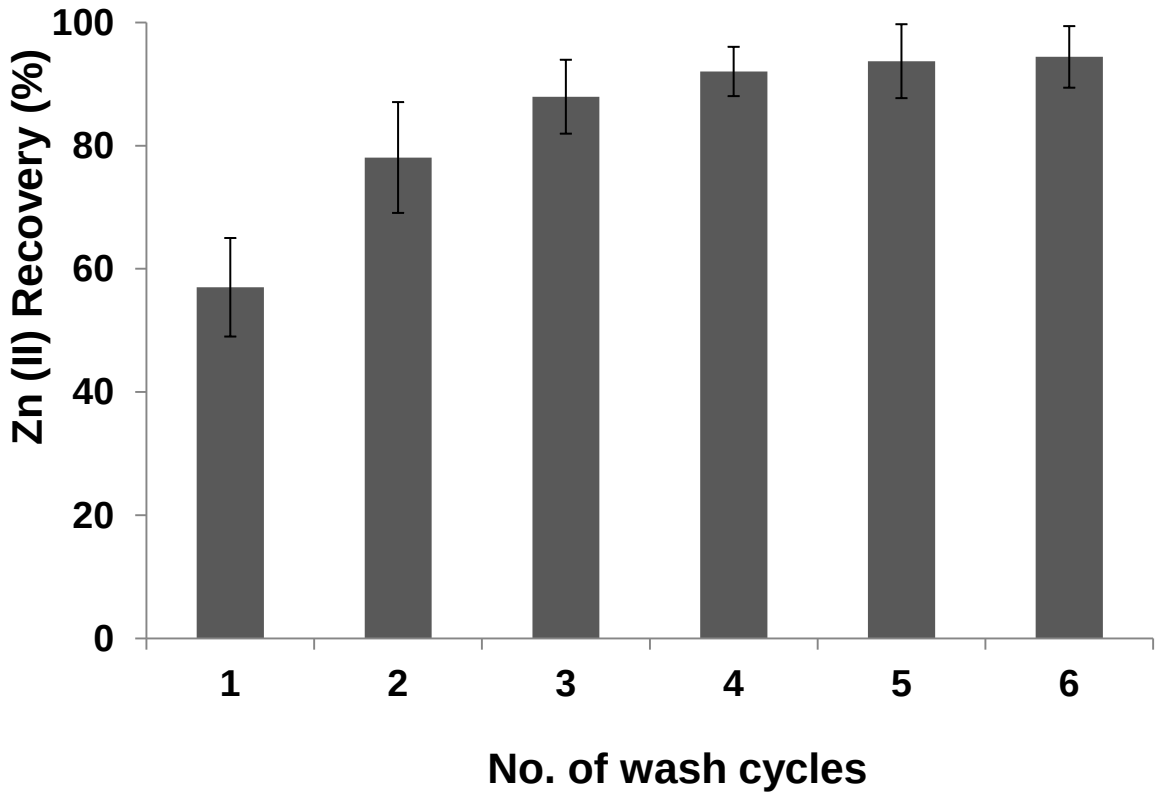


Figure 6.10 Cumulative Zn (II) recovery with successive wash cycles. SDBS concentration for recovery at the end of each wash cycle: 4 mM; pH: 6; temperature: 30^{o}C; contact time for each wash cycle: 120 min. Error bars represent the standard deviation of triplicate measurements.

Cumulative Zn (II) recovery employing SDBS was about 94% (± 5%) after 6 successive wash cycles (Figure 6.10). However, the increase in Zn (II) recovery (%) of each individual wash declined with increasing number of washes. A possible explanation for this is that bound zinc on the periphery of the biofilm was easier to remove than that bound in the inner depths of the biofilms. Since the initial washes may have removed peripheral zinc, the latter washes would have targeted inner zinc, which may have needed either longer contact time

with the surfactant or a more effective surfactant. Hence, industries focusing on recovering metals from biofilms using this surfactant would have to optimise their washing method at the end of each wash cycle to recover the maximum possible amount of zinc. Consequently, this could also reduce the amount of surfactant used and hence decrease the operational costs of the process, making metal recovery using biotechnology more feasible.

