Development of a Bioreactor System Using a Pine Bark Matrix for the Removal of Metal Ions from Synthetic Aqueous Solutions

by

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Abstract

Many industries use, or produce, metal-containing solutions which must be treated for reuse or discharge to sewer. One such treatment is biological and both living and dead materials have been investigated for the abstraction of metal ions from solution. Studies on systems containing only a single biosorbent are well documented, and mostly involve optimisation of biosorption capacities and metal uptake rates through modification of Biological Support Particle (BSP) size and surface characteristics. Literature on dual biosorbent studies is sparse. The commercial application of biosorption technology in wastewater treatment remains largely unexplored and unexploited.

The primary objective here was to assess the potential of forced-upflow packed-bed bioreactors, containing dual biological sorbents, for treating a synthetic wastewater containing copper, zinc and cadmium, at both laboratory- and pilot-scale. Pine bark was selected as BSP since it is an abundant, relatively cheap, agricultural waste product in South Africa, and is known to sorb metal ions. Initial experiments aimed to optimise biofilm development on the pine bark surfaces, since microbial biomass is also known to sequester metal ions. Systems comprising either one, or both, these biosorbents were compared for their efficiency in metal removal. The effects of type, size, and state of decomposition, of the pine bark, the addition of supplementary nutrients (Voermolas) and the mixing conditions, on the metal biosorption capacity and reaction kinetics of the systems were also studied.

All experiments were conducted at an initial metal concentration of 100mg.ℓ^{-1} with both composted and uncomposted pine bark as BSP. The former supported microbial colonisation and resisted biofilm sloughing, but degraded rapidly causing engineering difficulties. Uncomposted pine bark showed the same ability, but was also physically more robust.

Organic compounds leached from the pine bark did not hinder microbial colonisation of the BSP; rather they served as additional nutrients. Literature studies suggest that these compounds would not significantly compromise the COD or increase the toxicity of the final effluent. Biofilms developed without

supplementary nutrients, but Cd^{2+} and Zn^{2+} were sorbed more effectively in bioreactors containing Voermolas (39% and 38% Cd^{2+} removal, 36% and 32% Zn^{2+}removal, in 0.2% and 0.1% Voermolas solutions respectively) than in unsupplemented systems (25% Cd^{2+} removal and 20% Zn^{2+} removal). Conversely, Cu^{2+} was removed most efficiently in the absence of supplementary nutrients. Based on biosorption of the target metal ions, 0.1% (v/v) Voermolas was the most effective concentration of supplementary nutrients.

Raw, un-colonised pine bark nuggets (16-24mm), and plastic bioballs (commercially available, bespoke BSP), were compared in laboratory-scale bioreactors by measuring the decrease in residual metal ion concentrations over time, and changes in the solution pH. These experiments showed that the two BSPs did not differ significantly in their performance as a support matrix, or as a metal sorbent (30.6% and 32.6% of metal ion remained in solution when using bioballs and pine bark respectively). However, the presence of a biofilm on both these BSPs, improved the overall performance of the bioreactors significantly (for the bioball BSP, residual metal ion levels decreased from 30.6%, in the absence of a biofilm, to 11.0% with a biofilm present. Similarly, for the pine bark BSP, residual metal ion levels decreased from 32.6%, in the absence of a biofilm, to 7.3% with a biofilm present). A cost comparison of the two BSPs showed that raw pine bark nuggets were available at less than 0.1% of the cost of the bioballs.

At pilot-scale, modelled kinetic data compared poorly with experimentally determined results, but minimum residual metal concentrations for Cu (1.7mg.ℓ^{-1}) and Zn (4.2 mg.ℓ^{-1}) were below South African (eThekwini Municipality) regulatory limits for discharge to sewer (5mg,ℓ^{-1} for both), and sea outfall (3mg.ℓ^{-1} Cu and 20mg.ℓ^{-1} Zn). However, for Cd the final residual metal concentration (5.6mg.ℓ^{-1}) was above the regulatory discharge threshold for any receiving system.

Although some of the effluents from the system investigated could not be legally released into the municipal sewer system without further remediation, the study showed that a system combining living and dead biomass in a single reactor is capable of significantly reducing dissolved metal concentrations in synthetic wastewaters without temperature or pH adjustment. Furthermore, such a system can operate at pilot-scale, where a pine bark matrix represents a significant cost saving over conventional plastic BSPs.

Declaration

I hereby declare that this work, conducted at the University of KwaZulu-Natal, Pietermaritzburg, is the result of my own investigations unless specifically indicated in the text and has not been submitted for a higher degree at any other institution.

Signed 

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I hereby certify that this statement is correct.

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List of Abbreviations and Acronyms

AAS	Atomic Absorption Spectrophotometry
BOD	Biological Oxygen Demand
BSP	Biofilm Support Particles
CEC	Cation Exchange Capacity
DWAF	Department of Water Affairs and Forestry
EDTA	Ethylene Diamine Tetra-acetic Acid
EDX	Electron Dispersive X-ray microanalysis
E_h	Redox Potential
EPS	Exopolysaccharides
ESEM	Environmental Scanning Electron Microscopy
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrophotometry
meq	Milliequivalents
mmol	Millimolar
q_e	Biosorption Capacity
RBC	Rotating Biological Contactor
Redox	Oxidation-Reduction
UN	United Nations

Chapter 1

Literature Review

1.1 Introduction

The scarcity of water resources is of global concern. Increased awareness of the effects of toxic wastes on the environment has stimulated various municipal regulations (Braum, 2004), national legislation (National Water Act, 36 of 1998; National Environmental Management Act, 107 of 1998) and international action (United Nations, 2009). The period 1981-1990 was the first international United Nations (UN) Decade on Water, 2003 was the International Year of Freshwater and 2005-2015 is the second UN International Water Decade which aims to reduce by half the number of people without a source of clean drinking water (United Nations, 2009). While domestic and international legislation governing the protection of the environment has become progressively stricter, developments in the field of scientific research have given rise to increasing numbers of options to manage and treat waste effluents.

Industries are now obliged to reuse water, and/or substantially detoxify water containing waste before release into the environment. This is important, not just in water scarce countries such as South Africa, but the world over. Even though more than 70% of the earth's surface is covered by water, it is undeniably the planet's most valuable natural resource. Yet pollution of surface water remains common (Vijayaraghavan and Yun, 2008). Therefore it stands to reason that managing wastewater should follow the generally accepted "Waste Management Hierarchy"

which articulates the position that every effort must be made to reduce the need for disposal of waste products (European Commission on Environment, 2008). The discharge of waters containing heavy metal wastes has historically been problematic and is known to have an adverse effect on the environment (Kotrba, 2011). Accordingly, in South Africa this practice results in additional and unnecessary stress on already sub-optimal water resources. In industries which source their processed water from a potable mains supply and, therefore, do not abstract surface water directly, reusing industrial wastewater will still benefit the natural environment and potentially realise financial cost savings. Therefore, it stands to reason that research should focus its efforts away from disposal and concentrate on methodologies for waste treatment for the beneficial reuse of industrial wastewaters. The research described in this thesis focussed on reclamation of heavy metal contaminated water by removal of the metal ions using biosorption.

Man's technological activities often lead to the release of metal ions into the natural environment. These mobilised, bioavailable ions tend to accumulate throughout the food chain (Holan and Volesky, 1995; Naja and Volesky, 2010a). Although bioaccumulation is potentially hazardous to the environment, it can also be used as an effective and beneficial method of metal removal and concentration. Therefore, if exploited in conjunction with appropriate recovery strategies, biosorption can serve as an effective remediation following the utilisation of heavy metals (Volesky and Holan, 1995; Naja and Volesky, 2010b). The use of microbial bioaccumulation strategies as a form of metal-contaminated wastewater treatment is well researched and documented (Paton and Budd, 1972; Gadd and Griffiths, 1978; Montes *et al*., 2003; Volesky 2004; Mhavi *et al*., 2005; Vijayaraghavan and Yun, 2008; Gadd 2009; Naja and Volesky, 2010b; Kotrba, 2011; Macek and Mackova, 2011), relating mainly to the use of nonliving biotic matter to adsorb metal ions through a variety of

complexing mechanisms (Montes *et al*., 2003; Gadd, 2009). These mechanisms include; ion exchange, chelation, physical adsorption (Volesky and Holan, 1995; Volesky, 2004) as well as mechanical entrapment of metal ions in interfibrillar and intrafibrillar capillaries and spaces of the structural polysaccharide network of biofilms (Volesky and Holan, 1995).

Most metal-using industrial activities have a metal disposal problem (Holan and Volesky, 1994; Braum, 2004; Naja and Volesky, 2010a; Kotrba, 2011). This problem is sometimes passed downstream to wastewater treatment facilities, where the metal ions tend to accumulate in the resultant sludges, often limiting options for the final disposal of the sewage sludges, (Naja and Volesky, 2010a). These situations constitute point-source pollution, making it possible to implement on-site, end-of-pipe treatment options (Banat *et al*., 1996). Three elements of particular concern to the electroplating industry are Cd^{2+} (Naja and Volesky, 2010a) Cu^{2+} and Zn^{2+}, all of which are suitable for biosorption (Atkinson *et al*., 1998; Naja and Volesky, 2006).

1.2 Characteristics of Heavy Metals

Heavy metals have been defined in terms of density, atomic weight and atomic number, although never by an authoritative body such as the International Union of Pure and Applied Chemistry (IUPAC) (Duffus, 2002; Naja and Volesky, 2010a). More recently the term “heavy metals” has become almost meaningless because of increased awareness of both the diversity and the similarities apparent among the metals, metalloids and semimetals (Duffus, 2002). However, since the term is still widely accepted in scientific literature it will be used in this document. Some generally accepted definitions of heavy metals are:

- A group of approximately 65 metallic elements, each with a density greater than 5 (Gadd, 1992a).
- Substances which form positive ions in solution and have a density 5 times greater than that of water (Naja and Volesky, 2010a).

Heavy metals are present in a variety of states in the environment; they are recalcitrant and have a tendency to be retained in biotic tissue (Abel, 1989; Baird, 1995; Naja and Volesky, 2010a). Because of their non-degradable nature, regardless of chemical form, when released into the environment heavy metals pose serious ecological risk (Kotrba, 2011).

While some heavy metals have no known interaction with metabolic functions, many are essential in trace quantities for plant and animal growth; but at high concentrations they can have toxic and carcinogenic effects (Gadd *et al*., 1988; Duffus, 2002; Nagajyoti *et al*., 2008; Naja and Volesky 2010a). Heavy metal pollution continues to be a significant environmental problem (Macek and Mackova, 2011).

Commonly proposed mechanisms of heavy metal toxicity include: blocking essential functional groups of bioorganic compounds, e.g. enzymes used in transport systems for essential nutrients; displacing and/or substituting essential cations in bioorganic compounds; disruption of cell membrane integrity (Collins and Stotzky, 1989; Gadd, 1990a; 1992a) inducing oxidative stress, and interfering with protein folding and function (Nies, 1999).

1.2.1 Characteristics of the Heavy Metals Investigated

1.2.1.1 Cadmium (Cd)

Considered a “toxic heavy metal” (Wang and Chen, 2006; Wang and Chen, 2009), cadmium (Cd) is a soft and ductile, silvery-white metal used primarily in the manufacture of batteries, paint and plastics. It is chemically similar to Zn^{2+} (DWAF, 1998; Naja and Volesky, 2010a), but is neither essential nor beneficial to plants and animals (Mhavi *et al*., 2005; Naja and Volesky, 2010a). Cd can be found in unpolluted water due to natural weathering processes, but usually in concentrations below 0.001mg.ℓ^{-1} (DWAF, 1998). However, a variety of industrial processes (e.g. electroplating, battery production and smelter operations) are responsible for the release of elevated levels of Cd into the environment (Naja and Volesky, 2010a). In areas where water has been in contact with galvanised surfaces, increased concentrations of Cd (up to 0.005mg.ℓ^{-1}) have been observed (Higham *et al.,* 1985; DWAF, 1998). If consumed at this concentration health effects such as gastroenteritis become apparent (DWAF, 1998).

1.2.1.2 Copper (Cu)

Copper is a typical toxic heavy metal with simple solution chemistry, existing predominantly as Cu^{2+} (cupric ion) in aqueous solution (Naja and Volesky, 2006; Wang and Chen, 2006; 2009). However, copper also occurs in nature in three other oxidation states: elemental copper Cu, Cu^{+} (cuprous ion) and rarely Cu^{3+} (Georgopoulos *et al*., 2001). The metal is orange in colour and is used extensively as a conductor of heat and electricity, as well as in pipes for domestic and industrial water reticulation. Copper is found in small quantities in the fatty covering of nerve fibre sheaths (DWAF, 1998). In unpolluted water sources, Cu concentrations are

typically below 0.1mg.ℓ^{-1}. Concentrations above 0.1mg.ℓ^{-1} generally occur when low pH waters corrode distribution pipes, usually giving the water a blue-green appearance (DWAF, 1998). Copper is also a common contaminant in mining and electroplating wastewaters (Naja and Volesky, 2006).

Below 1mg.ℓ^{-1} there are no noticeable effects caused by ingestion. However, at concentrations between 1mg.ℓ^{-1} and 2mg.ℓ^{-1} effects vary from slight to chronic in sensitive people and at concentrations above 2mg.ℓ^{-1} effects increase from chronic to acute, resulting in nausea and vomiting (DWAF, 1998).

1.2.1.3 Zinc (Zn)

As with many other heavy metals, Zn is an essential element for biological functioning. It is required for enzyme and hormone activity and for protein and nucleic acid synthesis (Wong *et al*., 1980). At high concentration Zn is considered to be toxic to humans (Wang and Chen, 2006; 2009). Upper limits for Zn uptake by males and females above 19 years of age is 40mg.day^{-1}, however adverse health effects, including vomiting and nausea, have been associated with Zn uptake of only 4mg.day^{-1} (Institute of Medicine, Food and Nutrition Board, 2001). Elevated Zn concentrations can be found in association with various industrial discharge waters, or leaching from Zn pipes or corrugated iron under acidic conditions (DWAF, 1998). Zinc is also released from fossil fuel combustion and during non-ferrous metal processing (Moore, 1991). Industrially zinc is commonly used in alloys, especially brass, and as a protective coating on iron and steel (Galvin, 1996; DWAF, 1998).

1.2.2 Environmental Discharge Limits for Heavy Metals in South Africa

In South Africa, the National Water Act, (Act 36 of 1998), instructs the Minister of Water Affairs and Forestry to regulate the discharge of domestic and industrial wastewaters into water resources (including the ocean). The limits pertaining to selected heavy metals are given in **Table 1.1**. General and special limits apply to wastewater discharges of up to 2000m^3 per day and are applied according to the receiving water resource as set out in the National Water Act.

Table 1. 1: Heavy metal concentration limit values applicable to domestic and industrial wastewaters discharged directly into water resources (Bailey, 2004)

Heavy Metal Determinant	General Limit (mg.ℓ^{-1})	Special Limit (mg.ℓ^{-1})
Dissolved Arsenic	0.02	0.01
Dissolved Cadmium	0.005	0.001
Dissolved Chromium (IV)	0.05	0.02
Dissolved Copper	0.01	0.002
Dissolved Iron	0.3	0.3
Dissolved Lead	0.01	0.0006
Dissolved Manganese	0.1	0.1
Mercury and its compounds	0.005	0.001
Dissolved Selenium	0.02	0.02
Dissolved Zinc	0.1	0.04
Boron	1	0.5

It is not common for industries to discharge directly to water resources. These discharges are generally to municipal sewer systems, and are therefore regulated by the Water Services Act (Act 108 of 1997) (Bailey, 2004). Therefore, as a result of point source discharges of treated effluent from municipal wastewater treatment operations, municipalities are regarded as significant wastewater stream dischargers. Discharge of treated effluent by the municipality to any water resource (including the ocean via sea outfall pipelines) is regulated and controlled via an effluent discharge permit from the South African National Department of Water Affairs and Forestry (DWAF) (Bailey, 2004).

The operation performance, and therefore resultant final treated effluent, is dependent on a number of criteria. Including, the nature of the activities in the wastewater treatment works catchment area and the concentration of contaminants in the received wastewater. Therefore, in order to ensure effective performance of the municipal sewerage system, municipalities apply and police bylaws outlining what may and may not be discharged into the municipal sewage systems (Bailey, 2004).

Tables 1.2 and 1.3 show permissible metal discharge concentrations as set out in the eThekwini Municipality Sewage Disposal Bylaws (Durban Metro, 1999).

Table 1. 2: Acceptance of trade effluent into eThekwini sewage disposal systems

Heavy Metal Determinant	**Large Works (>25Mℓ.day^{-1}) (mg.ℓ$^{-1}$)**	**Small Works (<25Mℓ.day^{-1}) (mg.ℓ$^{-1}$)**
Copper (as Cu)	50	5
Nickel (as Ni)	50	5
Zinc (as Zn)	50	5
Iron (as Fe)	50	5
Boron (as B)	50	5
Selenium (as Se)	50	5
Manganese (as Mn)	50	5
Lead (as Pb)	20	5
Cadmium (as Cd)	20	5
Mercury (as Hg)	1	1
Chrome (as Cr)	20	5
Arsenic (as As)	20	5
Titanium (as Ti)	20	5
Cobalt (as Co)	20	5
Total Metals	**100**	**20**

Table 1. 3: Acceptance of trade effluent for discharge into sea outfalls

Heavy Metal Determinant	**(mg.ℓ$^{-1}$)**
Arsenic (as As)	5
Cadmium (as Cd)	1.5
Total chrome (as Cr)	3
Copper (as Cu)	3
Lead (as Pb)	5
Mercury (as Hg)	0.05
Nickel (as Ni)	10
Zinc (as Zn)	20

1.3 Non-Biological Treatments of Heavy Metal Contaminated Industrial Wastewaters

Traditional treatment of water containing heavy metals often involves a chemical approach, such as ion exchange, oxidation-reduction or precipitation. However, these methodologies are not without significant disadvantages such as generally being considered either ineffective or uneconomical in treating aqueous solutions with metal concentrations below about 5mg.ℓ^{-1} (Shumate II *et al.,* 1978; Sag and Kutsal, 1995; Ahalya *et al.,* 2003). However, some methods, such as ion exchange using man-made synthetic organic resins, have been shown to reduce metal content in wastewaters to µg.ℓ^{-1} levels (Kotrba, 2011).

1.3.1 Conventional Methods for the Treatment of Heavy Metal Contaminated Industrial Wastewaters

Two major advantages presented by non-biological (traditional) treatments of heavy metal contaminated wastewaters are some ion selectivity and tolerance to variations in pH. This is true in the case of electrodialysis and membrane technologies such as reverse osmosis and ultrafiltration (Kotrba, 2011). A brief summary of some conventional non-biological heavy metal treatment methods is presented in **Table 1.4**.

Table 1.4: Some conventional methods of treating heavy metal contaminated wastewaters

Treatment	Description	Reference
Reverse osmosis	Pressure is used to force a solution through a selectively permeable membrane, retaining the solute on one side and allowing the pure solvent to pass to the other side.	Rostker, 2000; Ahalya *et al*., 2003; Naja and Volesky, 2010b
Electrowinning	Also called electroextraction, uses electrolysis to drive otherwise non-spontaneous chemical reactions in order to remove metal ions from solution. A current is passed from an inert anode through the metal solution so that the metal is extracted as it is deposited in an electroplating process onto the cathode.	Cowan, 1998; Naja and Volesky, 2010b; Kotrba, 2011
Evaporation	This is not a treatment strategy *per se*, but may perform a vital role in the treatment process by concentrating the contaminants, rather than removing them. Evaporation may take place under vacuum or atmospheric conditions.	Cowan, 1998
Chemical oxidation and reduction	The transfer of electrons between molecules or ions in order to alter the properties of the desired reactant (e.g. solubility).	Belhateche, 1995; Ahalya *et al*., 2003; Naja and Volesky, 2010b
Electrodialysis	Ions are separated by applying an electrical potential across an ionic solution and using semi-permeable ion-selective membranes to create cells of concentrated and dilute salts.	Ahalya *et al*., 2003
Ion Exchange	Ions held by electrostatic forces on an ion exchange resin are substituted for metal ions. This technology is only used for dilute solutions.	Ahalya *et al*., 2003;Naja and Volesky, 2010b; Kotrba, 2011
Chelation	This can be used to either adsorb or precipitate metals in the presence of organic ligands that form coordination complexes with metals.	Xu and Xu, 2008; Naja and Volesky, 2010b; Naja and Volesky, 2011

1.3.1.1 Metal Precipitation Treatments

According to Kotrba (2011), precipitation through alkalisation (usually with lime) is generally considered to be cost effective and is still the most commonly used treatment for heavy metal contaminated wastewater. Metals are commonly precipitated as insoluble metal-hydroxides from solution using coagulants (e.g. alum, lime, iron salts and organic polymers) (Harris and Ramelow, 1990; Ahalya *et al*., 2003; Naja and Volesky, 2010b; Kotrba, 2011).The most commonly used reagents for the formation of insoluble metal hydroxide precipitates on a commercial scale are lime and caustic soda (NaOH), depending on the application (Naja and Volesky, 2010b; Kotrba, 2011). These traditional technologies often require expensive equipment and monitoring systems, and do not always achieve complete removal of the metals (Harris and Ramelow, 1990; Kotrba, 2011). Furthermore, precipitation is limited by its specificity because metal-hydroxides precipitate at various pH values resulting in no single ideal value for a mixed-metal solution (Kanluen and Amer, 2001). In reality, a typical wastewater contains a variety of other substances such as: oil, grease, chelating/complexing agents and dissolved solids, which might interfere with precipitation (Kanluen and Amer, 2001).

1.3.1.2 Metal Adsorption Treatments

Adsorption is the adhesion of atoms, ions, or molecules from a gas, liquid, or dissolved solid to a surface (Brandt *et al*., 1993). This process creates a film of the adsorbate on the surface of the adsorbent. It differs from absorption, in which the absorbate permeates the absorbent (Cussler, 1997). Adsorption is considered to be a viable technique for the treatment of heavy metal contaminated wastewaters.

Activated carbon has for decades been widely used as an adsorbent in industry (Hassler,1963; Mattson and Mark, 1971; Meena *et al*., 2004). Activated carbon's adsorption capacity is a function of the pore structure and chemical nature of the carbon surface and a consequence of its preparation conditions (Hassler, 1974). Meena *et al*. (2004) reported 99.8% removal of a 3mg.ℓ^{-1} mercury solution when using activated carbon and up to 99.7% removal when treating the same solution with weathered coal.

More recently, hydroxyapatite, has been proposed as an alternative adsorbent to activated carbon for the commercial treatment of heavy metal contaminated wastewaters (Qian *et al*., 2008; Wang *et al*., 2009). Chen *et al*. (2010) reported maximum Cu^{2+} and Cd^{2+} adsorption to hydroxyapatite of 73.5mmol.kg^{-1} and 66.7mmol.kg^{-1} respectively. Manu *et al*. (2009) used the Sips isotherms to model Cu^{2+} adsorption by silica- and amino-functionalised silica gels. The monolayer copper adsorption capacities for the silica gels ranged between 0.515mmol.g^{-1} and 0.55mmol.g^{-1}; and a maximum of 1.05mmol.g^{-1} by the amino functionalised silica gels.