In order for SDBS to be a credible metal recovery option, it is essential that the zinc be separated from the SDBS. Methods for removing surfactants from aqueous effluents are established and include electrochemical removal, coagulation precipitation or adsorption on activated carbon (Basar et al., 2004; Aboulhassan et al., 2006; Onder et al., 2007). However, none of them were able to separate the two compounds. Cationic exchange resins could potentially be an option because theoretically the low pH should weaken the metal-surfactant bond and hence allow for the recovery of the metal ion (Rengaraj et al., 2003), but this requires further investigation. Shen et al. (2015) developed a novel treatment process that employs polymer-surfactant complexation and flocculation to remove dilute heavy metal ions from effluent streams. They also showed that metal ions contained in the polymer-surfactant aggregates can be recovered by acidification and the aggregates in the form of precipitates are then dissolved in a base solution to reclaim the removal agents (Shen et al., 2016). This method can be applied here to recover and concentrate the metal ions from the metal-surfactant complex, and also to regenerate the surfactant such that it can be recycled with little deterioration of removal ability.

6.4 Conclusions

The recovery of biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate (SDBS) surfactant is novel and has received no previous attention in the literature. The following conclusions can be drawn:

- Zn (II) recovery is affected by solution pH, temperature, SDBS concentration and salt presence. The recovery rate of Zn (II) was rapid, reaching equilibrium within 120 min.
- Cumulative Zn (II) recovery was about 94% (± 5%) after 6 successive wash cycles using 4 mM SDBS at pH 6. This can potentially be improved by optimising the protocol at the end of each wash cycle.
- SDBS recovery out-performed citric acid and EDTA methods over 4 successive biosorption-recovery cycles. Employing EDTA lead to loss in the dry weight of the biofilm over the 4 cycles (72.1 ± 9.3 %) which diminished its biosorption capacity. Relative to SDBS and EDTA (2.03 ± 0.01 mg and 2.16 ± 0.08 mg, respectively), lower amount of Zn (II) was recovered for citric acid (0.85 ± 0.05 mg).
- SDBS did not exhibit any inhibiting effects on the biofilm over the 4 successive biosorption-recovery cycles in terms of organic degradation and only slightly decreased metal recovery with increasing mass of biofilm. Again, recovery could be enhanced by developing effective wash strategy unique to each cycle.

Chapter Seven

7. Concluding Remarks and Future Work.

7.1 Concluding Remarks

The objective of this research was to gain better understanding of the interactions between microbes, metals and organics when present as chemically mixed waste streams to determine how metal recovery and organic bio-treatment are impacted by such chemical mixtures. Firstly, the work started with a comprehensive literature review of how the exploitation of bio-treatment in pollutant remediation can help to provide low-cost and sustainable solutions for metal recovery and wastewater treatment. Metalworking fluids (MWFs) was selected as a model effluent since 95% of it is water, with the remainder of the composition being composed of organics and metals and they are ubiquitously employed in engineering and manufacturing industries during machining and other processes. As a result, their end of life treatment is a challenge since they contain metals at concentrations often exceeding the drinking water standard limits. Thus the development of sustainable technologies for their treatment is getting increasingly urgent since current technologies are expensive and energy demanding. This need for cost-effective alternative technologies is further intensified with increasing environmental awareness and legal constraints being imposed on effluent discharge. To achieve this overall objective, immobilised MWF acclimated bacterial biofilms were investigated for their ability to remove Zn (II) and Mn (II) ions from synthetic aqueous solutions through biosorption,

whilst in parallel biodegrading the organic component thereby reducing their risk to the environment and recovering an increasingly precious resource.

Biofilms- the focus of the thesis

Immobilised biofilms are increasingly used for wastewater treatment; however, very few studies have explored their potential for biosorption of metals from mixed solutions co-contaminated by organics. Biofilms have the potential to both biodegrade organics whilst in parallel immobilising and recovering metals in multiple biosorption-recovery cycles. This is the focus of this study.