An evolution of adsorption strategies is biosorption. Gadd (2009) defines biosorption as "*any system where a sorbate (e.g. an atom, molecule, or molecular ion) interacts with a biosorbent (i.e. a solid surface of a biological matrix) resulting in an accumulation at the sorbate-biosorbent interface and therefore a reduction in the solution sorbate concentration*".

1.4 Biological Treatment of Heavy Metal Contaminated Waters

Biological treatment of heavy metal contaminated waters has captured the interest of scientists for decades because of its apparent advantages for use in developing countries. This technology is generally referred to in the literature as simple and low cost, when compared with most conventional treatments (Wood, 1992; Hobson and Poole, 1998; Ahalya *et al*., 2003; Vijayaraghavan and Yun, 2008; Gadd 2009; Macek and Mackova, 2011). However, these claims tend not to be substantiated either through cost or engineering comparisons.

The key disadvantages of biosorption are as follows:

a. Rapid saturation of biomass requiring desorption prior to continued use (Das, 2008). (Saturation/breakthrough points are typically reported in a matter of minutes).
b. There is no user control over the potential for biological alteration of the metal valence state (Das, 2008).

Biological treatment is often associated with bioremediation, where an undesired contaminant is degraded into low toxicity products. However, because metals cannot be biologically broken down into smaller constituents (Baird, 1995), they are accumulated into or onto organic matter through a variety of chemical and biological mechanisms. This is known as biosorption (Gadd and Griffiths, 1978; Gadd, 2009).The ultimate goal of biological treatment systems (for heavy metals) is providing economical, efficient systems capable of removing metal ions to below 5mg.ℓ^{-1}.

Biosorption is a rapid, metabolism-independent process resulting from mechanisms such as ion exchange, coordination, complexation and chelation and may occur in living and nonliving biologically derived materials (Duncan and Brady, 1992; Hutchins *et al*., 1986; Cobbett, 2000; Ahalya *et al.*, 2003; Ahalya *et al.*, 2007; Naja and Volesky, 2010b). Metabolic processes may affect the sorbate bioavailability and the physico-chemical biosorption mechanisms (Gadd, 2009).

Heavy metal biosorption and accumulation vary according to a number of parameters. For example, whether a mixed-metal ion solution or single ion solution is used, the specific metal used (Mhavi *et al*., 2005; Kotrba, 2011), the amount of adsorbent and the initial concentration of the metals in solution (Mhavi *et al*., 2005). Therefore, the volume of metal solution, in relation to the concentration, will also have a bearing on the total metal adsorbed (Gadd, 2009). Another factor for consideration is the nature of the biosorbent. Practically all biological material has an affinity for metal species and microbes in particular can interact with all elements in the periodic table (including actinides, lanthanides and radionucleotides) (Macek and Mackova, 2011).

The use of biological materials as adsorbents for the accumulation of metal ions is well documented due to the wide variety of suitable, readily obtainable and inexpensive biological materials available (Pagnanelli *et al*., 2002; Keskinkan *et al*., 2003; Gadd, 2009; Kotrba, 2011; Yun *et al*., 2011). In particular, considerable work has been undertaken on the use of organic waste products for metal ion removal from contaminated wastewater. These include: olive mill residues (Pagnanelli *et al*., 2002); *Myriophyllum spicatum* stems (Keskinkan *et al*., 2003); chick pea and pigeon pea husks (Ahalya *et al*., 2005; 2007); *Ulmas* leaves and their ashes (Mhavi *et al*.,

2005); *Casurina equisetifola* bark (Mohan and Sumitha, 2008) and, significantly, pine bark (Martin-Dupont *et al*., 2004; 2006; Oh and Tshabalala, 2007; Mun *et al.,* 2009).

1.4.1 Biosorption Capacity of Sorbents

Biosorption capacity is a measure of the maximum amount of a particular sorbate that may be sorbed by a given quantity of sorbent and is usually expressed as either $mmol.mol^{-1}$ or $mg.g^{-1}$ (Mhavi *et al*., 2008; Feng *et al.,* 2009; Pagnanelli, 2011). Some natural materials may demonstrate specific metal-binding affinities while others may be considered broad-spectrum biosorbents due to their non-specific metal binding tendencies (Naja and Volesky, 2011). Therefore, the biosorption capacity of a sorbent may be specific for a particular sorbate. The biosorption capacity (q_e) of a sorbent for a particular sorbate may be calculated from experimentally determined data by the following equation (Feng *et al.,* 2009):

$$q_e = \frac{(\rho_0 - \rho_e)V}{m}$$

(**Equation 1.1**)

Where $\boldsymbol{\rho_0}$ and $\boldsymbol{\rho_e}$ are the initial and equilibrium M^{2+} ion concentrations respectively ($mg.\ell^{-1}$); **V** is the volume of the solution (ℓ) and **m** is the amount of sorbent (g).

There is increasing acceptance that biosorption involves a high degree of cation exchange (Naja and Volesky, 2011) and, therefore, may be modelled mathematically. The literature suggests that biosorption behaviour usually follows adsorption isotherm-type behaviour and, therefore, the models may contain a variety of isotherm equations (Viraraghavan and Srinivasan, 2011). The isotherm models most commonly applied to biosorption are those of Langmuir, Freundlich, and Brunauer-Emmett-Teller (BET) (Viraraghavan and Srinivasan, 2011) and are given as described by Weber (1972) in **Equations 1.2 – 1.4**.

The Langmuir Equation:

$$q = \frac{q_{max} b C_e}{1 + b C_e}$$

(**Equation 1.2**)

Where q = the milligrams of metal sorbed per gram of biomass; q_{max} = the maximum amount of metal ion sorbed per gram of biomass; b = ratio of adsorption and desorption rates; C*e* = the equilibrium concentration of metal ion in solution after biosorption has occurred.

The Freundlich Equation:

$$Q = K C_e^{1/n}$$

(**Equation 1.3**)

Where Q = the metal uptake capacity of the biomass; *K* = the biosorption equilibrium constant indicative of biosorptive uptake capacity; Ce = the equilibrium metal ion concentration.

The Brunauer-Emmett-Teller (BET) Model:

$$Ce/(Cs - Ce) = 1/BQ^{o} + [(B - 1)/BQ^{o}](Ce/Cs)$$

(**Equation 1.4**)

Where Cs = the saturation concentration of the metal ion; Q^{o} = the amount adsorbed per unit weight of biomass for monolayer biosorption; B = constant relating to the energy of interaction with the surface.

These mathematical relationships basically do not reflect the physico-chemical underlying principles of the sorption process which, in most cases, may not even be well understood. For all practical purposes they are just mathematical models capable of sometimes describing the experimentally observed relationship between sorbent and sorbate. None of these models offer any important clues as to the sorption mechanism nor could they be sensitive to external process variables (such as pH, ionic strength, etc.) (Volesky, 2003).

While the Langmuir adsorption model is valid for a single-layer adsorption, the BET model represents sorption isotherms reflecting apparent multi-layer adsorption. Both equations are limited by the assumption of uniform energies of adsorption on the surface. The Freundlich relationship is an empirical equation. It does not indicate a finite uptake capacity of the sorbent and can thus only be reasonably applied in the low to intermediate concentration ranges (Volesky, 2003).

Biosorption capacities, both measured and modelled, for various biological materials as they relate to Cu^{2+}, Zn^{2+} and Cd^{2+} are given in **Tables 1.5 – 1.7**. [as adapted from Vijayaraghavan and Yun, 2008; Naja, Volesky and Murphy, 2010 and Yun *et al*., 2011). The biosorption capacity of a biological material for a particular sorbate, may also be determined by modelling biosorption kinetics (Pagnanelli, 2011)

Table 1. 5: Biosorption of copper by various bacterial species. (Adapted from Vijayaraghavan and Yun, 2008; Naja, Volesky and Murphy, 2010 and Yun *et al*., 2011)

Organism	pH	Temp (°C)	Operating Conditions	Uptake ($mg.g^{-1}$)	Uptake ($mmol.g^{-1}$)	Reference
Bacillus sp. (ATS-1)	5.0	25	M=2g.ℓ^{-1}, t_{eq} =2h	16.3 (E)	0.26	Tunali *et al*., 2006
Bacillus subtilis IAM 1026	5.0	25	M=0.5g.ℓ^{-1}, t_{eq} =1 h	20.8 (L)	0.33	Nakajima *et al*., 2001
Enterobacter sp. J1	5.0	25	t_{eq} =24 h	32.5 (L)	0.51	Lu *et al*., 2006
Pseudomonas cepacia	7.0	30	NA	65.3 (L)	1.03	Savvaidis *et al*., 2003
Pseudomonas putida	6.0	NA	NA	6.6 (L)	0.10	Pardo *et al*., 2003
Pseudomonas putida	5.5	30	M=1g.ℓ^{-1}, t_{eq} =24 h	96.9 (L)	1.52	Pardo *et al*., 2003
Pseudomonas putida CZ1	4.5	30	M=1 g.ℓ^{-1}, t_{eq} =24 h	15.8 (L)	0.25	Chen *et al*., 2005
Pseudomonas stutzeri IAM 12097	5.0	25	M=0.5g.ℓ^{-1}, t_{eq} =1 h	22.9 (L)	0.36	Nakajima *et al*., 2001
Sphaerotilus natans	6.0	NA	M=3g.ℓ^{-1}; t_{eq} =0.5 h	60 (E)	0.94	Beolchini *et al*., 2006
Sphaerotilus natans [b]	5.5	30	NA	5.4 (L)	0.08	Beolchini *et al*., 2006
Streptomyces coelicolor	5.0	25	M=1g.ℓ^{-1}; t_{eq} =8 h	66.7 (L)	1.05	Öztürk *et al*., 2004
Thiobacillus ferrooxidans [a]	6.0	37	M=0.2g.ℓ^{-1}; t_{eq} =2 h	198.5 (L)	3.12	Ruiz-Manriquez *et al*., 1997
Bacillus sp. (ATS-1)	5.0	25	M=2g.ℓ^{-1}; t_{eq} =2 h	16.3 (E)		Tunali *et al*., 2006
Enterobacter sp. J1	5.0	25	t_{eq} =24 h	32.5 (L)		Lu *et al*., 2006
Geobacillus thermoleovorans sub.sp. *stromboliensis*	5.0	60	M=2.5g.ℓ^{-1}; t_{eq} =1 h	41.5 (L)		Ozdemir *et al*., 2009
Geobacillus toebii sub.sp. *decanicus*	4.0	60	M=2.5g.ℓ^{-1}; t_{eq} =1 h	48.5 (L)		Ozdemir *et al*., 2009

(E) = Experimental uptake
(L) = Uptake predicted by the Langmuir model;
M = Biomass dosage,
t_{eq} = Equilibrium time,
NA = Not available.
a Chemically modified.
b Immobilised.

Table 1. 6: Biosorption of cadmium by various bacterial species. (Adapted from Vijayaraghavan and Yun 2008, Naja, Volesky and Murphy, 2010 and Yun et al., 2011)

Organism	pH	Temp (°C)	Operating Conditions	Uptake ($mg.g^{-1}$)	Uptake ($mmol.g^{-1}$)	Reference
Aeromonas caviae	7.0	20	M=1g.ℓ^{-1}; t_{eq} =2 h	155.3 (L)	1.38	Loukidou *et al*., 2004
Bacillus circulans	7.0	30	M=0.5g.ℓ^{-1}; t_{eq} =2 h	26.5 (E)	0.24	Yilmaz and Ensari, 2005
Enterobacter sp. J1	6.0	25	t_{eq} =24 h	46.2 (L)	0.41	Lu *et al*., 2006
Pseudomonas aeruginosa PU21	6.0	NA	M=1-2g.ℓ^{-1}; t_{eq}=24 h	42.4 (L)	0.38	Chang *et al*., 1997
Pseudomonas putida	6.0	NA	NA	8.0 (L)	0.07	Pardo *et al*., 2003
Pseudomonas sp.	7.0	NA	M=1g.ℓ^{-1}, t_{eq} =1.5 h	278.0 (L)	2.47	Ziagova *et al*., 2007
Staphylococcus xylosus	6.0	NA	M=1g.ℓ^{-1}, t_{eq} =1.5 h	250.0 (L)	2.22	Ziagova *et al*., 2007
Streptomyces pimprina [a]	5.0	NA	t_{eq} =1 h	30.4 (L)	0.27	Puranik *et al*., 1995
Streptomyces rimosus[a]	8.0	NA	M=3g.ℓ^{-1}	64.9 (L)	0.58	Selatnia *et al*., 2004

(E) = Experimental uptake
(L) = Uptake predicted by the Langmuir model;
M = Biomass dosage,
t_{eq} = Equilibrium time,
NA = Not available.
a Chemically modified.

Table 1. 7: Biosorption of zinc by various bacterial species. (Adapted from Vijayaraghavan and Yun 2008, Naja, Volesky and Murphy, 2010 and Yun et al., 2011)

Organism	pH	Temp (°C)	Operating Conditions	Uptake ($mg.g^{-1}$)	Uptake ($mmol.g^{-1}$)	Reference
Aphanothece halophytica	6.5	30	M=0.2g.ℓ^{-1}, t_{eq} =1 h	133.0 (L)	2.03	Incharoensakdi and Kitjaharn, 2002
Pseudomonas putida	7.0	NA	NA	6.9 (L)	0.11	Pardo *et al*., 2003
Pseudomonas putida CZ1	5.0	30	M=1g.ℓ^{-1}; t_{eq} =24 h	17.7 (L)	0.27	Chen *et al*., 2005
Streptomyces rimosus	7.5	20	M=3g.ℓ^{-1}	30.0 (L)	0.46	Mameri *et al*., 1999
Streptomyces rimosus[a]	7.5	20	M=3g.ℓ^{-1}	80.0 (L)	1.22	Mameri *et al*., 1999
Streptoverticillium cinnamoneum [a]	5.5	28 ±3	M=2g.ℓ^{-1}, t_{eq} =0.5 h	21.3 (E)	0.33	Puranik and Paknikar, 1997
Thiobacillus ferrooxidans [a]	6.0	25	M=0.2g.ℓ^{-1}, t_{eq} =2 h	82.6 (L)	1.26	Celaya *et al*., 2000

(E) = Experimental uptake
(L) = Uptake predicted by the Langmuir model;
M = Biomass dosage,
t_{eq} = Equilibrium time,
NA = Not available.
a Chemically modified.

1.4.2 Metabolism-Independent Mechanisms of Metal Biosorption

Metabolism-independent accumulation occurs as a result of physico-chemical interaction between the metal ions and the functional groups present on the microbial cell surface and, therefore, does not require the presence of metabolically functional organisms (Ahalya *et al.,* 2003; Naja and Volesky, 2006; Gadd, 2009). Although a variety of metabolism-independent mechanisms can account for metal accumulation, these seldom exist in isolation; achieved sorption is usually attributed to a combination of mechanisms (Macek and Mackova, 2011). Ion-exchange, however, appears to be the dominant mechanism (Naja and Volesky, 2011). However, metabolism-independent biosorption is largely governed by the effects of environmental conditions such as pH and temperature on the state and effectiveness of the binding sites (Naja and Volesky, 2011).

1.4.2.1 Biosorption through Adsorption

Adsorption involves the bonding or physical adherence of ions or molecules onto the surface of another molecule (Gadd, 2009). Two types of adsorption can occur, i.e., physical and chemical adsorption. Physical adsorption (physisorption) occurs due to van der Waals interactions between a sorbate and substrate (Ahalya *et al*., 2003). These bonds are weak and, therefore, easily reversed. Chemical adsorption (chemisorption) usually occurs as a result of stronger covalent bonds with the ions occupying sites that maximise their coordination with the substrate (Atkins, 1998).

Under normal conditions cell walls have an overall negative charge brought about by the presence of functional groups such as –SH, -OH and -COOH on their surface

(Gadd, 2009). This net negative charge ensures that the cell wall components act as chelators. Anionic polymers, e.g. teichoic or teichuronic acid, and other cell wall constituents, such as peptidoglycan polysaccharides or polymer matrices, derive their negative surface charge from deprotonated organic ligands, e.g. $RCOO^-$, S^- and RO^- (Doyle, 1989; Birch and Bachofen, 1990; Gadd, 2009). These functional groups differ in their affinity and specificity for metal binding (Sahoo *et al*., 1992).

Because adsorption is a function of cell surface characteristics, recently there has been an increased focus on increasing or activating existing binding sites on cell surfaces, in order to enhance biosorption capacity (Yun *et al*., 2011). Methods for enhancing biosorption capacity include chemical modification of biosorbents, which in recent years has become extremely prevalent (Yun *et al*., 2011; Kotrba *et al*., 2011b); chemical pretreatments, which may enhance the action of existing binding sites or convert other, less important functional groups into active binding sites (Yun *et al*., 2011). Such chemical modification of biosorbents, usually through pretreatment of the biomass, involved mainly the use of acid, alkali, ethanol or acetone (Goksungur *et al*., 2005; Vijayaraghavan and Yun, 2007). These processes may increase biosorption capacity several fold (Yun *et al*., 2011). An emerging strategy for enhancing metal biosorption is cell surface engineering, whereby novel biosorbents are constructed by anchoring various functional proteins and peptides on cell surfaces (Kuroda and Ueda, 2011; Kotrba *et al*., 2011b)

1.4.2.2 Biosorption through Absorption

Absorption is *"the incorporation of a substance in one state into another of a different state"* (Gadd, 2009). This usually implies the entry of a material though the surface to an inner matrix and may occur in both metabolically active and inactive cells (Gadd and White, 1986; McHale and McHale, 1994; Ahalya *et al*., 2003; Naja and Volesky,

2006). Metal ions may be absorbed into cells by diffusion through the cell membrane (Gadd *et al*., 1988; Ahalya *et al*., 2003).

1.4.2.3 Biosorption by Cellular Components

Several nonliving surface components of living cellular structures are capable of metal ion accumulation. However, they are not present in metabolically inactive cells. Heavy metal resistance in a particular microorganism may result from the presence of one, or a combination, of these substances (Naz *et al*., 2005).

1.4.2.3.a Extracellular Polymers

Blenkinsopp and Costerton (1991) stated that sessile cells are more resistant to adverse environmental conditions than planktonic cells. This is primarily due to the action of the exopolysaccharides (EPS) of the microbial glycocalyx (matrices) that have an affinity for metallic cations, therefore acting as a molecular sieve. This is supported by Watnick and Kolter (2000) who state that biofilm-associated cells are more resistant to many toxic substances than free living cells, due to protection offered by reduced diffusion into the biofilm and interaction with biofilm specific substances such as EPS. The EPS layer also contains a variety of other constituents including: proteins, glycoproteins, glycolipids and, often, extracellular DNA which may interfere with and, therefore, reduce the effects of toxic substances (Flemming *et al.,* 2007).

1.4.2.3.b Metallothioneins

Metallothioneins are small cysteine-rich polypeptides capable of binding both essential and non-essential metals (Gadd, 1992b; Courbot *et al.,* 2004). Copper metallothionein (Cu-Mt produced by *Saccharomyces cerevisiae*.) is responsible for the mediation of Cu resistance (Gadd, 1992b). Research shows that metallothioneins tend to be nonspecific and are, therefore, capable of binding several metals

including: Cd, Zn, Co and Au (Courbot *et al.,* 2004). The production of exudates in microbial cultures in response to metal exposure has been documented (Birch and Bachofen, 1990; Courbot *et al.,* 2004) and some of these exudates contain metallothioneins (Courbot *et al.,* 2004).

1.4.2.3.c Phytochelatins

Similar to the metallothioneins, the phytochelatins are short cysteine-rich γ–glutamyl peptides found in certain fungi and yeasts, as well as plants and algae (Gadd, 1992b). Phytochelatin synthesis is activated by the presence of metal ions (Cobbett, 2000). Ha *et al. (*1999) found that plant phytochelatins played an important role in the detoxification of Cd^{2+} and AsO_4^{3-}, a minor role in the detoxification of Cu^{2+}, Hg^{2+} and Ag^{2+} and were ineffective against Zn^{2+} and Ni^{2+}. However, the evidence to suggest that they are effective in detoxifying ions other than Cd^{2+} and arsenate is scarce (Cobbett, 2000). Genetic engineering of microbial cells for increased phytochelatin production has been shown to improve Cd accumulation by up to 25 times (Kang *et al.,* 2007).

1.4.2.3.d Siderophores

Siderophores are strong Fe^{3+} coordination compounds that are excreted under conditions of poor iron availability (Gadd, 1992b; 2004). These low molecular weight compounds are virtually specific for Fe^{3+} but can also complex a variety of other metals ions, e.g. Ni^{2+}, Al^{3+}, Cu^{2+}, Mn^{2+} and Ca^{2+} (Brierly *et al.*, 1986; Gadd, 1988; 2004).

Siderophores are produced by microbial cells specifically for improved uptake of metal ions, by enhancing their availability through complexation of insoluble ions,

thereby forming a soluble complex, which may be subsequently transported into the cell (Birch and Bachofen, 1990; Gadd, 1992b; 2004).

1.4.3 Metabolism-Dependent Mechanisms of Metal Biosorption

In addition to the biosorption mechanisms previously described, living cells may continuously produce and excrete various metabolites that directly influence their immediate surroundings. Therefore, changes in microenvironments around some cells may provide conditions that support further metal removal. Actively metabolizing cells have the potential to bind metal cations by physico-chemical interactions such as chelation (Gadd, 1990b; 2009) and then engage a transporter system, allowing the microorganisms to internalize or absorb the metal ions (Hetzer *et al*., 2006; Hudek *et al*., 2009). The methods and mechanisms of metal accumulation in microorganisms are species specific, varying between different microorganisms (Gadd, 1988; 1990b; Hetzer *et al*., 2006), and are often associated with an active defense mechanism (Ahalya *et al*., 2003). Metabolism-dependent intracellular metal accumulation and related processes are mostly irreversible and, therefore, require cellular lysis if metal recovery is intended (Gadd, 1990b).

1.4.3.1 Active Transport of Metal Ions across the Cell Membrane

In some cases adsorption of metal ions is followed by active transport of the adsorbed ions across the cell membrane. This usually brings about the accumulation of essential metal ions from the external environment, but is also often the route of uptake for non-essential or toxic metal ions (Beveridge and Doyle, 1989; Birch and Bachofen, 1990; Ahalya *et al*., 2003; Hudek *et al*., 2009). The extent of internalisation of metal ions varies between microorganisms (Hudek *et al*., 2009). A known

mechanism for this internalisation of certain metal ions by bacteria, archea and baker's yeast is the Mn^{2+} transport system (Belliveau *et al*., 1987). Cd^{2+}, Zn^{2+} and Mn^{2+} are all like-charged, similar size ions and are quickly and efficiently accumulated by the CorA Mn^{2+} uptake system. Conversely, arsenate transport is via the Pit-phosphate system while chromate enters the cells via the sulphate uptake systems (Nies, 1999). Microbial systems dedicated to the uptake of Zn^{2+} have also been studied in bacteria and filamentous fungi (Hudek *et al*., 2009). Active transport of metal ions across a cell membrane is generally a slower process than adsorption and may often be a rate limiting step (Hudek *et al*., 2009). However, this process usually results in increased metal accumulation compared with metabolism-independent biosorption (Gadd, 1990a; Hudek *et al*., 2009).

1.4.3.2 Entrapment of Metal Ions by Cellular Components

Absorbed metal ions can be trapped and removed from solution by intracellular polymers (Wood and Wang, 1983; Hudek *et al*., 2009). *Escherichia coli* has been shown to synthesize inducible Cd^{2+} binding proteins (Gadd, 1988; 1990b). The bound metal ions may then be incorporated into biochemical pathways, compartmentalised within specific organelles, or converted to more innocuous forms through further binding or precipitation (Ford and Mitchell, 1992). Polyphosphate granules have been shown to sequester intracellular metal ions, including Zn^{2+} (Andrade *et al*., 2004).

1.5 Environmental Factors Affecting Bioaccumulation

The surrounding environment plays a significant part in biosorption of metals, because environmental conditions affect the reaction chemistry of the metal ions and the cells' receptive sites (Gadd, 1986; 2009). Furthermore, because metal bioaccumulation may occur as a result of either metabolically driven or metabolism-independent interactions, any environmental factors that affect the chemical speciation, mobility or other physico-chemical properties of a metal, will influence its bioavailability and toxicity. These include pH, oxidation-reduction potential, hydrolysis and complexation (Babich and Stotzky, 1978a; Collins and Stotzky, 1989; Ahalya *et al*., 2003; Naja and Volesky, 2010a). Therefore, microorganisms growing in the presence of high metal concentrations may be able to do so because of environmental factors diminishing or limiting the metals' toxic effects (Gadd, 1992d).

1.5.1 Effect of pH on Bioaccumulation

The pH of an environment can affect the toxicity of metals to microorganisms by affecting the physiological state and biochemical activities of the receiving organisms (Collins and Stotzky, 1989) through affecting the protonation of various functional groups (Feng *et al*., 2009). The chemical speciation of the metal ions and their complexation with various environmental constituents may also be influenced by their environment's pH (Collins and Stotzky, 1989). Although some biosorption activity has been shown to be independent of pH (Garnham, 1997), this generally is still possibly the most important physico-chemical factor affecting toxicity (Ahalya *et al*., 2003; *Wang et al*., 2007; Gadd, 2009). Changes in metal ion toxicity can occur in a variety of ways by affecting net charges and valence states or inter-ion competition and solubility (Gadd, 2009).

Whether functional groups on the cell surface are protonated or deprotonated is strongly influenced by the pH of the environment and, therefore, the affinity of the cell surface for metal ions will be affected by changes in pH (Collins and Stotzky, 1989). However, microorganisms have a tendency to alter the pH of the environment as a consequence of their growth. Therefore it is often necessary in industrial microbiological processes to buffer the culture (Brock and Madigan, 1991).

An acidic pH causes an increase in the concentration of free hydrogen ions in the medium. These free hydrogen ions compete effectively with heavy metal ions for attachment to microbial cells due to their small ionic radius and the establishment of a proton gradient across the cell membrane, hence improving bioavailability (Babich and Stotzky, 1980; Bux *et al*., 1997). Conversely, an alkaline pH gives rise to an abundance of hydroxyl ions which tend to promote the formation of insoluble, multiple-hydroxylated heavy metal species, thereby reducing toxicity (Hahne and Kroontje, 1973; Said and Lewis, 1991). The specific pH at which these hydroxylated species form varies among metals and the different hydroxylated forms of a metal also have different toxicities (Collins and Stotzky, 1989).

Bioavailability generally increases with decreasing pH due to the presence of phosphoric, sulphuric and carbonic acids which solubilise bound metals (Macek and Mackova, 2011). Roane *et al*. (2005) state that metal solubility is increased in soil surface layers where the effects of moisture, plant exudates and microbial activity lower the environmental pH. However, Hetzer *et al.* (2006), Wang *et al*. (2007) and Feng *et al*. (2009) found that in general, heavy metal biosorption increases with an increase in pH. The correlation between increased biosorption with increased pH is attributed to decreased competition between H^+ ions and metal ions (such as: Cu^{2+}, Zn^{2+} and Cd^{2+}) (Hetzer *et al.,* 2006; Gadd, 2009) or a change in the electrostatic

properties of the sorbent surface (Feng *et al*., 2009). Bux *et al. (*1997) stated that below pH 4 biosorption may be negligible. Hetzer *et al.* (2006) found that maximum biosorption of Cd^{2+} by thermophilic microorganisms occurred in the pH range 3.5 - 5.5, depending on the biomass used. Feng *et al*. (2009) found that sorption of Cu^{2+} to orange peel, that had been chemically modified with sodium hydroxide and calcium chloride increased with increasing pH, maximum sorption (95%) occurring at pH values between pH 4.5 and pH 6.0. However, Boyanov *et al. (*2003) found that *Bacillus subtilis* is capable of adsorbing Cd^{2+} at pH 3.4 and attributed this to chelation by phosphoryl ligands, whereas at higher pH (5.0 – 7.8) carboxylic ligands were the dominant functional moieties.

1.5.2 Effect of Temperature on Bioaccumulation

Temperature is one of the most important environmental factors affecting the growth of microorganisms. Within its specific temperature range, a microorganism's function and metabolism is directly related to temperature (Brock and Madigan, 1991). Martinez and Casadevall (2007) found that temperatures outside the normal growth range for *Cryptococcus neoformans* affected the ability of the organism to form a biofilm. The relationship between temperature and heavy metal toxicity is thought to be the result of the former's effect on the physiological state of the microbes, rather than on the chemical speciation or bioavailability of the metals (Collins and Stotzky, 1989). Most research relating to heavy metal biosorption has been done on mesophilic microorganisms, but the cell walls of thermophilic microorganisms differ from most other microorganisms and, therefore, will interact differently with heavy metal ions. This should be taken into account when comparing heavy metal toxicity levels in different types of microorganisms (Hetzer *et al.,* 2006).

Babich and Stotzky (1978b) found Zn^{2+} to be more toxic to the growth of *Aspergillus niger* at 25^{o}C than at 37^{o}C. This was attributed to reduced physiological activity of the fungus at the lower temperature. While metabolism-dependent uptake of the metal would be favoured at the higher temperature and inhibited at the lower temperature, excessively high temperatures could compromise the functioning and integrity of the cell membranes and, therefore, hinder compartmentalisation of metal ions, leading to increased toxicity and reduced bioaccumulation (Duncan and Brady, 1992; Gadd, 2009).

1.5.3 Oxidation-Reduction (Redox) Potential

The redox potential (E_h) of an environment is determined by the availability of electrons. A negative E_h favours reduction, while oxidation is favoured in environments with a positive E_h. This can affect the availability of heavy metals by altering their valence state as well as that of other matter in the surrounding environment (Babich and Stotzky, 1980). Reducing environments tend to favour metal-sulphide precipitation in the presence of ionic sulphur thus reducing the toxicity of various reactive metal ions in solution (Collins and Stotzky, 1989).

The valence state of metal ions is an important factor to consider in terms of their solubility and mobility (Gadd, 1992a). Cuprous ions (Cu^{+}) were found to be more toxic to *Escherichia coli* than cupric ions (Cu^{2+}) (Babich and Stotzky, 1980). Gadd (1993) suggested that this might be due to the existence of biological resistance mechanisms specific to individual ions, some mechanisms being more efficient than others. However, changes in redox state as a consequence of biological activity may lead indirectly to increased toxicity. Gaetke and Chow (2003) found that redox cycling of copper could lead to the generation of reactive oxygen species which caused

degeneration in cells' lipid membranes. Francis and Tebo (2002) reported that metabolically dormant spores of a variety of *Bacillus* species were able to oxidise soluble Mn^{2+} to insoluble Mn^{4+} enzymatically.

1.5.4 Presence of Counter Ions

Industrial wastewaters typically contain a variety of inorganic ions, including anions and light metal ions. These ions are likely to interfere with the biosorption processes (Vijayaraghavan and Yun, 2008).

1.5.4.1 Anions

Heavy metal ions form coordination complexes with a variety of anions and inorganic groups such as carbonate, sulphate and phosphate. These negatively charged coordination complexes generally have significantly lower affinities for net-negatively charged cell surfaces, with resultant reduced bioavailability, due to inherent electrostatic repulsion (Babich and Stotzky, 1978b; Atkins, 1998). Pandey *et al*. (2007) showed that fluoride and sulphate ions impeded the biosorption of Ni^{2+} on *Calotropis procera* root preparations. However, some heavy metal ions may form complexes with anions which result in compounds with higher affinities for sorbents than free metal ions (Vijayaraghavan and Yun, 2008).

Anions may also combine with metals to form soluble compounds such as metal-chlorides which decrease toxicity and, therefore, increase availability for biological interaction, e.g. *Agrobacterium tumefaciens* was found to be less susceptible to mercury as Hg complexes than as Hg^{2+} (Babich and Stotzky, 1979a). Some complex anions such as CO_3^{2-}, SO_3^{2-} and PO_4^{2-} react with metal ions to form insoluble salts which precipitate. Being biologically unavailable, these salts are consequently

generally less toxic than their corresponding free ions (Gadd, 2009). Anionic surface sites e.g. SiO^- and AlO^-, on minerals such as clays, can form inner sphere complexes with heavy metal cations, also preventing bioaccumulation (Oyanedel-Craver *et al.,* 2007). Tolerance of *A. giganteus* to lead improved on addition of CO_3^{2-} or PO_4^{3-} in an agar medium containing Pb^{2+} and this was attributed to the formation of insoluble $PbCO_3$ and $Pb_3(PO_4)_2$ (Babich and Stotzky, 1979b).

1.5.4.2 Cations

The presence of available cations can negatively influence the binding and intracellular accumulation of uncoordinated heavy metal ions by successfully competing with the metal ions for suitable binding sites on cell surfaces and transport systems, thereby reducing intracellular accumulation (Babich and Stotzky, 1980; Barabasz *et al*., 1990; Gadd, 1992b; Gadd 2000; 2009, Pagnanelli, 2011).

This competition is demonstrated by different organisms showing different capabilities for adsorbing/accumulating different metal ions e.g. *Chlorella vulgaris* tends to sorb metal ions in the order: $Al^{3+} = Ag^+ > Cu^{2+} > Cd^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} = Co^{2+} > Cr^{3+}$, whereas for *Vaucheria* sp., the order for metal ion affinity is: $Cu^{2+} > Sr^{2+} > Zn^{2+} = Mg^{2+} > Na^+$ (Garnham, 1997). Pandey *et al*. (2007) showed that Pb^{2+} ions impeded the biosorption of Ni^{2+} on *Calotropis procera* root preparations, whereas Cr^{2+} ions did not.

Two possible consequences of this competition may occur when the competing cations are both heavy metal ions. When the combined toxicity of the mixture is greater than that of each individual ion's toxicity this is referred to as synergism. Conversely, antagonism occurs where the overall toxicity of the combination/mixture is reduced below that of each of the individual ions (Ting *et al.*, 1991). For example,

the presence of Mg^{2+} has been shown to reduce the toxicity of both Zn^{2+} and Cd^{2+} towards *A. niger* (MacLeod and Snell, 1950), and no toxic effect of $CdCl_2$ toward *S. aureus* was observed with the introduction of 1g.mℓ^{-1} $CaCl_2$ (Kondo *et al*., 1974). Iron, as both Fe^{2+} and Fe^{3+}, reduced the detrimental effect of Cd^{2+} on *E. coli* (Babich and Stotzky, 1980).

1.5.5 Presence of Organic Matter

Heavy metal toxicity may be affected by the presence of dissolved and particulate organic matter because of their effects on the mobility and bioavailability of the heavy metal ions (Collins and Stotzky, 1989; Shi *et al.,* 2003; Oyanedel-Craver *et al.,* 2007; Gadd, 2009). Organic matter is a significant source of metal complexation; once complexed with organic compounds, heavy metals are often less toxic than the free forms of the metals (Babich and Stotzky, 1980; Gadd, 1993; Macek and Mackova, 2011), particularly where the organic complexing agents and metals form insoluble structures (Macek and Mackova, 2011).

Ethylene diamine tetra-acetic acid (EDTA) is a synthetic chelating agent which has been shown to counteract the toxic effect of copper on *Nitrosomonas* (Loveless and Painter, 1968). Chelation by amino acids reduces the toxic effects of copper as shown in *Candida utilis* (Avakyan, 1971). Methylated derivates of mercury showed lower toxicity to microorganisms than the non-methylated metal (Gadd, 1993). Different metal ions have differing capabilities to form complexes with dissolved organic matter e.g. Cu^{2+} has a higher affinity than Zn^{2+} for organic matter (Shi *et al.,* 2003).

1.5.6 Practical Considerations for the Application of Biosorption

Most biosorption literature presents claims to the numerous advantages of biological treatment of heavy metal contaminated waters over traditional physico-chemical methods (Wood, 1992; Hobson and Poole, 1998; Ahalya *et al*., 2003; Vijayaraghavan and Yun, 2008; Gadd 2009; Macek and Mackova, 2011). Yet biosorption technology has not progressed past laboratory-scale testing (Gadd, 2009). This may be because of the seldom reported disadvantages presented by biosorption when compared with conventional treatments.

Ingole and Dharpal (2012) report that chemically unmodified plant-derived biosorbents add lignin to the final effluent which has the effect of increasing the BOD. Such effluents may, therefore, require further treatment. Chemical modification might increase a biosorbent's efficiency but may also cause problems with final disposal (Ingole and Dharpole, 2012).

1.6 Biofilms

It is likely that in nature most microorganisms occur within biofilms (Hall-Stoodley *et al*., 2004; Moscosco *et al*., 2006). Dostalek (2011) states that biofilms represent the simplest form of biomass immobilisation, yet the structure of some mature biofilms is highly complex.

A biofilm may be broadly characterised as a heterogeneous community of microorganisms housed within a biopolymer gel (Christensen and Characklis, 1990; Dostalek, 2011). Biopolymers include proteins, polysaccharides, lipids, lipoproteins and nucleic acids (Jang *et al*., 2001). A biofilm has been metaphorically described as

a “City of Microbes” (Watnick and Kolter, 2000). Both of these descriptions suggest a complex set of roles, behaviours and interactions between the individual cells and groups of microorganisms present. Watnick and Kolter (2000) summarised some of these behaviours and interactions: in a single species biofilm the following apply: “stepwise construction of the biofilm, intercellular signalling, profiles of gene transcription distinct from planktonic cells. Additionally, in a naturally occurring mixed microbial biofilm, cells share genetic material at high rates, stay and leave with purpose and fill distinct niche roles within the biofilm”.

Biofilms are spatially diverse, covering a surface either uniformly or discontinuously, and consisting of either a single layer or a matrix of cells as thick as 300 - 400mm, with mature biofilms containing as many as 10^{10} cells.cm^{-3} (Chou *et al*., 1990). Superficially, there exist only two major constituents to a biofilm, i.e. the microbial cells and the exopolymers they produce. Consequently these two basic constituents determine the physical properties of the biofilm (Bryers, 1987; Gilbert and Allison, 1993; de Beer *et al*., 1994). Biofilms have, however, been shown to be structurally complex, with channels, micro-pores and macro-pores present (Characklis, 1990a; de Beer *et al*., 1996; Stoodley *et al*., 2002). These features contribute to maintenance of the biofilm by affecting porosity, density, water content, sorption properties and mechanical stability (Flemming and Wingender, 2002).

At a more sophisticated level, a biofilm may be considered to consist of as many as five components: the substratum, base film, surface film, bulk liquid and the gas phase (Characklis, 1990a). The biofilm may also contain other unique, functional, extracellular components, including extracellular vesicles. Vesicles formed by sessile Gram negative *Pseudomonas aeruginosa* differed both quantitatively and qualitatively from those formed by their planktonic counterparts (Schooling and

Beveridge, 2006). As mentioned previously, the EPS component of a biofilm contains not only a wide variety of polysaccharides, but also proteins, glycoproteins, glycolipids and often extracellular DNA (Flemming *et al*., 2007).

1.6.1 Attachment and Formation

Biofilm formation is the net result of a number of physical, chemical and biological processes (Characklis, 1990a), all of which are affected by a variety of environmental factors. These factors include osmolarity [the measure of solute concentration, defined as the number of osmoles (Osm) of solute per litre (ℓ) of solution (osmol/ℓ or Osm/ℓ) (Widmaier *et al*., 2008)], and nutrient composition and concentration (Kristich *et al*., 2004). Biofilm formation involves phases of cell attachment; growth and polysaccharide production; maturation; and the import of organic compounds (Wimpenny *et al*., 1993; Rice *et al*., 2005). These processes are detailed below and illustrated in **Figure 1.1.**

The initial stage of any biofilm development is the conditioning of the support matrix by the formation of a conditioning film, which involves molecular adsorption to a surface, (Blenkinsopp and Costerton, 1991; James *et al*., 1995). The particular conditioning film formed is dependent on the type of substratum and the molecules adsorbing to it, and this has a significant effect on subsequent bacterial colonisation (Blenkinsopp and Costerton, 1991).

Bacterial adhesions occur when planktonic microbial cells come into contact with the conditioning film. A wide variety of attachment strategies may be employed, depending on the nature of the surface and the organism; for example, *Vibrio cholera* cells use flagella or type IV pili to select and attach to suitable sites (Watnick and

Kolter, 2000; Mueller *et al*., 2007). Initial adhesions are reversible and may not be sustained because of various mechanical, environmental or physiological interactions occurring at the microbe-conditioning film interface, which may cause the bacteria to detach (James *et al*., 1995). Cells may also use type IV pili or flagellar motion to move along the support surface until other bacteria are encountered (Watnick and Kolter, 2000). Irreversible adhesion usually occurs following the synthesis of extracellular polymers which bind the cells to the substratum (James *et al*., 1995; Flemming *et al.,* 2007). However, biofilm formation, which infers irreversible adhesion, has been reported in the absence of EPS (Kristich *et al*., 2004). Furthermore, some EPS may negatively influence further attachments (Moscosco *et al*., 2006).

The irreversible adhesion of a particular bacterial species to a surface directly influences subsequent bacterial adhesions. This effect can be positive, negative or neutral.

a. Positive interactions occur either indirectly as a consequence of cellular modification of the conditioning film, or as a consequence of direct cell-to-cell contact (James *et al*., 1995). Primary colonisers may subsequently attract other cells from the planktonic phase (Blenkinsopp and Costerton, 1991; Watnick and Kolter, 2000).
b. Negative or inhibitory interactions are possible as a result of the production of inhibitory substances which, when adsorbed onto the conditioning film, modify it in such a way that it becomes unfavourable for further foreign microbial attachment (James *et al*., 1995).
c. Neutral interactions can arise when a substratum has separate binding sites for each species (James *et al*., 1995).

The immobilised cells grow and replicate, forming microcolonies of sister cells and extraneous macromolecules within a stabilised polymer matrix (Costerton *et al.,* 1987; Watnick and Kolter, 2000). The stability results from cross linking by multivalent cations, hydrophobic interactions and entanglements of biopolymers (Flemming and Wingender, 2002).

As the biofilm develops, other substances adhere to and become entrained in the biofilm itself and intercellular signalling may be used to recruit new microorganisms (Watnick and Kolter, 2000). This occurs until the biofilm becomes too thick or micro-environmental conditions become unfavourable, resulting in portions of the biofilm then detaching and becoming re-entrained in the liquid phase. (Bishop and Kinner, 1986; Bryers, 1987; Blenkinsopp and Costerton, 1999; Bitton, 1999, Watnick and Kolter, 2000; Rice *et al*., 2005).

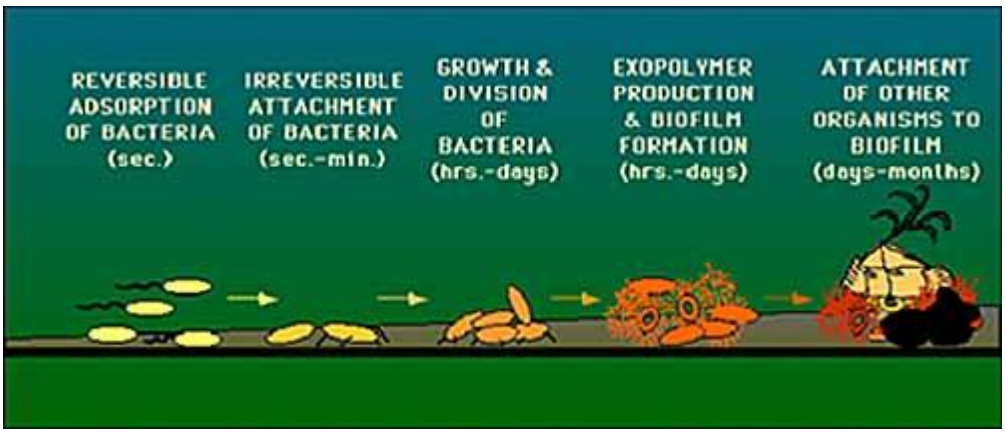

Figure 1.1: Steps in the formation of a typical biofilm.
http://www.biofilmsonline.com/cgi-bin/biofilmsonline/ed_how_primer.html (2009)

1.6.2 Industrial Biofilm Morphology

The architecture and structure of biofilms is largely governed by their EPS matrix and these characteristics contribute to the biofilms' general resilience (Schooling and Beveridge, 2006). Biofilm shape is partially determined by the shape of the support material and the surrounding physical conditions. Thus in industrial applications such as fixed-bed bioreactors, the shape of the biofilm is influenced by the flow of the medium over the bed, whereas in fluidised or expanded bed bioreactors it is influenced by particle movement within the reactor (Atkinson *et al*., 1980). In industrial applications the support particle size determines the biofilm surface, which is important in diffusion-limited conditions (Callander and Barford, 1983).

In attempts to increase bacterial densities, surface areas for bacterial attachment have been maximised on many commercially available support matrices (Callander and Barford, 1983). Substances which have been divided into smaller particles provide higher surface area to volume ratios and thus larger surface areas for cell attachment. However, due to the cohesive forces holding a biofilm together, a minimum particle size and density is prescribed in order to prevent washout (Switzenbaum, 1983). In the absence of environmentally dictated structural growth, two general biofilm morphologies are reported; either flat and undifferentiated, or more structurally complex differentiated forms. The latter has been described for *Pseudomonas aeruginosa* consisting of towers and mushroom shapes with channels and voids, which presumably serve to deliver nutrients to, and remove waste products from, the cells (de Beer *et al*., 1996; Stoodley *et al*., 2002). Factors such as alginate expression, rhamnolipid production, and quorum sensing have been shown to be important in controlling both the three-dimensional architecture and stress resistance of biofilms (Hentzer *et al.,* 2001; Rice *et al*., 2005).

Although a function of a biofilm is to provide protection from external stresses, high cell densities within a biofilm may result in stressful environments characterised by a scarcity of nutrients and oxygen, nonoptimal pH, and accumulation of metabolic by-products (de Beer *et al*., 1996; Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004). When a biofilm's surface area to volume ratio is high cell activity may be limited by mass transfer rates of substances into and out of the biofilm, a function largely governed by its thickness (Christensen and Characklis, 1990; Gadd, 2009). The thickness may, however, vary considerably over a given substratum, due to topographical features of the biofilm (Rice *et al*., 2005).