Initial experiments were undertaken to determine the influence of temperature, pH, initial metal ion concentration and contact time on biofilms' metal removal performance. Maximum metal uptake was detected for both metals at pH 6. Application of the Freundlich model demonstrated a better fit for the adsorption process when compared to Langmuir model, suggesting that the biofilm has a heterogeneous surface with biosorption sites having variable biosorption energies. The maximum metal uptake was 24.60 mg/g and 5.23 mg/g for Zn (II) and Mn (II), respectively, for an initial metal concentration of 80 mg/L. The pseudo-second order rate was used to model the kinetic profiles. The biosorption of binary system was found to be competitive in nature; Zn (II) sorption decreased up to 87% whereas the Mn (II) removal decreased up to 66%. Metal uptake by free-living cells achieved equilibrium more rapidly, in fact within 90 min compared to biofilms, which took 300 min. Biofilms were able to immobilise 12 times more zinc (mg/g biofilm) at initial metal ion concentration of 80 mg/L, confirming that the bacterial biofilms can sorb greater amounts of

heavy metals as compared to their free-living counterpart cells. Scanning Electron Microscopy (SEM) analysis of biofilm before and after sorption experiments revealed morphological differences for zinc-loaded and manganese-loaded biofilms, when compared to control. The results revealed that immobilised MWF acclimated bacterial biofilm had a great potential application for the removal of Zn (II) and Mn (II) ions from aqueous solutions.

UV- a novel analytical tool for inactivating biofilms

In order to understand how biofilms function and to quantify the relative contributions of metabolic activity and physicochemical processes on pollutant removal, many researchers have used different techniques (heat, formalin, uncouplers, etc.) to attempt to unravel the functioning of biofilms (Tsezos and Bell, 1989; Wang and Grady, 1994; Klein and Kennedy, 1997; Johnson et al., 2007; Das and Guha, 2009). In this study, a comparative assessment of chemical (sodium azide) and physical (UV) methods of cell inactivation was undertaken to investigate the relative roles of biodegradation and biosorption in pollutant removal, and to determine the extent these were active and passive processes. This is the first study, as far as we are aware, to report that UV has potential as an analytical tool for determining mechanisms of pollutant removal by biofilms.

Respiration, post-exposure recovery and SEM analysis demonstrated that both ultraviolet (UV) treatment (300 mJ/cm^2) and sodium azide (10 mM) completely inhibited metabolic activity at 5 and 24 hour of exposure,

respectively, whilst not damaging the integrity of the biofilms. Chemical inhibitors such as formalin and dinitrophenol caused biofilm structural damage and cell lysis, whereas UV provided a relatively non-invasive tool for inhibiting metabolic activity of biofilms. Biofilm inhibition with UV and sodium azide was found to be equally effective at inhibiting biofilms for treatment of triethanolamine (TEA) and benzotriazole (BTA): the results confirming that the dominant removal mechanism was biodegradation. However, the rates of glucose removal by sodium azide-inhibited biofilms were similar to the controls, suggesting that chemical inhibitors were not effective for distinguishing the removal mechanisms of simple sugars. There were no statistical differences between amounts of metal removed by biofilms treated with UV and sodium azide in zinc, copper and cadmium single-systems: the results indicated that the removal mechanism was predominantly a passive biosorption process.

The influence of organics on biofilm metal immobilisation

Organics and metals compete for the biofilm active sites, so potentially interfering with each other in both passive and metabolic removal mechanisms. The influence of co-contaminating water borne organics on metal removal by microbial biofilms was investigated in chemically mixed systems to determine the most effective biological treatment strategy. The effect of chelation was by far the most inhibitory on the passive metal removal mechanism, reducing the zinc biosorption by 60% (± 5%) in fully chelated Zn-EDTA complex system. Interestingly, the non-chelated zinc was preferentially removed relative to the chelated form by biofilms in partially chelated systems. Addition of biocides

negatively impacted metal removal: zinc removal decreased to 4.77 mg/g biofilm (± 0.56) in the presence of 0.05% BIT (toxic) from 7.75 mg/g biofilm (± 0.51) when exposed to 0.025% BIT (non-toxic). Nonionic surfactants, in contrast, were beneficial in terms of metal removal: octylphenol ethoxylate (Triton X-100) accelerated the zinc biosorption process by reducing the equilibrium time by half. This was possibly due to the reduction of surface tension within the system. Metabolic removal of organics increased the zinc biosorption, confirming our hypothesis that the active sites of the biofilm became more available for free Zn (II) ions in the solution as the organics were biodegraded.