In industrial applications, biofilm thickness is a function of a number of variables: microbial type, species, biofilm age, type of substrate, substrate loading rates, reactor configuration and operating conditions under which the biofilm is cultivated (Timmermans and van Haute, 1984; Fan *et al*., 1987; Hentzer *et al.,* 2001; Stoodley *et al*., 2002;). As the concentration of essential chemicals in the liquid phase is increased, the depth of penetration of substrate into the biofilm increases (Atkinson and Fowler, 1974). This depth of penetration is an important parameter as it represents the distance through which mass transfer takes place to maintain the microorganisms in an active state (Atkinson and Fowler, 1974). The ideal thickness is equal to the penetration depths of the substrates and/or electron acceptors, since both solute uptake and growth increase until this critical thickness is reached (Wimpenny *et al*., 1993). Kristich *et al. (*2004) found that *E. faecalis* cells monitor their intracellular physiological state and the surrounding environmental conditions, using this information to regulate biofilm development.

Changes in biofilm thickness across a support matrix will, therefore, result in a number of different microenvironments. In mixed biofilms, microorganisms distribute themselves in accordance with microenvironments most suited to their particular needs. This is due to the existence of various biological and physiochemical gradients (O_2, CO_2 and pH) arising from processes such as the influx of nutrients and efflux of metabolic wastes (Watnick and Kolter, 2000; Stoodley *et al*., 2002). These gradients influence mass transfer mechanisms and diffusion coefficients within the biofilm (Characklis, 1990a; Zhange and Bishop, 1994). This facilitates the creation and maintenance of a microcosm within which conditions may differ completely from the adjacent phases (Massol-Deya *et al*., 1995; Watnick and Kolter, 2000; Parsek and Fuqua, 2003). For example, despite a thickness of only a few millimetres, a vertical zonation of respiratory processes in relation to an O_2 gradient can be found in biofilms (Kuhl and Jorgensen, 1992).

As microorganisms grow, the biofilm increases in thickness and at a critical point the available O_2 will be entirely consumed before penetrating to the deeper layers of the biofilm, thereby creating an anaerobic layer between the oxygenated biofilm and the support surface (Blenkinsopp and Costerton, 1991). This has also been observed in relation to nutrient gradients where cells within deeper layers are subjected to restricted nutrient levels and, therefore, may be metabolically inactive. This may be advantageous because these cells are protected from antimicrobial agents and environmental stressors (Parsek and Fuqua, 2003). However, these starving cells in the deeper layers may commence endogenous respiration and as a consequence lose their ability to cling onto the support surface, resulting in sloughing of the biofilm (Bishop and Kinner, 1986; Bryers, 1987). The depth where this occurs is generally 50

- 150μm above the substratum, depending on a variety of factors such as substrate concentration and the kind of organisms forming the biofilm (James *et al.*, 1995).

In industrial applications, biofilm thickness may be controlled by harvesting or the mechanical scraping of the surface by abrasive forces arising from physical contact between solid surfaces (Black and Pinches, 1981). If the particles are kept in suspension, such as in a fluidised bed bioreactor, frequent collisions between the particles cause the film to attain a thickness that is essentially constant. Excess film is removed from the BSP, becoming flocs within the liquid phase (Black and Pinches, 1981).

1.6.3 Biofilm Uses

Microbes in nature commonly occur within biofilms (O'Toole *et al*., 2000). As previously stated, biofilms can provide survival advantages and protection under a range of environmental conditions (Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004; Rice *et al*., 2005). Biofilms facilitate colonisation of, and persistence in, inhospitable environments, often giving rise to chronic problems. These include anaerobic corrosion in water pipelines (McLean *et al*., 1994) and persistent infections in patients due to growth on medical implants because of the cells' increased resistance to antimicrobial agents (Donlan and Costerton, 2002). However, biofilm persistence also allows for beneficial applications through biotechnology. In general, biofilm growth has found a number of industrial applications through the development of reactors designed to enhance processes involving organisms in stressful /adverse conditions, or which grow too slowly for continuous suspended culture operation (Characklis, 1984). Biofilms have been extensively applied in high-rate water treatment processes in which cell retention is critical (Senior, 1990). In addition to wastewater treatment, uses for biofilms include the production of ethanol (Kuduru and Pometto, 1996), vinegar (Silva *et al*., 2007) and other chemicals (Li *et al*., 2006).

1.6.4 Support Surface

In order for a biofilm to form it requires a support surface or substratum (Matrix). The nature of the matrix plays a major role in biofilm development, by influencing the rate and ease of cell accumulation, as well as initial cell micropopulation distribution (Callander and Barford, 1983; Characklis and Marshall, 1990; Martinez and Casadevall, 2007). Substrata may accumulate conditioning films differing in composition, due to variances in their surface properties (Characklis, 1990b). These variances include charge, surface area, chemical toxicity, hydrophobic/hydrophilic

tendencies and critical surface tension (Characklis, 1990b). Matrix surface roughness promotes microbial adhesion by diminishing shear forces, while physico-chemical properties promote the attachment of microorganisms to a solid support (Baker, 1984; Characklis and Marshall, 1990). The compatibility of a microorganism with a particular support surface is important to consider, Martinez and Casadevall (2007) found that *Cryptococcus neoformans* adhered to a wide variety of materials, including glass and polyvinyl, due to the dynamic nature of its polysaccharide capsule, which allowed the cells to interact favourably with a variety of substrata and promote attachment.

The results of early work undertaken by Bruce and Hawkes (1983), Characklis (1984) and Bonastre and Paris (1989) suggested that ideal support surfaces should possess the following characteristics: a high surface area to volume ratio; exposed surfaces over which the liquid can flow and on which biofilms can develop; a rough texture, to permit bacterial adhesion since microbial colonisation appears to increase with corresponding increasing roughness of the substratum; biological inertness; mechanical strength; economical in material and design.

As well as the physical characteristics, physico-chemical properties are also important to consider: adherence and proliferation of bacteria have been shown to be promoted when a hydrophilic and porous support matrix is used (Murray and van den Berg, 1981; Klein and Ziehr, 1990; Pirbazari *et al*., 1990). In addition to providing support, surfaces should be non-toxic to the cells and should not influence cellular metabolism (Kolot, 1988). Further considerations may be the weight and handling capabilities, availability, and whether or not the material is environmentally friendly to allow for safer disposal (Volesky and Naja, 2005).

1.6.5 Development of Biosorbent Particles

An alternative to growing a biofilm on traditional biofilm support particles (BSP) is to form the biosorbent into granules for direct application to bioreactors (Dostalek, 2011). The two most common methods for this being; immobilisation by cross linkage and immobilisation by entrapment. The former employs the use of cross linkers e.g. formaldehyde, glutaric dialdehyde and divinalsulfone to form stable cellular aggregates (Veglio and Belochini, 1997), and is commonly used to immobilise algae. Immobilisation by entrapment involves cells (living or dead) being trapped in soft gels (Dostalek, 2011). Marseaut *et al*. (2004) successfully embedded yeast cell wall envelopes on a silica matrix for the biosorption of cadmium.

1.7 Bioreactors

Bioreactors are vessels or tanks in which immobilised or planktonic cells are used to bioremediate wastewaters of various types, including metal-contaminated liquids (Erikson, 2004). Successful microbiological wastewater treatment depends on the development and maintenance of a system containing an appropriate, active, mixed microbial population (Barnes *et al*., 1981). Mixed consortia have been shown to be more resilient to physicochemical fluctuations and stresses within bioreactors and often perform better than single species applications (Kotrba *et al*., 2011a). Successful treatment also requires physical contact between the target contaminant and the desired organism/s for an appropriate period of time, to allow the organism/s to modify or remove the target pollutant (Winkler, 1983). In order to achieve these objectives, bioreactors are often designed and operated to optimise environmental conditions for biological activity.

Bioreactors can be classified either on the basis of the method of feeding and removal of media, gasses and product e.g. batch, fed-batch or continuous; or, alternatively, on their physical/operational design e.g. stirred-tank, bubble-column or packed-bed (Williams, 2002; Erikson, 2004).

Traditionally, microbiological treatment processes comprised suspended growth systems involving planktonic microorganisms distributed throughout the bulk liquid medium (Winkler, 1983). This strategy exploits the tendency of microorganisms to flocculate, enabling the organisms to be separated from the treated wastewater cheaply, rapidly and efficiently by means of settling. This also allows for physical pollutant removal, because suspended colloids remove some dissolved materials from the wastewater by adsorption onto the flocs (Winkler, 1983). These materials

may be removed and treated separately. An example of this system is the activated sludge process found in most commercial/municipal sewage treatment works.

Alternatively, wastewater treatment systems may rely on the activities of attached microorganisms in the form of biofilms. These systems operate in fixed-film wastewater treatment bioreactors (Bryers and Hamer, 1987; Hao *et al*., 1990). The rate of reaction is usually directly proportional to the concentration of microbial mass within the bioreactor, therefore, the primary design objective of such systems is to increase the amount of biomass available within the reactor, thereby allowing an increased loading rate (Erikson, 2004). Wastewater treatment systems using attached microbial systems may achieve high population levels, up to 10^9 - 10^{10} cells.g^{-1} of support medium, thus allowing for high rates of chemical degradation (Hallas *et al*., 1992).

1.7.1 Attached-Film or Biofilm Reactors

Fixed-film bioreactors have several advantages over suspended-growth systems. These include: prevention of biomass washout; easier operation since there is no need to separate biomass from the bulk liquid; higher biomass production per volume of reactor; extension of cell longevity; increased resistance in the cell population to toxic loading because most of the biological activity takes place on the surface, thus protecting the subsurface microorganisms. Also some microorganisms grow better when immobilised, than when cultured in suspended cell reactors; higher rates of degradation (reduction of toxic components in the system) are possible; greater microbial diversity is achievable; continual removal of reaction inhibitors for a specific biomass is possible without loss of the latter; a more stable gene pool may be provided, resulting in enhanced rates of genetic transfer; emissions of toxic vapours

are minimised since most immobilised reactors are closed systems (Wheatley, 1984; Bruce and Hawkes, 1983; Pezzuto and Popielarski, 1998).

Lazarova and Manem (1994) divided biofilm reactors into two main categories: “fixed-in-place media”, in which wastewater flows over a biofilm attached to the support e.g. fixed-bed reactors; and, “media in motion” in which the support surface moves through the wastewater, for example Rotating Biological Contactors (RBCs). Within these categories a wide variety of different bioreactor systems are found, none of which are entirely unique. Some examples are: completely mixed systems - the liquid in the reactor is completely mixed and biological utilisation is brought about by a biofilm or sorbent which uniformly contacts the liquid, e.g. RBCs as used by Costley and Wallis (2001): fixed bed reactors - the biofilm is attached to an immobile solid medium and utilises substrate as the liquid passes through the reactor (usually downwards) in a plug-flow manner. Volesky and Prasetyo (1994) found that a packed-bed contacting column maximised contact between sorbate and substrate. Volesky and Naja (2005) state that fixed-bed reactors can remove metals to a final concentration of 10-50µg.ℓ^{-1}. In expanded-bed and fluidised-bed systems, the biofilm and solid support medium are mixed and distributed throughout the reactor, which is operated at a velocity sufficiently high to maintain the dispersed solids in suspension (Volesky and Naja, 2005). Higher velocities are needed over expanded systems to fluidise particles. A major advantage of this system is that the feed stream does not need to be particle free. However, because of the high degree of mixing, the system cannot make maximum use of the biosorbent charge to create a concentration gradient between the solid and liquid phases (Volesky and Naja, 2005).

1.7.2 Bioreactor Configuration and Operation

1.7.2.1 Downflow Configuration in Bioreactors

This configuration involves the addition of influent at the top of the reactor vessel and the removal of the treated or partially treated liquid at the bottom (Hall, 1992). Mixing may be provided by the counter current action of rising gas bubbles (e.g. air introduced via a sparger at the bottom of the reactor) and/or effluent recycle and, therefore, tends to be at a maximum at the top of the reactor (Hall, 1992). The extent of the mixing is dependent on reactor operating procedures (discussed in 1.8.2.3). Bubbles rising against the flow can aid in effective distribution of the liquid medium without an expensive or complex distribution arrangement (Senior, 1990; Williams, 2002).

Mixing contributes towards efficient removal of suspended solids by reducing their physical hold-up, allowing the solids to move between the BSP, as they are carried down with the liquid flow and out of the reactor (Hall, 1992). Thus, systems operating in downflow configuration retain only attached microorganisms and can be regarded as “true fixed-film processes” (Hall, 1992). Because of this, such systems may require subsequent separation of the biomass from the bulk liquid (Williams, 2002; Volesky and Naja, 2005).

The amount of biomass retained in a downflow fixed-film reactor is dependent on, and limited by, the support matrix area. Washout of sloughed biofilm and suspended solids may result in a poorer effluent quality, particularly when the influent contains high proportions of insoluble material (Hall, 1992). Kennedy *et al. (*1988) found that because of the increased mixing taking place in downflow reactors (compared with upflow reactors), these reactors are more efficient at treating wastewater with a high

suspended solids concentration. Williams (2002) and Volesky and Naja (2005) state that packed-bed bioreactors operating in downflow configuration are more efficient because they operate at a lower volume and, therefore, have greater contact between the solid and liquid phases.

1.7.2.2 Upflow Configuration of Bioreactors

When a fixed-film reactor is operated in the upflow mode, a high biomass is maintained because unattached cells are retained within the interstices between the biomass support particles by settling and physical contact with the medium (Callander and Barford, 1983). In general, upflow bioreactors cannot match the flow velocities achieved in downflow systems because of the increased risk of biomass washout associated with upflow configurations (Kennedy *et al*., 1988).Therefore, upflow reactors tend towards a hybrid operational mode, since treatment results from the continued activities of both attached and suspended biomass (Hall, 1992).

High-rate upflow anaerobic bioreactors are, however, widely used for the purification of industrial wastewaters with the upflow anaerobic sludge bed being one of the most common types of bioreactors (Frankin, 2001; Diaz *et al.,* 2006). These high-flow velocity systems, such as fluidised-bed reactors, are used where washout of the suspended biomass from the reactor is not considered to pose any operational problems, because the support matrix should physically impede washout during hydraulic shock loads (Colleran *et al*., 1982). A particular advantage of fluidised-bed reactors is that suspended solids are less likely to cause problems than in packed-bed reactors, therefore the liquid feedstock need not be entirely particle free (Volesky, 2003).

A wide range of upward flow velocities have been reported, e.g. 0.4m.h^{-1} (Sales and Shieh, 2006) to 60m.h^{-1} (Tarre and Green, 2004). The high biomass retention resulting from accumulation in the matrix's interstitial sites may result in preferential flow paths or "channelling" (Hall, 1992). This is undesirable as it gives rise to dead volume because the liquid passing through the preferential flow channels bypasses the remainder of the medium in the chamber, resulting in reduced contact between the microorganisms/biofilm and the liquid within the reactor, thus leading to gross inefficiency (Hall, 1992). Indicators of excess biomass within the reactor include high levels of suspended solids in the reactor effluent, or deterioration in the residence time distribution characteristics (Hall, 1992).

1.7.2.3 Aeration of Bioreactors

Supplementary aeration may be provided to encourage aerobic growth by increasing dissolved oxygen concentrations in the mixed liquor, with consequent higher biological oxygen demand (BOD) removal rates (Surampalli and Baumann, 1997). Oxygen has a potent influence on the activity of physiological and biochemical pathways in bacteria. For example, Ahn *et al. (*2009) found that transport of glucose, fructose or mannose by the sugar:phosphotransferase system in *Streptococcus muta* was significantly enhanced under aerobic growth conditions.

Secondary and tertiary effects may result as the bubbles produced by aeration have an abrasive action on the biofilm, resulting in reduced biofilm thickness which allows more of the biofilm to remain aerobic (Hall, 1992). Ahn *et al. (*2009) reported that aeration altered *S. muta* biofilm architecture. Further mechanical advantages of supplementary aeration include providing agitation and turbulence in the reactor, resulting in improved mixing and, therefore, reduced channelling (Senior, 1990; Williams, 2002; Erikson, 2004).

1.7.2.4 Bioreactor Contact Times

Biosorption is generally considered in terms of the mass or concentration of sorbent (biomass) used and the residual concentration of the sorbate at equilibrium. However, in commercial applications, the rate of reaction and, therefore, the time taken to treat the wastewaters are likely to be important considerations. Sorption times for copper, cadmium and zinc by various bacterial species, as achieved by various researchers, are shown in **Tables 1.5 to 1.7.** However, real-world biotransformation rates of pollutants are likely to vary considerably, depending on the nature and biodegradability of the pollutants (Byrns, 2001).

1.7.3 Biosorption Kinetics

Pagnanelli (2011) described biosorption kinetics as one of the most important factors to consider in adsorption system design because it controls residence times and reactor dimensions. Various empirical models, with different degrees of complexity, have been derived to predict adsorption rates. Among these, two of the most popular are: Lagergren's pseudo-first-order rate equation which describes adsorption rate (*dq/dt*) based on the concentration of solute in solution and the pseudo-second-order model, which takes account of the number of binding sites present (Pagnanelli, 2011).

Lagergren's pseudo-first-order rate equation:

$$\frac{dq}{dt} = k_1(q_e - q)$$

(**Equation 1.5**)

Where q is the metal concentration at time, t, q_e is the equilibrium metal concentration and k_1 is the first-order rate constant.

The pseudo-second-order model:

$$\frac{dq}{dt} = k_2(q_e-q)^2$$

(**Equation 1.6**)

Where q is the metal concentration at time, t, q_e is the equilibrium metal concentration and k_2 is the second-order rate constant (Pagnanelli, 2011).

The pseudo-first-order model is reported to apply only early on in a sorption process and does not fit well over prolonged metal/sorbent contact time. Pandey *et al*. (2007) showed poor linear correlation when applying this equation to Ni^{2+} uptake by unmodified biomass over 180min, whilst Gupta and Rastogi (2008), also investigating Ni^{2+}, found a good correlation between their experimental data and the pseudo-first-order model up to 150min. A pseudo-second-order model is generally considered to be more appropriate than a pseudo-first-order model to represent kinetic data of heavy metal biosorption in batch reactors because the former, unlike the latter, assumes a second-order dependence of the sorption rate on the number of binding sites available (Pagnanelli, 2011).

1.8 Pine Bark

Plant-based biosorbents derived from agricultural wastes have been gaining increasing interest for use in metal sequestration (Demirbas, 2008; Sud *et al*., 2008; Ribé, 2009). Bark is a large-scale waste product of timber industries. Mills in Quebec, Canada have generated up to 1200t of waste bark daily (Frigon *et al.,* 2003), and in 2002, mills in the USA produced an estimated 2,200,000t of tree bark waste (McKeever and Falk, 2004). Although bark is typically used as a low quality fuel at mills to mitigate disposal issues, and has found some other applications, such as

decorative uses and plant potting medium, it still remains an abundant, underutilised, low-value waste product (Tshabalala *et al.,* 2003).

1.8.1 Suitability of Pine Bark as a Metal Sorbent

The suitability and advantages of various tree barks as sorbents for heavy metal ions have been well studied (de Vasconcelos and Beca, 1992; Villaescusa *et al*., 2000; Martin-Dupont *et al*., 2002, 2004, 2006; Tshabalala *et al*., 2004; Sekar *et al.,* 2004; Shin and Rowell, 2005; Oh and Tshabalala, 2007). Being of plant origin, pine bark contains many negatively charged organic functional groups ($R\text{-}COO^-$) able to bind cations on its surface (Tucker, 2005). Pine bark is also permeable, allowing the liquid medium to diffuse through the bark, thereby providing additional surface area for metal ion binding compared with impermeable substrates. Another important consideration of a sorbent is its cation exchange capacity (CEC), which is the capacity of a material (usually soil) to exchange and retain cations and is expressed in terms of milliequivalents per 100 grams ($meq.100g^{-1}$) (Virginia Department of Health, 2005). This is important as it allows quantification of the sites suitable for cation binding on the sorbent. Tucker (2005) states that pine bark typically has a CEC in the range of 10-13 $meq.100mg^{-1}$. This concurs with the findings of The Virginia Department of Health (2005), which gives a value of approximately 10,6$meq.100mg^{-1}$ CEC for pine bark.

Under naturally occurring conditions pine bark has an acid pH. Tucker (2005) reported a pH range for pine bark of pH 3.4 – 4.5, while Krewer and Ruter (2009) reported a pH range of pH 4.0 – 5.0 which varied according to the state of decomposition of the bark. The low pH environment effected by the pine bark should favour the solubilisation of most metals and may potentially aid biosorption. However, it may not provide optimal conditions for the bioaccumulation of certain metal ions.

For example, Volesky and May-Phillips (1995) showed that the uptake of uranium, zinc and copper ions by *S. cerevisiae* was optimal in a medium with a pH of 4 – 5. Feng *et al*., 2009 reported maximum Cu^{2+} uptake by orange peel occurring at pH 4.5. However, as discussed in **Section 1.5.1** an acid medium introduces competition between protons and metal cations for attachment sites on the sorbent, which may hinder biosorption. Villaescusa *et al*. (2000), found that maximum sorption of Cu^{2+} and Ni^{2+} by cork and yohimbe bark wastes occurred at pH 6 – 7. In addition, pine bark may also introduce competitive metal cations into the system, such as manganese (Krewer and Ruter, 2009).

1.8.2 Suitability of Pine Bark as a Biofilm Support Surface

Organic BSPs are sometimes preferred to inorganic substrates because they provide a greater variety of reactive groups, such as carboxyl, amino and hydroxyl groups, on their surfaces (Kolot, 1988). Additionally, it is also important that the support matrix is able to retain a high microbial loading (Ahalya *et al*., 2005). BSPs with high porosity (such as pine bark) permit internal colonisation, thus providing sheltered attachment sites for cells which facilitates rapid recolonisation following sloughing of the biofilm after severe system shocks (Messing and Oppermann, 1979). Krewer and Ruter (2009) state that 40-45 % of the overall volume of a pine bark particle is made up of internal pore spaces, and when packed into a container another 40 % of the volume is inter-particle pore space, also available for microbial colonisation. Pine bark contains significant quantities of water soluble organics, including soluble tannins which, when leached, may be toxic to microorganisms (Tshabalala *et al.,* 2004; Oh and Tshabalala, 2007). Although pine bark has been proved to leach significant quantities of dissolved organic carbon, only a small fraction of this was found to be phenols (Ribé *et al*., 2009). Furthermore, ecotoxicological tests, conducted using

Daphnia magna, showed that the toxic effects of the pine bark leachate were due to decreased pH and no toxicity was observed in pH adjusted toxicity tests (Ribé *et al*., 2009). These released organic molecules may also negatively impact on the biosorption capabilities of the system (Macek and Mackova, 2011). In order to prevent this release of organics, the bark may be treated. For example, Vázques *et al.* (2002) used acidified formaldehyde to bind the organics in the pine bark, while Haussard *et al.,* 2003 treated bark biologically or chemically and saturated it with transition metal ions to avoid the release of soluble organic compounds. Although pine bark is mechanically fragile and, therefore, potentially unsuitable for use in certain bioreactor applications, such as fluidised-bed reactors, it is high in lignin and, therefore, more resistant to decay than cellulosic organic substrates (Krewer and Ruter, 2009). **Table 1.8** lists the desired properties of a BSP and the suitability, or otherwise, of pine bark for this purpose (after Bruce and Hawkes, 1983; Characklis, 1984; Bonastre and Paris; 1989; Naja and Volesky, 2010).

Table 1.8: Suitability of pine bark as BSP (Al-Asheh and Duvnjak, 1997; Krewer and Rutler, 2009; Mun *et al*., 2009)

Desired properties of BSP	Suitability of Pine bark
High surface area to volume ratio	Yes
A multiplicity of exposed surfaces	Yes
Rough texture	Yes
Biologically inert	No
Mechanically strong	No
Economical in material and design	Yes
Non-toxic to cells	No
Non-interfering with cellular metabolism	No
Lightweight and easy to handle	Yes
Environmentally friendly	Yes

1.9 Project Objectives

Biosorption is well studied and recognised as a viable option for the treatment of heavy metal contaminated wastewaters, particularly using either plant or microbial biomass (living or dead) as the sorbent. Of particular interest is the use of either optimally growing biofilms or abundant waste products such as pine bark as biosorbents. However, in all reported literature where pine bark or other plant materials were used, this material had undergone various forms of mechanical, biological, or chemical pretreatment in attempts to produce a more efficient sorbent. These modifications unfortunately add engineering complexity (such as containment

of fine particles or acid washing) and, therefore undoubtedly, cost to the system. Unmodified pine bark would be preferable as it can be supplied more quickly (without delays caused by pretreatment) and, as it is a freely available waste product, often with costs limited only to transportation of the bark. Also, the generation of additional undesirable by-products associated with the modified derivates is avoided.

The operation of attached-film-bioreactors is also well understood. Many researchers have already studied various aspects of biofilm systems, including reactor choice and BSP options, to optimise sorption of metal ions to the biomass in order to achieve beneficial reuse of contaminated wastewaters.

To my knowledge a system combining a nonliving organic sorbent and a living biofilm for the remediation of waters containing Cu^{2+}, Zn^{2+} and Cd^{2+} has not been previously reported, and all reported investigations into the use of plant materials required substantial modification of the sorbents. Furthermore, Gadd (2009) states that although many metal biosorbent systems have been investigated and reported, only a handful have found application at pilot or industrial scale. It is suspected that this may be because of onerous engineering requirements for using finely divided materials which reach saturation within a few minutes. Hence, the primary objective of this investigation was to develop a simple, low-tech method to remove metal ions from wastewaters, using such a dual biosorbent system in an attempt to overcome some of the barriers to industrial adoption of biosorption for the treatment of metal contaminated wastes. Therefore, the feasibility of a pilot-scale upflow attached-film-bioreactor system using raw pine bark nuggets, as both microbial biomass support matrix and sorbent, to treat a synthetic metal-contaminated wastewater was investigated.

1.9.1 Research Aims:

- Engineer a suitable laboratory-scale forced-upflow fixed-bed bioreactor.
- Investigate the use of pine bark as a biofilm support matrix.
- Test the feasibility of a system combining matrix and biofilm adsorption by evaluating:
    - The effectiveness of biofilm growing on plastic as a sorbent of Cu^{2+}, Zn^{2+} and Cd^{2+};
    - The effectiveness of sterilised, uncomposted pine bark as a sorbent of Cu^{2+}, Zn^{2+} and Cd^{2+};
    - The effectiveness of a combination of biofilm growing on uncomposted pine bark as a sorbent of Cu^{2+}, Zn^{2+} and Cd^{2+}.
- Establish a pilot-scale forced-upflow bioreactor based on the laboratory-scale model.
- Investigate the operating conditions of the bioreactors, such as mixing and recycle rates, to optimise the system for heavy metal bioremediation.

Chapter 2

General Materials and Methods

2.1 Source and Characterisation of the Inoculum Culture

A sample of activated sludge from the Hammarsdale Sewage Works in KwaZulu-Natal was used to provide an inoculum for the experiments. This facility has a capacity of 27Mℓ/day with an influent chemical oxygen demand of 750mgO_2/ℓ and was selected because the plant serves a highly industrialised catchment area. Results of routine analysis on the inoculum are shown in **Tables 2.1 and 2.2.**

Table 2. 1: Analysis of inoculum sample taken the Hammarsdale Sewage Works

Determinants	**Sample**	**Operational Limit**
Total Helminth's (count. dry g^{-1})	6	1 viable Helminth ova
E.coli in colonies (per 100mℓ)	0	<10,000
pH	8.4	
Total Solids	16.8 %	
Volatile Fatty Acids (mg.ℓ^{-1} CH_3COOH)	188	
Volatile Suspended Solids	51.7 %	

Table 2. 2: Metal content analysis of inoculum sample taken the Hammarsdale Sewage Works

Determinants (mg.ℓ^{-1})	Sample	Limits (Class A sludge)
Arsenic	0.984	<40
Cadmium	0.433	<40
Chromium	17.020	<1,200
Copper	71.150	<1,500
Lead	16.730	<300
Mercury	0.571	<15
Nickel	7.380	<420
Zinc	585.710	<2,800

The samples were prepared using an aqua-regia digestion. The digests were analysed in triplicate by ICP-OES. Average results are presented in **Table 2.2**.

2.2 Enrichment of the Inoculum Culture

Voermolas (Voermol Voermolas Reg. no V10257, N-FF0562) is a molasses based liquid animal feed supplement generally used to increase palatability and reduce dustiness of feeds (Voermol, 2012). The composition of Voermolas is shown in **Table 2.3**.

Table 2. 3: Composition of Voermol Voermolas Reg. no V10257, N-FF0562

Crude Protein (Min)	33
Moisture (Max)	300
Calcium (Min/Max)	6/9.2
Phosphorus (min)	0.8
Energy (MJ ME/kg)	9

The Voermolas was purchased from NCD agricultural suppliers in Pietermaritzburg, KwaZulu-Natal at a cost of ZAR 49.95 for a 25ℓ container.

Preliminary experiments showed that 0.1% - 5% (v/v) Voermolas solutions were capable of sustaining microbial growth in submerged conditions. Enrichments were performed in 0.1% (v/v) Voermolas solutions initially spiked with 10mg.ℓ^{-1} copper, 10mg.ℓ^{-1} zinc, and 10mg.ℓ^{-1} cadmium (**Section 2.3**). Nine further subcultures were performed in a series of stepwise increases in concentration (10mg.ℓ^{-1}each metal) until the metal concentrations in the growth medium reached 100mg.ℓ^{-1} for each metal. The cultures were incubated at 25^{o}C for the duration of the procedure.

2.3 Metal Salts Used for Enrichments and the Production of the Synthetic Wastewater

Metal Salts (Analar Grade) used were:

Cu: $CuCl_2.2H_2O$

Zn: $ZnCl_2$ and $(CH_3COOH)_2Zn.2H_2O$

Cd: $CdCl_2.H_2O$ and $(CH_3COOH)_2Cd.2H_2O$

Initially enrichment media contained Analar Grade $CdCl_2.H_2O$ and $ZnCl_2$ as Cd and Zn sources respectively; however, due to supply issues, the chloride salts were substituted with Analar Grade $(CH_3COOH)_2Cd.2H_2O$ and $(CH_3COOH)_2Zn.2H_2O$.

2.4 Preparation of the Synthetic Wastewaters

The preparation of the synthetic wastewaters is described in the relevant chapters, with all liquid media prepared using municipal tap water. The metal content of the municipal water supply in the laboratory, as determined by Atomic Absorption Spectroscopy, was 0.45 (±0.04)mg.ℓ^{-1} Cu, 0.31(±0.03)mg.ℓ^{-1} Zn and 0.01(±0.00)mg.ℓ^{-1} Cd.

2.5 General Construction of Forced-Upflow Bioreactors

Three different bioreactor designs, all forced-upflow, were used in this research.

a. Five identical 37ℓ laboratory-scale bioreactors were constructed for use in the experiments presented in **Chapters 3 and 4**.
b. Six identical 40ℓ laboratory-scale bioreactors were constructed for use in the experiments described in **Chapter 5**.
c. Two identical 1000ℓ bioreactors were constructed and used in the pilot-scale experiments discussed in **Chapter 6**.

All the bioreactors were engineered to similar principles; all were forced-upflow-attached-film bioreactors. The synthetic wastewater was pumped from a reservoir into an influent chamber from which the liquid medium flowed to the bottom of each bioreactor chamber. The liquid then passed over the BSP in an upward direction.

2.6 Liquid and BSP/Biofilm Sampling

Liquid and biofilm sampling procedures differed in each experiment. Detailed sampling protocols are described in the relevant chapters.

2.7 Determination of Residual Metal Ion Concentration

In preliminary experiments, residual metal concentrations were determined using methods such as a copper ion selective electrode or a reflectoquant (Merck RQ Flex) to monitor treatment progress. However, as these methods gave poor repeatability, they were abandoned, and all residual metal ion concentrations were subsequently determined using either Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) or Atomic Absorption Spectroscopy (AAS). These methods are described in the appropriate experimental chapters.

Chapter 3

Evaluation of Pine Bark as BSP in Forced-Upflow Bioreactors

3.1 Introduction

In general, heavy metals occur in solution as positively charged ions (Naja and Volesky, 2010a), subject to various environmental conditions. These ions are attracted to negatively charged functional groups on the surfaces of biotic matter (Gadd and Griffiths, 1978; Gadd, 2009). Any fixed-bed wastewater treatment apparatus using a biological solid matrix should, therefore have the capacity to attract and retain heavy metal ions, thereby removing them from solution.

Because the reactive functional groups are found on the exposed external and internal surfaces of the pine bark matrix, and the target metal ions are contained within the liquid medium, the bioreactor should be designed to maximise contact between matrix surfaces and the wastewater (Switzenbaum, 1983). Increased contact may be achieved by using a smaller matrix particle size, resulting in a desired increase in the surface area to volume ratio (Characklis, 1984; Erikson, 2004).

Biofilms also display an affinity for attracting and retaining heavy metal ions (Gadd, 2009). As such, systems using biofilms supported by a solid matrix have been shown to provide viable systems for the removal of heavy metal ions from aqueous solutions (Costley and Wallis, 2001).

The ease of cell attachment and biofilm development is markedly influenced by the nature of the BSP, which largely determines the rate of cell accumulation and the initial micropopulation distribution (Characklis and Marshall, 1990; Martinez and Casadevall, 2007). Hence, desirable characteristics of support surfaces include high surface area to volume ratios, and an uneven surface texture to reduce shear forces and permit bacterial adhesion (Characklis and Marshall, 1990; Martinez and Casadevall, 2007).

Overall, biofilm shape is largely determined by the shape of the support material and the surrounding physical conditions (Atkinson *et al*., 1980). However, the architecture and structure of biofilms is largely governed by their EPS matrix (Schooling and Beveridge, 2006). The three-dimensional architecture of biofilms is an essential contributor to microbial stress resistance (Hentzer *et al.,* 2001; Rice *et al*., 2005).

Just as for living biomass, dead biomass also has the ability to sequester metal ions (Mongar and Wassermann, 1949; and Haug, 1959). Pine bark has this ability as documented in a number of studies (Tshabalala *et al*, 2004; Martin-Dupont *et al*., 2004; Martin-Dupont *et al*., 2006; Oh and Tshabalala, 2007, Mohan and Sumitha, 2008; Mun *et al*., 2009). Therefore, a packed-bed reactor containing pine bark as a matrix, or a biofilm supported on inert BSP, would present little novelty for the treatment of aqueous heavy metal bearing wastes. However, a review of current and historical literature has failed to unearth any studies using both forms of metal sequestering components simultaneously, viz. pine bark as BSP and an attached viable microbial biofilm, for application in wastewater treatment processes. Therefore, the use of colonised pine bark in packed-bed bioreactors appears to be untested.

The research reported here aimed to establish the suitability of various types (grades) of pine bark (varying in particle size and state of decomposition) for use as BSP in forced-upflow packed-bed bioreactors.

3.2 Experimental Procedure

3.2.1 Source of the Inoculum Culture

A sample of activated sludge was taken from the Hammarsdale municipal sewage treatment plant in KwaZulu-Natal, and enriched as described in **Chapter 2**.

3.2.2 Preparation of Liquid Medium

A single 200ℓ volume of liquid medium was prepared as follows:

- 195.8ℓ tap water
- 200mℓ undiluted Voermolas
- 4ℓ enriched inoculum

Because this experiment was set up only to establish some basic criteria for bioreactor operation, pH was not measured nor controlled. Ideally, a 10% inoculum (20ℓ) would have been used; however, because of the nature of the inoculum (Sewage based), maximum culture volume was limited to 5ℓ, to minimise laboratory health, safety and manual handling risks. Of the enriched 5ℓ stock culture, 4ℓ was used in the experiment and the remainder was retained for subculture and further experiments.

No literature pertaining to the use of Voermolas as a liquid growth medium for microbiological applications could be sourced. Therefore, preliminary experiments were conducted in order to investigate the suitability of Voermolas as a liquid growth medium for microbial growth under submerged aquatic conditions. The preliminary experiments

indicated that Voermolas concentrations, ranging between 0.1 and 5% (v/v), sustained growth of the autochthonous microbial community in the sewage inoculum, following suspension in aqueous solutions. Hence, an arbitrary value of 0.2% (v/v) final Voermolas concentration was selected for this experiment.

3.2.3 Operational Temperature Range

The experiment was conducted in a modified greenhouse with elementary temperature control (wet wall and circulatory fan). With the exception of a few mid-day highs that reached 40°C, ambient daytime temperatures were maintained at 30°C ±6°C for the duration of the experiment. No attempt was made to control the temperature of the liquid medium because this was unlikely to be feasible in any practical application.

3.2.4 Construction of Bioreactors

A single glass, domestic aquarium was divided into five identical bioreactor chambers. Each bioreactor comprised an inlet chamber with an adjacent BSP-containing chamber separated by a vertical baffle plate. The baffle plate was suspended 25mm above the bioreactor floor, thus allowing the influent to pass under the baffle plate to the bottom of the BSP chamber. The BSP chamber housed a fibreglass BSP container, the top and bottom of which were perforated to allow the liquid medium to flow vertically upwards through the container. The BSP containers were sealed against the internal walls of the bioreactor to prevent liquid by-passing the BSP. The liquid medium exited at the top of the BSP chamber and was led, via an outflow pipe, back to the reservoir for mixing and reintroduction to the top of the inlet chamber. This constituted a closed-loop recirculation system.

Figure 3.1 shows a schematic representation of a cross section of a single bioreactor.

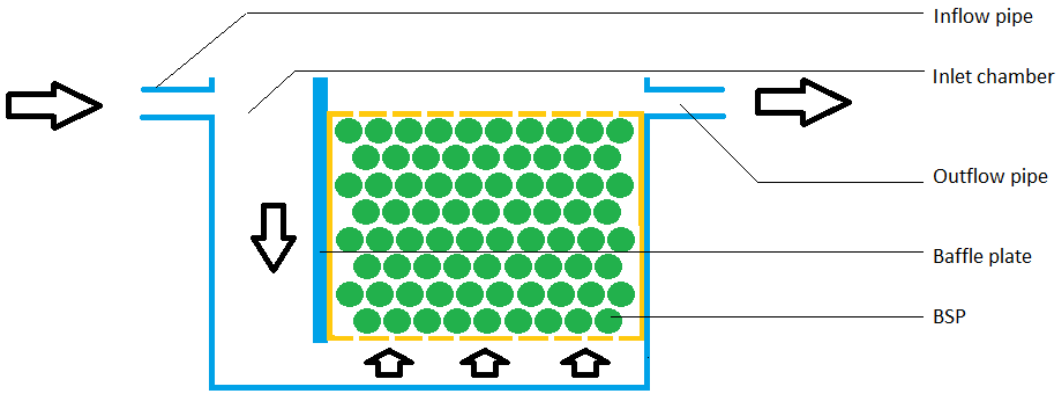

Figure 3. 1: Diagram showing a single bioreactor as used in all laboratory-scale experiments. Arrows show the passage of the bulk liquid through the bioreactor

The reservoir and outflow pipes were made from high-density-polyethylene (HDPE) and all tubing was plastic based. Circulation was provided by identical Project-Step 2 (220-230 V, 6W) submerged electric water feature pumps. An initial flow-through rate of 2.0ℓ.min^{-1} was selected because Singh and Mishra (1990) reported maximum biosorption of phenolic compounds on waste saw dust occurred at this rate. Flow-through rates were measured by recording the volume of liquid that passed through the reactor outflow in five minutes.

3.2.5 Configuration of Bioreactors

Both composted and uncomposted pine bark BSP were investigated. All pine bark was supplied free-of-charge by Gromed Organics (no longer trading) located in Crammond, KwaZulu-Natal. The two forms of pine bark used were commercially available as potting medium for plants (composted bark) and decorative pine bark (uncomposted). Both forms of bark were available to purchase ready-for-use at garden centres.

Each bioreactor contained a unique pine bark BSP based on particle size range and its state of decomposition.

The bioreactors were packed as described in **Table 3.1**.

- The composted pine bark used in Bioreactors A and B was available in two grades (grouped according to particle size), i.e. <16mm and 16mm-25mm.
- The uncomposted pine bark used in Bioreactors C, D and E was available in three grades (grouped according to particle size), i.e. <16mm, 16mm-25mm and 25mm-50mm.

Table 3.1: Bioreactor matrix type (composted/uncomposted pine bark) and particle size (mm), together with initial flow rates used in the pine bark evaluation experiment

Bioreactor	Support matrix type	Particle size	Initial flow rate* ($\ell.min^{-1}$)
A	Composted pine bark	<16mm	1.99
B	Composted pine bark	16-25mm	1.98
C	Uncomposted pine bark	<16mm	2.01
D	Uncomposted pine bark	16-25mm	2.01
E	Uncomposted pine bark	25-50mm	2.00

* Initial rates of liquid passage through the reactor were measured at the outflow pipe.

3.2.6 Operation of Bioreactors

In order to ensure that the liquid medium was identical, at least initially, in all the bioreactors, each bioreactor was fed from a common 200ℓ reservoir at a uniform rate of 2.0ℓ.min^{-1}. The effluent from all the bioreactors was returned to the reservoir for mixing with the feedstock. The medium in the reservoir was continuously mixed using a Biowave 2000 aquarium pump operating at 4000ℓ.h^{-1}. The experiment ran continuously for 20 days.

3.2.7 Characterisation of the Biofilms

3.2.7.1 Sampling Procedure

At termination of the experiment ten individual pine bark nuggets were selected at random from the BSP chamber of each bioreactor (half for biofilm thickness determinations, the remainder for ESEM analysis). All samples were stored in liquid medium taken from the shared reservoir and kept at 4^{o}C until analysis.

3.2.7.2 Determination of Biofilm Thickness

Biofilm thickness was determined for all BSP types by measuring random cross sections of biofilm attached to random samples of bark. Five samples from each bioreactor were examined. Cross sections were measured under 5x magnification, using a precision ruler. Triplicate measurements, taken from different locations, were obtained for each cross section.

3.2.7.3 Electron Microscopic Analysis of the Biofilm Morphology

Traditional scanning electron microscopes require samples to be dehydrated and coated with a conductive, electron-dense covering, e.g. gold/palladium sputter coating (Bruton, 2000). Conversely, an Environmental Scanning Electron Microscope (ESEM) allows specimens to be viewed uncoated and fully hydrated. A Phillips XL30 ESEM was used to study the surface morphology of randomly selected biofilm samples under high magnification (650x - 6500x).

The respective biofilms on five colonised pine bark nuggets from Bioreactor B (composted pine bark) and five pine bark nuggets from Bioreactor D (uncomposted pine bark) were ESEM analysed. Corresponding samples of fresh, composted pine bark (16-25mm) and uncomposted pine bark (16-25mm), were also examined for comparative purposes. Specimens, approximately 5 x 5mm, were carefully removed from the pine bark samples and mounted in carbon paste for immediate analysis in the microscope. Microscope settings are indicated on each electron micrograph.

3.3 Results and Discussion

3.3.1 Biofilm Development: Initial Observations

A biofilm was visible on the BSP surfaces in all bioreactors after 20 days. The results of the pine bark grade evaluation experiment are presented in **Table 3.2**.

Table 3.2: Results of the BSP evaluation experiment after 20 days

Bioreactor	Biofilm thickness (mm)	Observations	Initial flow rate ($\ell.min^{-1}$)	Final flow rate ($\ell.min^{-1}$)
A Composted pine bark <16mm	Not measured	A thick biofilm formed over the entire matrix. BSP were held together by the biofilm. All the interstitial spaces were occluded by microbial biomass.	1.99	0.31
B Composted pine bark 16-25mm	Not measured	A thick biofilm formed over the entire matrix. BSP were held together by the biofilm. All the interstitial spaces were occluded by microbial biomass.	1.98	0.27
C Uncomposted pine bark <16mm	3.5	Void spaces between the BSP were visible i.e. the interstices were not occluded by biomass. The flow rate remained unchanged, although liquid flow through the bioreactor chamber was impeded. This was evidenced by the 42mm disparity in the heights of the liquid columns in the inlet and outlet (See **Figure 3.2**).	2.01	2.01
D Uncomposted pine bark 16-25mm	2.0	Void spaces between the BSP were visible i.e. the interstices were not occluded by biomass. Liquid flow through the chamber was not impeded.	2.01	2.01
E Uncomposted pine bark 25-50mm	2.0	Void spaces between the BSP were visible i.e. the interstices were not occluded by biomass. Liquid flow through the chamber was not impeded.	2.00	2.00

During the first two days of operation, some of the matrix particles in Bioreactors A and B were too small and light to be retained in the BSP chambers and, therefore, were washed out. However, the surfaces of the BSP remaining in these two bioreactor chambers were covered with confluent microbial growth. This was so prolific that interstitial spaces were no longer present and the entire BSP in both these bioreactors had formed solid “cakes”. This indicated that composted pine bark readily supported biofilm development, which was not unexpected since composting is a microbially mediated process (Alexander, 1961). Therefore, it could be expected that the bark had an extensive resident microbial community before exposure to the inoculum and liquid medium. However, because of the small particle size (<5mm) and consequent small interstitial spaces, the latter were rapidly filled by microbial biomass, thus severely restricting liquid percolation through the bioreactor. This is supported by the differences in initial and final flow-through rates shown in **Table 3.2**. These restrictions to liquid flow through the BSP chambers in Bioreactors A and B eventually caused their respective inlet chambers to overflow, because the liquid medium was unable to enter or exit the BSP chamber at a rate matching that of the inflow.

A biofilm with thickness varying from 1mm - 5mm (average 3.5mm) developed on the BSP in Bioreactor C. Although the rate at which liquid returned to the reservoir at the end of the experiment closely matched that at the start, a difference in liquid head height of 42mm had formed between the inlet chamber and the outlet from the BSP chamber (**Figure 3.2**).