In mixtures of two or more components in a solution, the synergistic or antagonistic interaction occurring between the components may affect their own interaction with the microbial component of the system. The negative impact of metals on the biodegradation of organic pollutants has been extensively studied (Sandrin and Maier, 2002 & 2003; Gadd, 2010), however the effects of the organic compounds present in mixed effluents on metal recovery has received little attention. The findings of this study demonstrate that metal chelators, toxic organics and ionic surfactants hinder biosorption and therefore they should ideally be removed from industrial effluents before any biological treatment can be applied. In contrast, nonionic surfactants could prove to be beneficial in the metal removal process as long as they do not exhibit toxic or chelating effects on the biofilms and the metals, respectively, present in the effluents. This information can be beneficial for industries developing effective biological treatment strategies for chemically mixed wastes.

Surfactants- a novel method for metal recovery from biofilms

Finally, the potential of using sodium dodecylbenzene sulphonate (SDBS) as a measure for recovering metals immobilised from biofilms was investigated by examining the parameters influencing the process. The effect of initial pH, temperature, surfactant concentration, contact time and salt presence on the recovery of Zn (II) from biofilms in bioreactors was investigated in batch mode. SDBS performed well in terms of achieving high and stable zinc recovery over four successive biosorption-recovery cycles when compared to the commonly used metal recovery agents: ethylenediaminetetraacetic acid (EDTA) and citric acid. Relative to SDBS and EDTA (2.03 ± 0.01 mg and 2.16 ± 0.08 mg, respectively), lower Zn (II) recovery was obtained for citric acid (0.85 ± 0.05 mg). EDTA destroyed the biofilm at the end of four cycles, reducing the dry weight of the biofilm by 72.1 ± 9.3 % and diminishing its biosorption capacity. An additional advantage of SDBS is that it did not have an inhibitory effect on organic microbial degradation confirming its potential to contribute to metal recovery whilst not interfering with organic biotreatment in bioreactors. Moreover, over six successive wash cycles, 94% (± 5%) of Zn (II) was cumulatively recovered, confirming that SDBS is a potential zinc recovery agent for biofilms.

SDBS recovery outperformed citric acid and EDTA methods over four successive biosorption-recovery cycles. The recovery of biosorbed Zn (II) from microbial biofilms using sodium dodecylbenzene sulphonate (SDBS) surfactant is novel and has received no previous attention in the literature.

7.2 Future Work

There exist many exciting possibilities for future work stemming from this study:

- Surfactant regeneration from the surfactant-metal complex after recovering metals from microbial biofilms is important to concentrate and recover metals as well as to keep the costs low and to make the overall process sustainable. This needs to be investigated.
- Sodium dodecylbenzene sulphonate (SDBS) needs to be investigated in terms of its interaction with more metals as its potential for their recovery remains unknown, and its ability to recover more than one metal simultaneously is certainly poorly studied.
- Further research on metal recovery is required to discover other surfactants which might possess greater metal recovery ability, when compared to SDBS. Many studies have focused on oil and heavy metal recovery employing biosurfactants, but none on recovering metals from microbial biofilms.
- Metal removal and organic degradation have been modelled separately however, combining both models might help researchers to better understand the underlying mechanisms and to optimise the process as well as the design.
- Genetic engineering has the potential to improve or redesign microorganisms. Common biosorbents lack specificity in metal-binding, which reduce the amount of desired metal(s) that can be recovered. This could be resolved by genetic modification of the consortium, to have a

higher intrinsic capacity as well as specificity and greater resistance to environmental conditions (Mejáre and Bülow).

- More information on biosorption from real industrial effluents is required because it provides a more realistic assessment when compared to synthetic waste solutions, which are often used in laboratory studies. Despite the fact that industrial effluents contain several pollutants simultaneously, the effects of multiple metals and organics on the metal removal ability of biofilms is poorly understood and has rarely been the subject of research.
- Large scale experiments are important before the process can be replicated for industrial scale and hence experiments in 5L and 20 L reactors can provide useful information about its scalability.
- Metal support is usually not the preferred material for bacterial immobilisation due to low biomass concentration; however, in lab scale it provided an even distribution of biofilm which was essential for microscopic analysis. Other support matrices should be researched which may yield higher biomass concentration and can be used at industrial scale.
- An investigation into biofilm reactor configuration on adsorption and recovery potential should be conducted; for example, packed-bed columns under the continuous flow conditions are more useful in large scale water treatment systems.

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