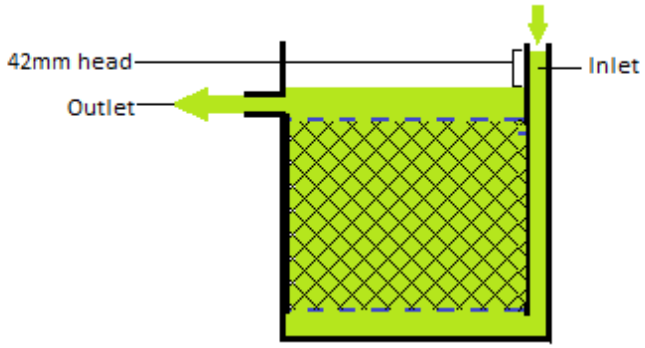

Figure 3. 2: Diagram showing the difference in liquid level between inlet and outlet of Bioreactor C, indicating a restriction to flow through the matrix.

Because both chambers were open to the atmosphere, the difference in head height indicated that increased pressure at the base of the BSP chamber was required to maintain the outlet flow rate at 2.01$\ell.min^{-1}$. Thus, as with Bioreactors A and B, the flow of liquid through the BSP chamber had been arrested, in this case by microbial growth in the smaller interstitial void spaces. This forced the liquid to follow preferential flow paths through the larger pores in the matrix. The establishment of preferential flow paths (channelling) through packed-bed bioreactors, is a frequent problem experienced following excessive microbial growth. This reduces contact between sorbent and sorbate and consequently leads to gross inefficiencies in the system (Hall, 1992).

The BSP in Bioreactors D and E supported biofilms similar to those observed in Bioreactor C, varying in thickness from 1mm - 3mm (average of 2mm). In the BSP chambers of these bioreactors some interstitial spaces became completely occluded by biomass. This occurred because the liquid velocity through the chambers was not sufficient to generate the shear forces required to regulate biofilm thickness (Characklis and Marshall, 1990).

The prolific microbial biomass produced over the 20 day period of the experiment are likely due to the combined effects of the relatively high day time laboratory temperatures (30-40^{o}C)

and the nutrient rich nature of the liquid medium, which contained 0.2% (v/v) Voermolas. Another factor could be the provision of additional organic nutrients leaching from the pine bark BSP as described by Tshabalala *et al.* (2004), Oh and Tshabalala, (2007) Ribe *et al*. (2011) and Ribe *et al*. (2012).

3.3.2 Biofilm Development: Environmental Scanning Electron Microscopy Investigation

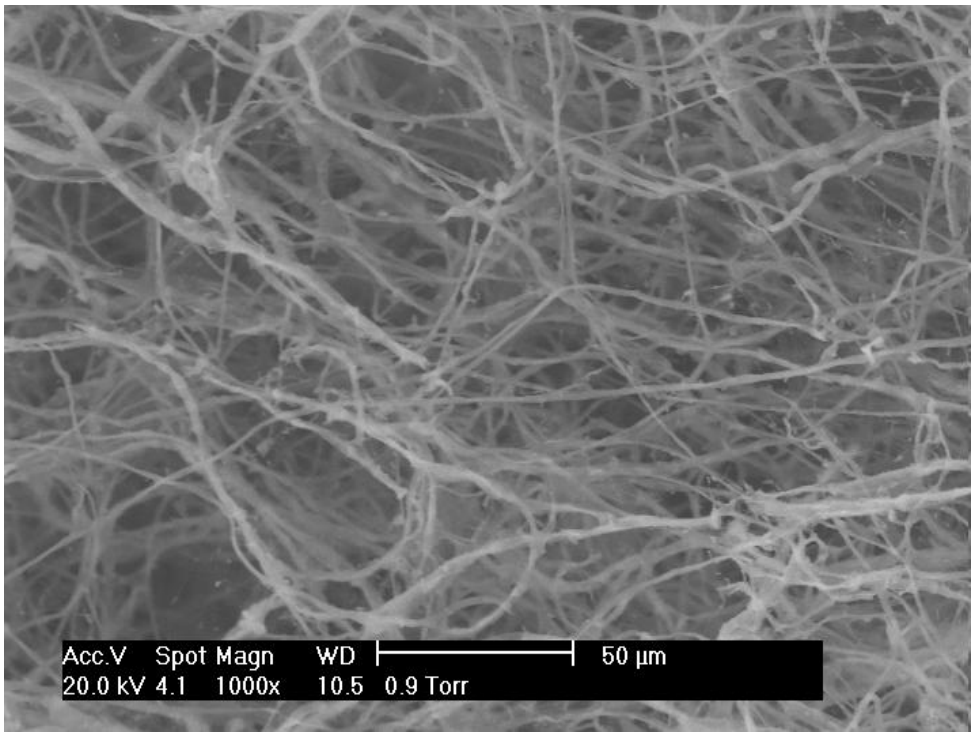

Plate 3.1: ESEM Image of composted pine bark (16 – 25mm) before use as BSP

Plate 3.1 shows an interwoven mat of filamentous microorganisms covering the entire surface of a composted pine bark nugget before use in the bioreactors. None of the nuggets examined showed any uncolonised surfaces. As stated previously, this was not surprising since the composting process is microbially mediated (Alexander, 1961).

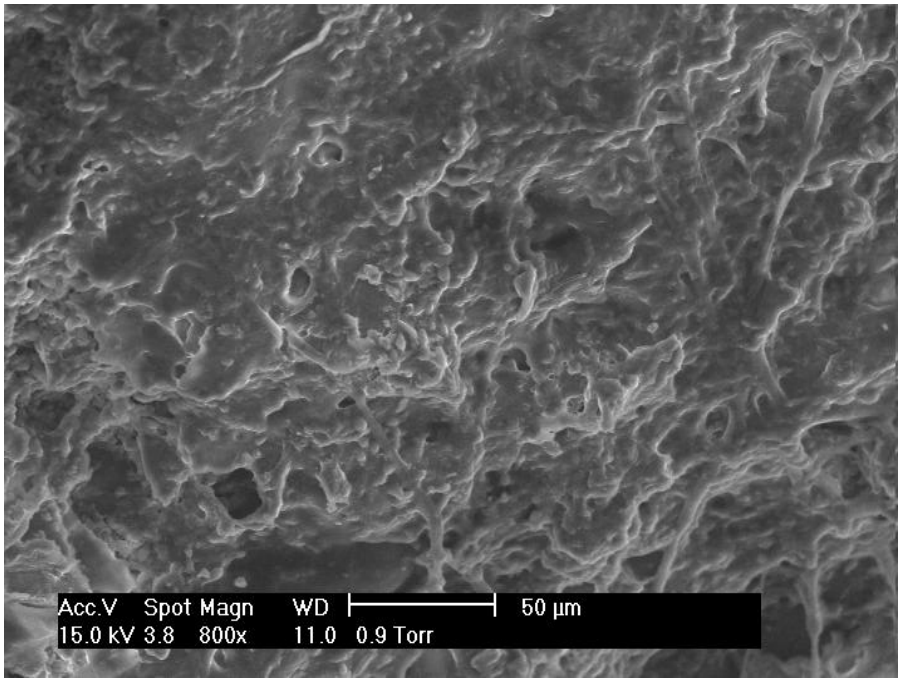

Plate 3.2: Surface view of the biofilm that had established on a composted pine bark (16 - 25mm) nugget from Bioreactor B after 20 days

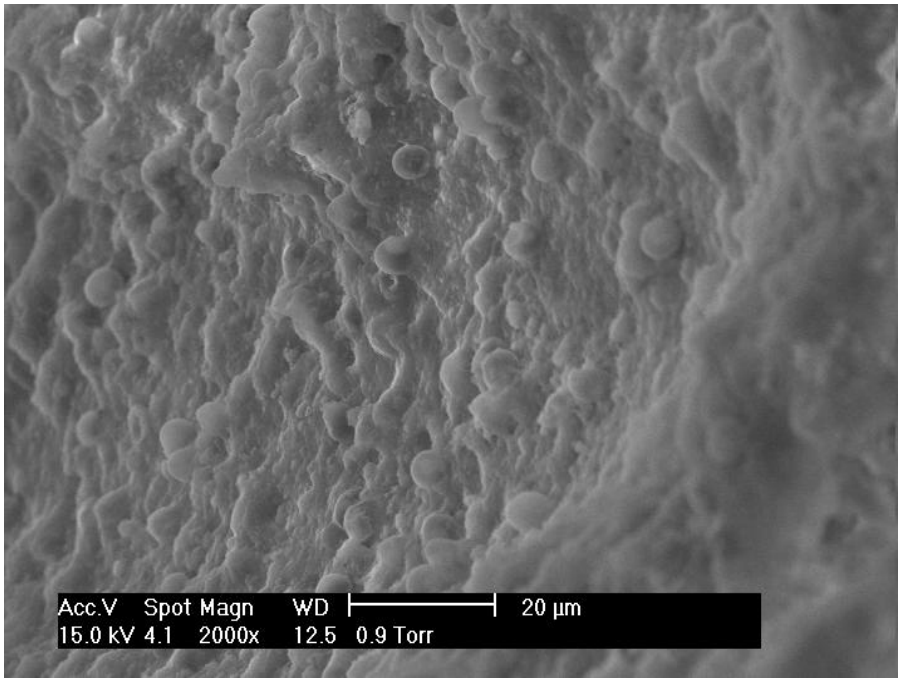

Plate 3.3: Area of continuous biofilm that had established on a composted bark nugget (16 – 25mm) from Bioreactor B after 20 days of continuous operation.

Plates 3.2 and 3.3 show typical views of the biofilm surface in Bioreactor B at low and high magnifications, respectively.

The biofilm appeared to be continuous, with no gaps/breaks in the EPS visible in any of the samples viewed. At 800x magnification, filamentous structures and individual cells were discernible underlying the EPS layer (**Plate 3.2**). **Plates 3.2 and 3.3** both show the irregular, rather than flat, surface architecture of the biofilm, suggesting an increased surface area for metal ion adsorption. This is particularly evident in **Plate 3.3** which shows a number of spherical bodies, possibly groups of cells, on the surface of the biofilm. Subsurface architecture of the biofilm was not investigated as no suitable cross sections through the biofilm could be produced and no uncolonised pine bark surfaces were seen. However, a successful method was developed subsequently allowing cross sections of biofilm to be made (**Chapter 5)**.

Initial colonisation of the composted bark surfaces by filamentous organisms (**Plate 3.1**) and the resultant established biofilm (**Plates 3.2** and **3.3**), indicate that if such bark was used as BSP in bioreactor applications, the establishment of a conditioning film, or provision of primary colonisers, as described by Blenkinsopp and Costerton, (1991) and James *et al*. (1995), would not be necessary. The same would apply, however, were previously uncolonised BSP to be used. Additionally, the use of composted pine bark in bioreactor applications could possibly lead to reduced start-up times.

Plates 3.4 - 3.7 show representative samples of uncomposted bark.

Plate 3.4 shows the irregular surface characteristics of uncomposted pine bark nuggets. This is regarded as advantageous when establishing a biofilm in submerged conditions (Bruce and Hawkes, 1983; Characklis, 1984; Bonastre and Paris, 1989), because such irregularities serve to diminish liquid shear forces adjacent to the support's surface, thereby promoting microbial adhesion, and the establishment of a conditioning film (Baker, 1984; Characklis and Marshall, 1990).

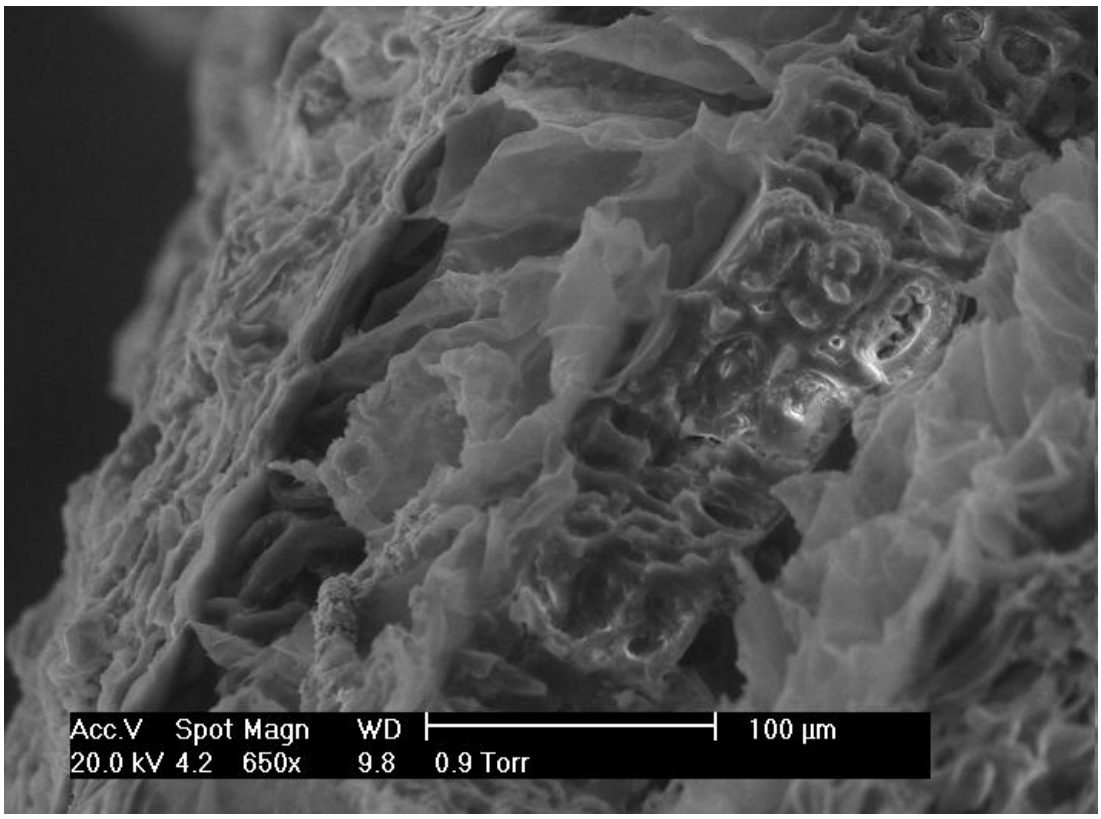

Plate 3.4: A typical example of the surface topography of 16-25mm uncomposted pine bark nuggets prior to use as BSP

In contrast to composted pine bark (Plate 3.1), no microorganisms were seen initially on the surfaces of any of the uncomposted pine bark nuggets examined. This suggested that, unlike composted pine bark, in order to develop a viable biofilm on uncomposted pine bark matrix the full biofilm start-up process shown in **Figure 1.1** (**Section 1.6.1**), including the establishment of a conditioning film and primary colonisation (Blenkinsopp and Costerton, 1991; James *et al.*, 1995), would be necessary. However, this also suggests that up to 100% of the uncomposted pine bark surface would be available for metal ion adsorption at bioreactor start-up.

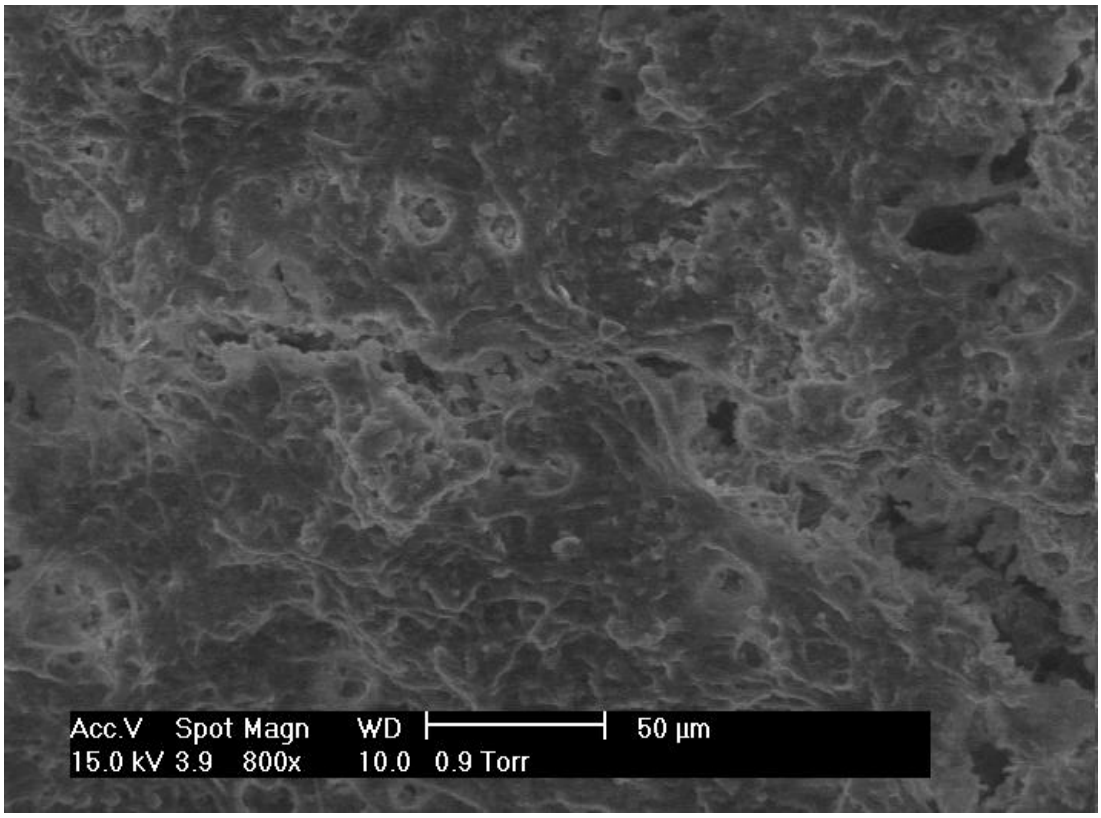

Plate 3.5: The surface characteristics of the biofilm that developed on uncomposted pine bark nuggets (<16mm) in Bioreactor C after 20 days. Channels and voids in the EPS are visible

Plate 3.5 shows that, except for occasional fissures and small (<20 µm) gaps in the EPS layer, [which Telgman *et al*. (2004) described as "channels" and "voids" respectively] the biofilm that formed on the uncomposted pine bark BSP was morphologically not dissimilar to that found on the composted bark (**Plate 3.2**). Telgman *et al*. (2004) state that the channels and voids are early indicators of the biofilm sloughing process, initiated by localised liquid shear forces acting on the biofilm surface. Biofilm sloughing was not investigated here, but it is likely that the final flow-through rate of 0.27ℓ.min^{-1} in Bioreactor B was too slow to generate shear forces sufficient to cause sloughing of the biofilm.

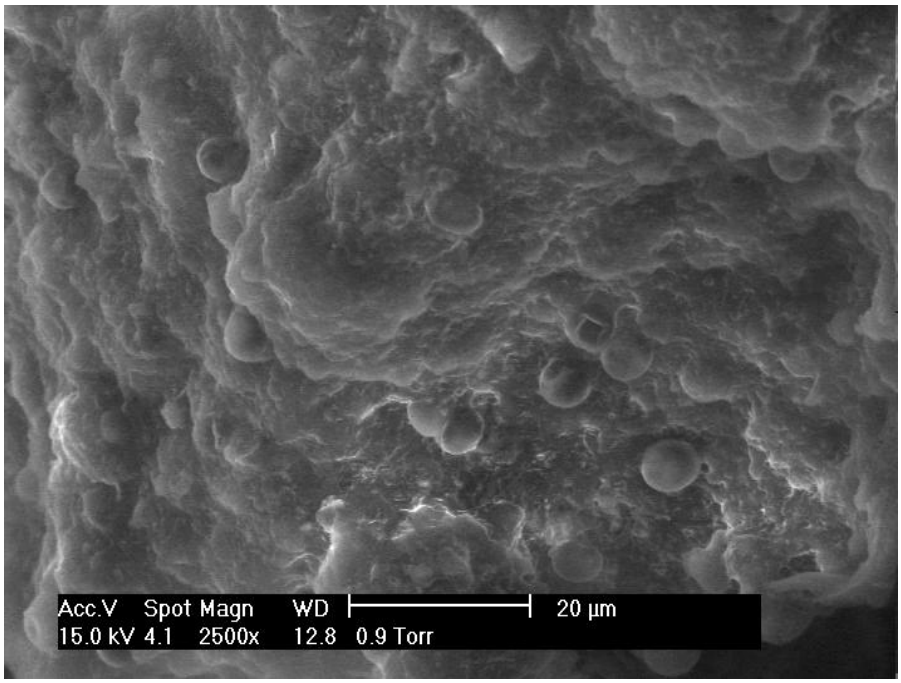

Plate 3.6: An area of continuous biofilm which formed on the surface of an uncomposted pine bark BSP nugget (16 – 25mm) from Bioreactor D after 20 days

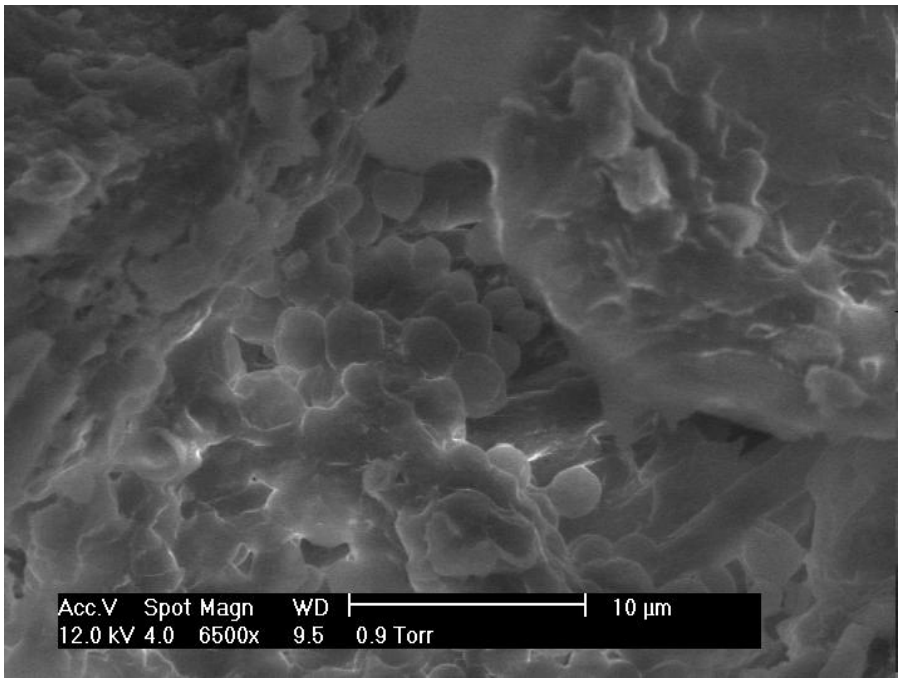

Plate 3. 7: View inside a void space in the biofilm which had formed on an uncomposted bark BSP nugget (16 – 25mm) from Bioreactor D after 20 days. Note the large number of cells underlying the surface EPS layer

Plates 3.6 and 3.7 show representative samples of an area of continuous biofilm and a void in the EPS, respectively. The area of continuous biofilm on the uncomposted pine bark from Bioreactor D, shown in **Plate 3.6**, was visually indistinguishable from that which occurred on the corresponding composted bark in Bioreactor B. This was not unexpected because although the composted bark in Bioreactor B was already colonised, macro-environmental conditions were the same in all the bioreactors because they shared a biologically and nutritionally homogenous liquid feedstock, and experienced identical ambient temperatures.

Plate 3.7 shows the only surface feature differentiating the biofilms in Bioreactors B and D, i.e. a void in the EPS, visible only on the uncomposted pine bark BSP taken from Bioreactor D. In all cases the predominant residents of the biofilm void spaces were clusters of individual cocci. However, some filamentous structures were also discernable. Although irreversible cellular adhesion has been reported in the absence of EPS (Kristich *et al*., 2004), filamentous biofilm structures tend not to show any signs of sloughing (Wagner *et al*., 2010). The apparent complete colonisation of the composted BSP by filamentous microorganisms prior to commencement of the experiment may, therefore, also account for the apparent lack of sloughing of the biofilm in Bioreactor B (as indicated by the lack of fissures or voids in the EPS surface).

3.4 Conclusions

- Composted pine bark supported biofilm growth. However, it proved to be unsuitable for use as BSP because the degraded particles caused operational difficulties by escaping from the BSP chamber and blocking the bioreactor plumbing.
- The filamentous microorganisms which colonised the composted pine bark prior to use as BSP are likely to have significantly reduced sloughing of the biofilm from the BSP, thereby giving rise to preferential flow paths through the BSP and ultimately causing the bioreactor to fail.
- Uncomposted pine bark adequately supported microbial attachment and biofilm development.
- Over time, uncomposted pine bark supported copious biofilm growth, and, in addition, proved sufficiently physically robust for use in the laboratory-scale forced-upflow bioreactors.

Chapter 4

Effect of Supplementary Nutrient Concentration on Metal Biosorption in Forced-Upflow Bioreactors with Pine Bark as BSP

4.1 Introduction

Pine bark presents some distinct advantages for use in wastewater treatment (discussed in Chapter1). Therefore, in recent years interest in using this material for treating a wide variety of wastewaters has been increasing. Lens *et al. (*1994) used pine bark to treat domestic waste water in percolator columns; Vasquez *et al*. (2002) sorbed cadmium and mercury with pine bark. Bras *et al*. (2005) presented pine bark as a natural, abundant sorbent for hydrophobic organic compounds such as pentachlorophenol, and Nehrenheim *et al*. (2008) used pine bark to remove metals from low strength landfill leachate. However, the use of pine bark and a living biofilm together in a dual-sorbent system has not yet been reported.

When designing and commissioning wastewater treatment systems using living biomass to remove heavy metal ions from solution, a wide variety of factors must be considered. Among these the continued development and growth of the biofilm is important (Gadd, 2009; Naja and Volesky, 2010b). In this regard, a potential disadvantage arising when using pine bark for this purpose is its tendency to release organics into the liquid medium (Vasquez *et al.,* 2002; Haussard *et al.,* 2003; Tshabalala *et al.,* 2004; Oh and Tshabalala, 2007). Not only

could this cause secondary treatment problems, but such organics may also produce toxic effects in the resident microorganisms. Conversely, it is possible that these organics may provide a nutrient source for a suitably adapted microbial community (Tshabalala *et al.,* 2004; Oh and Tshabalala, 2007).

The availability of nutrients is of paramount importance to the growth and development of any living biomass. The macro-effects of increased biomass, as a direct result of cellular growth and polymer synthesis, are that more cellular surface area is available for adsorption of metal cations (Wood and Wang, 1983; Gadd and White, 1986; McHale and McHale, 1994). In biotechnological applications, the rate of intracellular metal uptake is directly influenced by the surrounding medium, including the availability of a suitable energy source to support continued cellular activity (Brock and Madigan, 1991).

The research reported in this chapter aimed to establish some nutritional criteria to optimise the removal of copper, zinc and cadmium ions from a synthetic industrial wastewater using upflow, packed-bed bioreactors with pine bark as BSP.

4.2 Experimental Procedure

Two separate experiments were conducted.

- Experiment 1 investigated the level of supplementary nutrition required to establish a biofilm in a new bioreactor start-up on a fresh (uncolonised) uncomposted pine bark BSP in the presence of Cu^{2+}, Zn^{2+} and Cd^{2+}, each at 100mg.ℓ^{-1}.
- Experiment 2 was conducted to optimise the levels of supplementary nutrients required for sustained optimal metal ion removal in bioreactors with an established biofilm in place.

4.2.1 Construction of the Bioreactors

The five forced-upflow bioreactors used here have already been described (**Chapter 3**. However, each bioreactor was fed by, and drained into, its own individual reservoir as shown in **Plate 4.1.** **Figure 3.1** shows a schematic cross section of a single bioreactor.

Plate 4. 1: Elevated side view showing the five identical bioreactors A (Background), each with its own reservoir B (Foreground). Also visible are the influent (I) and effluent (E) pipes and the electrical cables to the submerged pumps in the reservoirs (B)

4.2.2 Experiment 1: Investigation of the Nutritional Requirements for Establishing a Biofilm on Uncomposted Pine Bark BSP for the Removal of Metal Ions from Aqueous Solution

4.2.2.1 Configuration of the Bioreactors (Experiment 1)

One bioreactor served as a control with no inoculum or supplementary nutrient sources added. Four experimental bioreactors were established to investigate the effects of 0.1% and 0.2% (v/v) Voermolas on the removal of copper, zinc and cadmium from aqueous solution, using fresh pine bark as BSP. Duplicate experiments were run for each Voermolas concentration. The experimental conditions are shown in **Table 4.1**.

Table 4.1: Bioreactor operating conditions (Experiment 1)

Bioreactor	A (Control)	B	C	D	E
Nutritional supplement	0	0.1%(v/v) Voermolas[c]	0.1%(v/v) Voermolas[c]	0.2%(v/v) Voermolas[d]	0.2%(v/v) Voermolas[d]
BSP dry mass (kg)	3.04	2.97	2.99	3.01	2.99
Pump speed (ℓ.min-1)	2	2	2	2	2
Hydraulic retention time[a] (min)	2.5	2.5	2.5	2.5	2.5
Inoculum (%)	0	10	10	10	10
Calculated heavy metal ion starting concentration[b] (mmol)	$[Cu^{2+}]$ 1.33 $[Zn^{2+}]$ 1.53 $[Cd^{2+}]$ 0.89	$[Cu^{2+}]$ 1.33 $[Zn^{2+}]$ 1.53 $[Cd^{2+}]$ 0.89	$[Cu^{2+}]$ 1.33 $[Zn^{2+}]$ 1.53 $[Cd^{2+}]$ 0.89	$[Cu^{2+}]$ 1.33 $[Zn^{2+}]$ 1.53 $[Cd^{2+}]$ 0.89	$[Cu^{2+}]$ 1.33 $[Zn^{2+}]$ 1.53 $[Cd^{2+}]$ 0.89
Total volume (ℓ)	37	37	37	37	37

a Hydraulic retention time was measured at the start of the experiment
b 100mℓ aliquots of the metal stock solution containing all three metals as described in **Chapter 2** were added to each bioreactor
c 37mℓ Voermolas in 37ℓ synthetic wastewater
d 74mℓ Voermolas in 37ℓ synthetic wastewater

4.2.2.2 Description of the Inoculum Culture (Experiment 1)

A sample of activated sludge from the Hammarsdale municipal sewage treatment plant (enriched with copper, cadmium and zinc), as described in **Chapter 2,** was used in the experimental bioreactors only.

4.2.2.3 Preparation of Liquid Media (Experiment 1)

Synthetic wastewaters were formulated using 0.2% and 0.1% (v/v) (final concentration) Voermolas solutions and appropriate aliquots of (Analar Grade) $CuCl_2.2H_2O$, $ZnCl_2$ and $(CH_3COOH)_2Cd.2H_2O$ to yield final metal ion concentrations of 100mg.ℓ^{-1}. All metal salts were dissolved into a single 1ℓ stock solution; their individual masses were calculated to yield 100mg.ℓ^{-1} of each metal when a 100mℓ aliquot of the 1ℓ stock solution was diluted to the total volume of a bioreactor chamber and reservoir system (37ℓ). The masses of metal salts used are shown in **Table A1** (**Appendix A)**.

No literature pertaining to the use of Voermolas as a nutrient liquid growth medium for microbiological applications could be sourced. The findings reported in **Chapter 3** showed that a biofilm could be successfully established in less than 20 days on an uncomposted pine bark BSP in a 0.2% (v/v) Voermolas solution. However, the amounts of microbial biomass produced at this Voermolas concentration were considered excessive; therefore a final concentration of 0.1% (v/v) Voermolas was selected for the current experiments. The bulk liquid described in **Chapter 3** received additional organic and biomass contributions from the degrading composted pine bark, and the microorganisms comprising the biofilms were not subjected to any inhibitory effects of metal ions. Since the experiments reported here used uncomposted pine bark, 0.2% (v/v) (final concentration) Voermolas solutions were also tested, for comparative purposes. The liquid medium used in the experimental bioreactors comprised 10% inoculum, and was supplemented with 100mℓ aliquots of the metal stock solution containing all three metals.

4.2.2.4 Pine Bark BSP Selection (Experiment 1)

Uncomposted pine bark (particle size <16mm) was selected for use in this experiment. However, the pine bark was washed and sieved to remove all particles smaller than 6mm. Thus the particles ranged in size from 6mm to 16mm. The prepared bark was air dried for 24h before being placed in the BSP chambers.

4.2.3 Experiment 2: Optimisation of the Nutritional Requirements for Sustained Biofilm Activity on Uncomposted Pine Bark BSP for the Removal of Metal Ions from Aqueous Solution

4.2.3.1 Inoculum Culture (Experiment 2)

Because a biofilm was already established in the experimental bioreactors (Experiment 1), no additional inoculum was added to the liquid medium in any of the bioreactors.

4.2.3.2 Preparation of the Liquid Medium (Experiment 2)

To ensure the same heavy metal concentration in all five bioreactors, the remaining 500mℓ of the stock metal ion solution (**Chapter 2**) was made up to 185ℓ with municipal tap water in a sterilised container. Thirty seven litres of this solution was used to fill the control bioreactor. Thereafter, the remaining 148ℓ was divided in half and amended with appropriate aliquots of Voermolas, to give final concentrations of 0.1% and 0.2%, and distributed among the experimental bioreactors.

4.2.3.3 Configuration of the Bioreactors (Experiment 2)

The same bioreactors described in Experiment 1 were used in this experiment. However, they were all completely drained after Experiment 1 and filled with the fresh medium described in **Section 4.2.2.4**. The BSP chambers were not opened or interfered with in any way. The pump speed was left unchanged at 2ℓ.min^{-1} and the bioreactors were operated as described in **Table 4.2** for 216h. Again, one bioreactor served as a control and duplicate bioreactors were run for each of the two Voermolas concentrations.

Table 4. 2: Reactor operating conditions (Experiment 2)

Bioreactor	**A (Control)**	**B**	**C**	**D**	**E**
Nutritional supplement	0	0.1%v/v Voermolas[c]	0.1%v/v Voermolas[c]	0.2%v/v Voermolas[d]	0.2%v/v Voermolas[d]
BSP dry mass (kg)[a]	3.04	2.97	2.99	3.01	2.99
Pump speed (ℓ.min^{-1})	2	2	2	2	2
Hydraulic retention time[b] (min)	2.5	2.5	2.5	2.5	2.5
Inoculum (%)	0	0	0	0	0
Calculated heavy metal ion starting concentration[b] (mmol)	[Cu^{2+}] 1.33 [Zn^{2+}] 1.53 [Cd^{2+}] 0.89	[Cu^{2+}] 1.33 [Zn^{2+}] 1.53 [Cd^{2+}] 0.89	[Cu^{2+}] 1.33 [Zn^{2+}] 1.53 [Cd^{2+}] 0.89	[Cu^{2+}] 1.33 [Zn^{2+}] 1.53 [Cd^{2+}] 0.89	[Cu^{2+}] 1.33 [Zn^{2+}] 1.53 [Cd^{2+}] 0.89
Total volume (ℓ)	37	37	37	37	37

a The BSP was not changed from that used in Experiment 1, and therefore the dry mass of the BSP remained unchanged
b Hydraulic retention time was measured at the start of the experiment
c 37mℓ Voermolas in 37ℓ synthetic wastewater
d 74mℓ Voermolas in 37ℓ synthetic wastewater

4.2.4 General Experimental Conditions

4.2.4.1 Operational Temperature Range

Both experiments were conducted in a modified greenhouse. Ambient daytime laboratory temperature was maintained at 24°C ±6°C for the duration of the experiment.

4.2.4.2 Liquid Sampling and Sample Preparation

Samples (40mℓ) were drawn from the return-flow pipes of each reactor (between the reactor and its reservoir) for analysis. The samples were gravity filtered through fluted Whatman 3 µm filter paper and stored at 4^{o}C. Before quantitative analysis, 10x and 100x dilutions of the filtered samples were prepared using deionised water.

4.2.4.3 pH Determinations

The pH of the samples was measured with a Crison micro 2002 pH meter calibrated using pH 4.0 and pH 7.02 standards.

4.2.4.4 Determination of Residual Metal Ion Concentrations

A Varian 702 ES ICP-OES was used because it allows simultaneous identification and quantification of all metal ions present in the mixed-metal samples, over a wide range of concentrations. Samples to be analysed were drawn into the instrument where they passed through a nebuliser for atomisation. The atomised samples subsequently entered an argon plasma flame where they were broken down into their charged ionic constituents. Upon transformation into a gaseous atomic state, optical emission of the characteristic wavelength

specific light, emitted by the excited atoms, was used for quantitative metal analysis. The instrument measured the samples in triplicate but only mean values were provided.

Standard solutions of 0, 0.5, 1, 2.5, 5, 10 and 15mg.ℓ^{-1} Cu^{2+}, Zn^{2+} and Cd^{2+} were used to calibrate the spectrometer. The wavelengths used for detection of the three metals studied are shown in **Table 4.3**.

Table 4. 3: Wavelengths used for ICP-OES analysis

Element	Wavelength (nm)
Cu^{2+}	199.970
Zn^{2}	213.897
Cd^{2+}	214.439

4.2.4.5 Determination of Metal-Biomass Binding

Four different parameters were evaluated in order to compare bioreactor performance: Total M^{2+} sorbed, Relative M^{2+} sorption percentage, M^{2+} sorbed per g pine bark (µmol); and M^{2+} sorbed per g pine bark (mg). The respective formulae used were as follows:

Total mmol M^{2+} sorbed (Q_t) was calculated according to **Equation 4.1**:

$$Q_t = ([M]_i - [M]_f)V$$

(**Equation 4.1**)

Where V = solution volume; $[M]_i$ = initial metal ion concentration; and $[M]_f$ = final metal concentration.

Relative M^{2+} sorption percentage (Q_r) was calculated according to **Equation 4.2**:

$$Q_r = \frac{([M]_i - [M]_f)}{[M]_i}$$

(**Equation 4.2**)

Where $[M]_I$ = initial metal ion concentration; and $[M]_f$ = final metal concentration.

M^{2+} sorbed per g (Q_s) expressed in both (µmol) and (mg) were calculated according to **Equation 4.3,** using appropriate units.

$$Q_s = \frac{([M]_i - [M]_f)\ V}{m}$$

(**Equation 4.3**)

Where V = solution volume $[M]_i$ = initial metal ion concentration, $[M]_f$ = final metal concentration and m = the dry mass of BSP.

4.3 Results and Discussion

4.3.1 Experiment 1

The bioreactors were operated for 384h (16 days), in order to investigate the amount of supplementary nutrition required to establish, and maintain, a biofilm suitable for the removal of the heavy metal ions from the synthetic wastewater. Liquid samples were taken at predetermined intervals and the residual metal ion concentration of each sample was measured by ICP-OES. The averaged results, expressed as millimolar values, are reported in **Table 4.4**, Raw data are provided in **Appendix B, Tables B1 and B2**. The averaged data are also presented graphically in **Appendix B, Figures B1 – B7**.

Biosorption was evaluated by considering absolute indicators (the residual metal concentrations and the total moles of metal sorbed) as well as relative indicators (percentages biosorbed and metal biosorption relative to the amount of pine bark present). Cu^{2+} was sorbed most effectively in the control (no supplementary nutrients added) and least effectively in the bioreactors containing 0.2% (v/v) Voermolas. In contrast, both Zn^{2+} and Cd^{2+} sorption was greatest in the bioreactors containing 0.2% (v/v) Voermolas at bioreactor start-up, with biosorption levels lower under 0.1% (v/v) Voermolas start-up concentrations. These findings are summarised as follows:

Cu^{2+} biosorption occurred in the order:	Control >0.1% Voermolas > 0.2% Voermolas
Zn^{2+} biosorption occurred in the order:	0.2% Voermolas ≥ 0.1% Voermolas > Control
Cd^{2+} biosorption occurred in the order:	0.2% Voermolas > 0.1% Voermolas >Control

Table 4. 4: Average residual metal ion concentration after selected time intervals and corresponding pH values (Experiment 1). Mean values and variances are shown for the bioreactors containing 0.1% and 0.2 % (v/v) Voermolas

	Control				0.1% (v/v) Voermolas				0.2% (v/v) Voermolas			
Time (h)	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**
0	0.68	1.09	0.83	5.9	0.75 ±0.01	1.18 ±0.03	0.89 ±0.02	7.00 ±0.10	0.77 ±0	1.22 ±0.02	0.92 ±0.02	7.05 ±0.05
1	0.61	1.04	0.85	5.9	0.55 ±0.05	1.13 ±0.03	0.85 ±0	6.90 ±0	0.65 ±0.05	0.98 ±0.08	0.74 ±0.05	7.15 ±0.15
2	0.61	1.13	0.87	5.7	0.50 ±0.06	1.13 ±0.05	0.86 ±0.04	7.00 ±0	0.63 ±0.05	0.96 ±0.03	0.74 ±0.05	7.10 ±0.10
6	0.55	1.06	0.8	5.2	0.48 ±0.02	1.18 ±0.01	0.84 ±0.07	7.15 ±0.05	0.59 ±0.07	1.00 ±0.02	0.81 ±0.01	7.30 ±0.10
12	0.41	0.92	0.72	4.9	0.52 ±0.00	1.00 ±0.04	0.77 ±0.06	7.25 ±0.15	0.47 ±0.03	1.12 ±0.11	0.76 ±0.02	7.40 ±0.20
24	0.36	0.9	0.73	4.8	0.36 ±0.03	0.97 ±0.02	0.74 ±0.04	7.05 ±0.15	0.45 ±0.01	0.93 ±0.01	0.72 ±0.03	7.55 ±0.15
36	0.28	0.92	0.77	4.7	0.31 ±0.04	0.92 ±0.09	0.72 ±0.07	7.20 ±0.20	0.39 ±0.03	0.92 ±0.02	0.72 ±0.02	7.55 ±0.05
48	0.2	0.78	0.6	4.6	0.28 ±0.01	0.87 ±0.06	0.67 ±0.05	7.30 ±0.20	0.28 ±0.01	0.83 ±0.07	0.65 ±0.05	7.80 ±0.10
120	0.19	0.84	0.64	4.5	0.22 ±0.02	0.90 ±0.06	0.68 ±0.03	7.40 ±0.10	0.28 ±0.04	0.80 ±0.02	0.60 ±0	7.85 ±0.05
168	0.16	0.75	0.57	4.7	0.19 ±0.03	0.69 ±0.12	0.53 ±0.07	7.50 ±0.20	0.29 ±0.02	0.85 ±0.02	0.67 ±0.02	7.95 ±0.05
216	0.13	0.84	0.68	4.8	0.23 ±0.03	0.83 ±0.10	0.68 ±0.05	7.50 ±0.10	0.26 ±0.01	0.74 ±0.04	0.59 ±0.04	8.05 ±0.05
384	0.11	0.82	0.66	4.9	0.20 ±0.01	0.73 ±0.01	0.61 ±0.01	7.35 ±0.05	0.26 ±0	0.75 ±0.02	0.60 ±0.03	8.05 ±0.05
Change in conc.	**0.57**	**0.27**	**0.17**		**0.56** ±0.01	**0.45** ±0.02	**0.29** ±0.03		**0.51** ±0	**0.47** ±0.01	**0.33** ±0.01	
Total M^{2+} sorbed [a]	**21.1**	**9.99**	**6.29**		**20.55** ±0.15	**16.65** ±0.75	**10.56** ±0.94		**18.90** ±0	**17.40** ±0.40	**12.00** ±0.20	
% M^{2+} sorbed[b]	**84%**	**25%**	**20%**		**74%** ±0	**38%** ±0.01	**32%** ±0.02		**66%** ±0	**39%** ±0	**36%** ±0.02	
M^{2+} sorbed per g Pine Bark [c]	**6.94**	**3.29**	**2.07**		**6.89** ±0.04	**5.59** ±0.27	**3.54** ±0.32		**6.29** ±0.02	**5.80** ±0.15	**4.01** ±0.05	
M^{2+} sorbed per g Pine Bark [d]	**0.44**	**0.21**	**0.23**		**0.44** ±0	**0.37** ±0.01	**0.40** ±0.01		**0.40** ±0	**0.38** ±0.01	**0.46** ±0.01	

a Total M^{2+} sorbed = [Change in concentration] x [Total volume (37ℓ)] (mmol)
b Relative M^{2+} sorption = [Change in concentration] / [Initial concentration]
c M^{2+} sorbed per g Pine Bark (mmol $x10^{-3}$) = [Total mmol M^{2+} sorbed] / [Dry Mass of BSP]
d M^{2+} sorbed per g Pine Bark (mg) = [Total mg M^{2+} sorbed] / [Dry Mass of BSP]

In Experiment, 1 the initial (T0) metal concentrations and pH values differed across all bioreactors (**Appendix B, Table B2**). This occurred because each bioreactor was set up independently, using 100mℓ aliquots of a 1000mℓ metal stock solution as a source for Cu, Zn and Cd. Thus, even slight inconsistencies in the preparation of the liquid medium would account for these variations. Therefore, the relative sorption values, rather than absolute values, were considered most appropriate for discussion. The differences in pH meant that comparisons between the bioreactors regarding the influence of nutritional supplementation on metal sorption was difficult.

pH decreased in the control bioreactor but increased in all four experimental bioreactors. The pH increase was more noticeable in the bioreactors containing the 0.2% (v/v) Voermolas solutions (final pH of 8.05 ± 0.05) compared with those containing the supplementary nutrient at 0.1% (v/v) (final pH of 7.35 ± 0.05). Pine bark is naturally acidic and is known to influence medium pH to varying extents, depending on the state of decomposition of the bark (Tucker, 2005; Krewer and Ruter, 2009). Tucker (2005) and Krewer and Ruter (2009) report the pH of pine bark to be 3.4 - 4.5 and 4.0 - 5.0 respectively. It is, therefore, likely that the pine bark caused the pH of the medium to become increasingly acidic with time, as leaching proceeded in the control bioreactor. Conversely, pH increased in the experimental bioreactors throughout the experiment. Initially this could be attributed to the Voermolas possibly acting as a buffer. However, actively metabolising microorganisms have a propensity to alter the pH of their environments as a consequence of their growth (Brock and Madigan, 1991). Therefore, the increase in pH observed in the experimental bioreactors is likely to have occurred as a result of the microbial growth taking place. The more pronounced increase in pH observed in the bioreactors containing Voermolas at 0.2% (v/v), compared with those containing Voermolas at 0.1% (v/v), and the control bioreactor, are likely to have occurred as a consequence of the additional nutrients leading to increased

levels of microbial growth. Furthermore, biofilms are known to restrict molecular diffusion, thereby limiting mass transfer rates between the BSP and the bulk liquid (Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004). As the biofilms established and developed on the pine bark BSP, they are likely to have formed a physical barrier between the pine bark and the bulk liquid; thus impeding deprotonation of the pine bark. The above postulations assume that the levels of Voermolas were growth limiting in all bioreactors and, therefore, that microbial growth was directly proportional to the concentration of nutrients in the bioreactors; i.e. no/minimal growth in the control bioreactor and most vigorous growth occurring in the bioreactors containing 0.2% (v/v) Voermolas. However, as microbial growth (mass/biofilm thickness) was not measured in this experiment, it is difficult to speculate on these relationships.

The observed differences in bioreactor pH may also contribute to understanding the differences in metal sorption under the different nutritional conditions, i.e. copper was best sorbed under conditions of minimum nutrient supplementation (lowest pH amongst the bioreactors) whereas Zn^{2+} and Cd^{2+} sorption was favoured by increased supplementary nutrition with concomitant increased pH (and suspected increased microbial biomass). Kuyucak and Volesky (1988) suggested that pH affects the solution chemistry of dissolved metal ions as well as the activity of the functional groups in the biomass. This in turn affects the competitive behaviour of metal ions for the binding sites within biotic material. Matheickal *et al*. (1988) found that at pH values below pH 5, acidic functional groups of some polysaccharides were rendered labile by protons competing with metal ions for the binding sites, thereby decreasing sorption of the latter. This was also observed by Holan *et al*. (1993) who reported that cadmium biosorption by *A. nodosum* fell from 1.8mmol.g^{-l} at pH 4.9 to less than 0.3mmol.g^{-l} at pH 2.

When considering the percentage of metals sorbed, as well as the M^{2+} sorbed per g pine bark, in all reactors (i.e. irrespective of pH and nutrient level), the biosorption pattern was consistently: copper >zinc>cadmium. Garnham (1997) found that Cu^{2+} has a higher affinity for biotic matter than both Zn^{2+} and Cd^{2+}. The results are also consistent with the work of Shi *et al*. (2003) on dissolved organic matter. The paucity of literature on metal biosorption from multi-metal bearing solutions, and complete absence of data on multi-metal biosorption in dual biosorbent systems, makes a cogent explanation of the results difficult. However, the behaviours of the target metal ions as consequences of their environmental conditions have been studied and may be of relevance.

Biosorption from multi-metal solutions is considerably more complex than single metal sorption because of factors such as inter-ion competition for surface binding sites. Holan and Volesky (1995) found that the presence of copper and zinc ions in solution had a significant negative effect on cadmium biosorption by modified *A. nodosum*. Mongar and Wassermann (1949) and Haug (1959) found that algal biomass had higher selectivity coefficient values for copper ions [*L. hyperborea* (K=340) and *L. digitata* (K=230)] than for other metals, thus making Cu^{2+} a strong competitor for surface binding sites on biotic materials. Furthermore, the nature of the surface of the biomass itself may display selective binding for various metal ions. Ion selectivity by biological sorbents has been linked to the polysaccharide composition making up the cellular surface structures, as well as the ratios between them (Huang *et al*., 1967; Williams *et al*., 1997). Polysaccharide structure/composition is affected by pH as discussed previously.

Competition between metal cations reduces the capacity of a given biosorbent for any particular metal species. Tobin (1988) found that, in general, Cd competed poorly for biosorption sites. Cd biosorption decreased by as much as 70% in the presence of other

divalent cations. This may explain the relatively poor results obtained for Cd compared with the other metals in the present study. In addition, Cd biosorption has been shown to occur principally through favourable interactions with sulfhydryl groups, forming complexes with cysteine-rich proteins (Kagi and Vallee, 1961). Cadmium-polysaccharide binding occurs mainly due to the weak electrostatic interactions between the sulphate ester groups and the cation (Kagi and Vallee, 1961). Although the surface biochemistry of neither the pine bark nor the biofilms was analysed, it is possible that a scarcity of sulphate ester groups on the pine bark surface in the control, compared with a growing biofilm (and the associated EPS) in the experimental bioreactors, could also contribute to reduced biosorption of Cd in the control bioreactor compared with the experimental bioreactors.

In addition to surface binding, metabolism-dependant mechanisms for the internalisation of Cd^{2+} and, also Zn^{2+} are understood. Belliveau *et al. (*1987) state that absorption of Cd^{2+} and Zn^{2+} is via the Mn^{2+} transport system; all three are all like-charged, similarly sized ions which are quickly and efficiently accumulated by the CorA Mn uptake system present in many microorganisms. *Escherichia coli* has been shown to have inducible Cd^{2+} binding proteins (Gadd, 1988; Gadd, 1990b). Additionally, polyphosphate granules have been shown to sequester intracellular metal ions, including Zn^{2+} (Andrade *et al*., 2004), and dedicated microbial systems for the uptake of Zn^{2+} have been studied (Hudek *et al*., 2009).

Although some adsorption of Zn by biotic matter through ion exchange or electrostatic interaction is highly likely, Zn biosorption has been shown to occur mainly through metabolism-dependant bioaccumulation mechanisms (Chipman *et al*., 1958). These researchers demonstrated that living *Nitzchia closterium* cells readily accumulated zinc to levels 80x in excess of the metabolic needs of the organism. This may account for the poor

performance of the control (25% Zn removal) compared with the experimental bioreactors (38% [0.1% Voermolas] and 39% [0.2% Voermolas]) for the biosorption of zinc.

Crist *et al. (*1990) found that copper biosorption by *Nitzschia palea* was limited to cell walls and extracellular substances, indicating that metabolism-independent sorption mechanisms were responsible. Competition between protons and metal ions for metal binding sites on the algal surface indicated that ion exchange was responsible for the biosorption of copper by the biomass. Further experimentation showed that copper was adsorbed not only through H^+ ion exchange, but also by additional covalent binding with carboxyl groups. A decrease in solution pH, corresponding with copper biosorption, was also evident, indicating ion exchange between copper and protons at the sorption surface (Crist, *et al*., 1990). In the experiments reported here, metal sorption in the control bioreactor was also accompanied by a decrease in pH, suggesting possible ion exchange at the pine bark surface.

Therefore, in all the bioreactors, biosorption was likely to have occurred as a combination of metabolism-independent sorption to cellular and EPS surfaces and metabolism-dependent absorption. These experimental findings are consistent with those of Mhavi *et al. (*2005) who state that metal biosorption is subject to a number of parameters, including the amount of adsorbent and the initial concentration of the metals in solution. The low pH in the control, and selective surface binding of Cu ions (in preference to Zn and Cd) clearly favoured Cu binding in this bioreactor. Furthermore, phenolic groups (such as those leached from pine bark) contribute significantly to Cu binding in humic-type substances over a wide pH range (Vincent, 1960; Benedetti *et al*., 1996). The higher pH in the bioreactors containing Voermolas facilitated increased levels of cadmium and zinc biosorption and a concomitant decrease in copper sorption. Furthermore, the greater amount of biomass in the experimental bioreactors likely supported additional metal sorption, particularly of Zn^{2+} and

Cd^{2+}, via the various metabolic pathways present in actively metabolising biomass e.g. the CorA Mn^{2+} uptake system (Belliveau *et al*., 1987; Hudek *et al*., 2009). This is consistent with the findings of Erikson (2004) that the rate of metal ion sorption is usually directly proportional to the concentration of microbial mass within the bioreactor.

4.3.2 Experiment 2

The bioreactors were operated for 216h (9 days) to investigate the removal of the heavy metal ions from two synthetic wastewaters, each with a different level of supplementary nutrition, in the presence of established biofilms. Liquid samples were withdrawn at preordained times and the residual metal ion concentration was measured by ICP-OES.

Originally the experiment was scheduled to run for 384 days (i.e. the same duration as Experiment 1). However, after 280h, excess microbial growth in Bioreactor E [0.2% (v/v) Voermolas] severely restricted the flow of liquid through the pine bark matrix. This caused the inlet to overflow, resulting in bioreactor failure (similar to the composted bark experiments reported in Chapter 3). Hence, the experiment was terminated earlier and only the results obtained up to T10 (216h) were considered. The reduced duration of Experiment 2 (compared with Experiment 1) was unlikely to have had a significant effect on the overall findings because in Experiment 1 very little biosorption occurred between 216h and 384h. Less than 0.02mmol of the individual metals in the control bioreactors, and those containing 0.2% (v/v) Voermolas, occurred during this period, while ≤0.1mmol of the individual metals were biosorbed in the bioreactors containing 0.1% (v/v) Voermolas.

At the end of Experiment 2, the bioreactor chambers were opened, revealing the presence of an established biofilm on the pine bark surfaces in each bioreactor, including the control (Bioreactor A). Microbial biomass determinations were not made. The metal biosorption

results, expressed as millimoles, are presented in **Table 4.5**. Raw data are provided in **Appendix C, Tables C1 and C2**. The averaged data are shown graphically in **Appendix C, Figures C1 – C7**.

Table 4. 5: Average residual metal ion concentrations after selected time intervals and corresponding pH values (Experiment 2). Mean values and variances are shown for the bioreactors containing 0.1% and 0.2 % (v/v) Voermolas

	Control				0.1% (v/v) Voermolas				0.2% (v/v) Voermolas			
Time (h)	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**pH**
0	0.88	1.48	0.91	6.1	0.88 ±0.00	1.48 ±0.00	0.91 ±0.00	6.10 ±0.00	0.88 ±0.00	1.48 ±0.00	0.91 ±0.00	6.10 ±0.00
1	0.76	1.30	0.80	5.9	0.80 ±0.08	1.32 ±0.04	0.80 ±0.02	6.95 ±0.05	0.80 ±0.01	1.24 ±0.14	0.75 ±0.07	7.10 ±0.10
2	0.71	1.22	0.77	5.8	0.76 ±0.08	1.04 ±0.05	0.64 ±0.03	6.95 ±0.05	0.76 ±0.05	1.04 ±0.00	0.63 ±0.01	7.10 ±0.10
6	0.69	1.19	0.76	5.7	0.65 ±0.07	0.90 ±0.06	0.55 ±0.03	7.00 ±0.10	0.67 ±0.02	0.93 ±0.09	0.58 ±0.06	7.10 ±0.10
12	0.56	0.98	0.61	5.5	0.63 ±0.02	0.91 ±0.01	0.60 ±0.02	7.00 ±0.10	0.80 ±0.15	1.28 ±0.10	0.78 ±0.04	7.30 ±0.10
24	0.58	1.15	0.73	5.4	0.64 ±0.04	1.08 ±0.15	0.67 ±0.09	7.10 ±0.10	0.55 ±0.05	0.98 ±0.02	0.61 ±0.01	7.50 ±0.10
36	0.57	1.19	0.75	5.3	0.56 ±0.04	1.00 ±0.14	0.62 ±0.09	7.20 ±0.20	0.50 ±0.06	0.94 ±0.03	0.59 ±0.02	7.50 ±0.10
48	0.55	1.33	0.84	5.3	0.33 ±0.03	0.78 ±0.12	0.49 ±0.08	7.25 ±0.15	0.37 ±0.07	0.85 ±0.05	0.55 ±0.03	7.65 ±0.05
120	0.30	1.22	0.77	5.2	0.28 ±0	0.64 ±0.03	0.41 ±0.01	7.25 ±0.25	0.44 ±0.05	0.99 ±0.03	0.65 ±0.02	7.65 ±0.05
168	0.26	0.81	0.53	5.4	0.30 ±0	0.71 ±0.10	0.46 ±0.06	7.25 ±0.15	0.29 ±0.09	0.81 ±0.08	0.54 ±0.06	7.80 ±0.10
216	0.26	0.82	0.52	5.4	0.25 ±0.01	0.71 ±0.12	0.46 ±0.06	7.25 ±0.15	0.29 ±0.09	0.81 ±0.07	0.53 ±0.08	8.10 ±0
Change in conc.	0.62	0.66	0.39		**0.64 ±0.01**	**0.78 ±0.12**	**0.46 ±0.06**		**0.59 ±0.09**	**0.67 ±0.07**	**0.38 ±0.08**	
Total M^{2+} sorbed (mmol)[a]	22.9	24.4	14.4		**23.50 ±0.20**	**28.65 ±4.25**	**16.85 ±2.05**		**21.85 ±3.35**	**24.80 ±2.60**	**14.05 ±2.95**	
% M^{2+} sorbed[b]	70%	45%	43%		**73% ±0.01**	**53% ±0.08**	**50% ±0.06**		**67% ±0.10**	**46% ±0.05**	**42% ±0.09**	
M^{2+} sorbed per g Pine Bark [c]	7.55	8.03	4.75		**7.89 ±0.09**	**9.62 ±1.40**	**5.65 ±0.66**		**7.28 ±1.09**	**8.26 ±0.84**	**4.68 ±0.97**	
M^{2+} sorbed per g Pine Bark [d]	0.48	0.52	0.53		**0.51 ±0.01**	**0.63 ±0.10**	**0.64 ±0.08**		**0.46 ±0.07**	**0.54 ±0.06**	**0.53 ±0.11**	

a Total M^{2+} sorbed (mmol) = [Change in concentration] x [Total volume (37ℓ)]
b Relative M^{2+} sorption (%) = [Change in concentration] / [Initial concentration]
c M^{2+} sorbed per g Pine Bark = [Total mmol M^{2+} sorbed] / [Dry Mass of BSP)]
d M^{2+} sorbed per g Pine Bark = [Total mg M^{2+} sorbed] / [Dry Mass of BSP]

The results show that biosorption took place in all bioreactors, with relative biosorption rates ranging between 42% and 73%. In Experiment 1, T0 pH values and metal ion concentrations differed across all bioreactors because the bulk liquids were prepared individually. Therefore, for Experiment 2, a single volume of synthetic wastewater was prepared and sampled before distribution between the five bioreactors, ensuring that initial M^{2+} concentrations and pH (6.1) were standardised between all bioreactors.

The major differences in the performance of the Control (Bioreactor A) in Experiment 2, compared with Experiment 1, were likely due to the presence of a biofilm in Bioreactor A during Experiment 2. Although the biofilm was only detected at the end of this experiment, it is likely that it formed towards the end of Experiment 1. The increase in pH, from 4.5 to 4.9, that occurred between 120h and 384h is likely to have signalled the development of the biofilm because, as already discussed, microbial growth may alter medium pH (Brock and Madigan, 1991) and the physical presence of a biofilm may impede deprotonation of the pine bark (Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004).

The biofilm in the control bioreactors was likely to have developed later than in the experimental bioreactors because the inoculum, carbon and energy sources would have been derived solely from the pine bark. Pine bark contains and leaches water soluble organics, including soluble tannins and phenols (Vasquez *et al.,* 2002; Haussard *et al.,* 2003; Tshabalala *et al.,* 2004; Oh and Tshabalala, 2007; Ribé *et al*., 2009), some of which possibly served as carbon and energy sources for the microbial community present. Being indigenous to pine bark these organisms would be resistant to, or have adapted to, the soluble organic compounds in the pine bark.

However, microbial communities indigenous to pine bark are unlikely to have previously encountered metal ion concentrations approaching those used in these experiments. Therefore, a lag phase in microbial growth was expected to have occurred in the control reactor. This would probably not have been the case for the inoculum culture in the experimental bioreactors.

The release of dissolved organics from submerged pine bark was not unexpected but did cause concern because these substances could impart colour and contribute to an increase in COD, BOD or TOC. All of which would be undesirable in wastewater treatment applications. Trois *et al*. (2010a; 2010b) found that submerged pine bark released phenolic compounds and hydroxylated benzene rings when it was used as a sorbent to treat synthetic landfill leachate. The organics leached from the pine bark increased microbial acclimatisation times and inhibited denitrification. Although phenols are known to be toxic to various life forms, including microorganisms, toxicity tests using *Daphnia magna* and *Vibrio fischeri* showed that the organics leached from pine bark did not significantly alter liquid toxicity (Ribe *et al*., 2011; 2012). Diaz *et al*. (2003) also showed that untreated pine bark contributed COD to aqueous systems, but effluent COD always remained below regulatory limits (125mg/ℓ O_2) (European Directive 91/271).

The overall pH decrease in the control bioreactor was less dramatic in experiment 2 (pH 6.1 – pH 5.4) than in Experiment 1 (pH 5.9 – pH 4.9). As discussed previously, this was possibly due to the presence of the biofilm, which either physically restricted deprotonation of the BSP, or as a consequence of cellular metabolism. In Experiment 2 the pH in the control reactor increased from 5.2 at 120h to 5.4 at the end of the experiment (216h). A similar trend, viz. a slight increase in pH, occurred over the

same period in Experiment 1. It is possible that protons liberated through cation exchange between the protons on either biomass and the metals in the fresh liquid medium, could have masked any pH altering effects of biofilm metabolism. However, it is more likely that environmental stress or shock, as a consequence of the removal of the original medium (containing leached organics) and replacement with fresh medium, (devoid of dissolved organics) negatively impacted the development of native microbial communities, resulting in an additional lag phase (Brock and Madigan, 1991).

As in Experiment 1, the increase in pH in the experimental bioreactors corresponded to increased Voermolas levels. Averaged final pH values of 7.25 ±0.15 in the bioreactors containing 0.1% (v/v) Voermolas and pH 8.10 ±0 in those containing 0.2% (v/v) Voermolas were similar to those measured in Experiment 1 (pH 7.35 ±0.05) and (pH 8.05 ±0.05), respectively. This indicated that although initial conditions differed in Experiment 1 and Experiment 2, ultimately the environmental conditions were similar and, therefore, the final results obtained in the corresponding bioreactors were likely to be comparable. However, one contained a biofilm at the start of the experiment, whereas the other did not.

In Experiment 2, the control and both sets of experimental bioreactors all contained dissolved organic matter and living microbial biomass, but in different quantities. There was also a strong indication that a biofilm was present in the control bioreactor at the end of Experiment 1. In Experiment 2 final pH values also differed in the different treatments, despite the fact that all the bioreactors had the same starting pH of 6.1. However, the parallel decrease in residual metal ion concentrations indicated that the different levels of supplementary nutrition did not greatly affect metal biosorption. Biosorption levels in the control bioreactor, and in those containing the

0.2% (v/v) Voermolas solutions, were indistinguishable. Biosorption of all three metals was slightly higher in the bioreactors with 0.1% (v/v) Voermolas solutions. As in Experiment 1, copper biosorption was better than that of the other two metals which showed similar biosorption levels irrespective of nutrient concentration and pH value. A comparison of the hydrated ionic radii of copper, zinc and cadmium ions in solution (**Table 4.6**) indicates their very similar sizes. Electrostatic attraction of metal ions to fixed ionic groups on the sorbent materials may result in biosorption (Kagi and Vallee, 1961).

Table 4. 6: Properties of selected divalent metal cations in aquatic conditions

	Hydrated ionic radius (Å)	Polarisability ($10^{-24}cm^{-3}$)
Cu^{2+}	4.19	6.1
Cd^{2+}	4.26	7.2
Zn^{2+}	4.30	7.1

The strength of the electrostatic attraction is dependent on the ionic charges involved as well as the distance of closest approach between the dissolved metal ion and the fixed ionic groups in the biosorbent. For example, electrostatic attraction is inversely proportional to the square of the distance between charges, so that ions with smaller hydrated ionic radii are held more strongly (Boyd *et al*., 1947; Kressman and Kitchner, 1949). Hydrated Cu^{2+} has a smaller ionic radius than either Zn^{2+} or Cd^{2+}, which are similar in size. Therefore, Cu^{2+} should consistently form stronger electrostatic bonds with the biosorbent materials than the other metal cations investigated. This may explain why copper behaved differently from the zinc or cadmium, and why biosorption values for zinc and cadmium were consistently similar.

Another factor to consider is the polarisability of the cations investigated. The more polarised the counter ion, the smaller the distance of closest approach and, therefore, the greater the tendency to be retained within the biosorbent particle (Boyd *et al*., 1947; Kressman and Kitchner, 1949). However, the relatively similar polarisability values of the three metals under investigation (**Table 4.6**) makes use of this aspect to distinguish their absorbancies onto pine bark of little value.

In all cases, the values obtained for Total M^{2+} sorbed and M^{2+} sorbed per g pine bark (Experiment 2), exceeded those of Experiment 1, possibly because no desorption cycle had been instituted between the experiments. Heavy metal biosorption and accumulation is dependent on the availability of adsorbent (Mhavi *et al*., 2005). In Experiment 2 the systems would have benefitted from the continued development of the biofilms initiated in Experiment 1, and would have the various benefits of an attached system over a planktonic system (Stoodley *et al*., 2002; Hall-Stoodley *et al*., 2004; Rice *et al*., 2005) from the start of the experiment. The increased metal biosorption in the control in Experiment 2 was also likely due to the increased biomass available for sorption, resulting from the biofilm established during Experiment 1.

4.4 Conclusions

- Although pine bark is known to leach organics into the bulk liquid (Diaz *et al*., 2003; Trois *et al*., 2010a;), and some of these may be toxic to some microorganisms (Trois *et al*., 2010b; Ribe *et al*., 2011; 2012), they were not detrimental to the growth of the autochthonous microorganisms in the bioreactors. In fact, they provided a source of additional nutrients for the microbial; community.
- Microorganisms indigenous to the pine bark used were able to form biofilms in the control bioreactors which contained relatively high metal ion concentrations and no extraneous supplementary energy sources. This could reduce operating costs were the system to be used commercially. Therefore, in order to assess the contribution to metal removal made by the support matrix itself, the pine bark should be sterilised before use as a matrix in subsequent control bioreactors. In addition to contributing nutrients to the cells in those bioreactors not supplemented with Voermolas, the bark appeared to deprotonate, causing lowering of the solution pH.
- Copper biosorption was favoured over that of zinc and cadmium which showed similar biosorption levels to one another.
- Biosorption levels in the control bioreactor and in those supplemented with 0.2% (v/v) Voermolas were indistinguishable, while biosorption of all three metals was slightly higher in the 0.1% (v/v) Voermolas solutions.

Chapter 5

Contributions by the Biological Components of a Pine Bark-Biofilm System to the Removal of Metal Ions from a Synthetic Wastewater.

5.1 Introduction

Packed-bed bioreactors, such as forced-upflow designs, are commonly used in industrial wastewater treatment studies (Dostalek, 2011). Bioreactors used in the reported studies typically contain a living biofilm supported on an inert BSP (Dostalek, 2011). The efficacy of these types of systems is easy to quantify and compare in isolation by measuring either the total metal ion biosorption or relative metal ion biosorption (percentage sorbate sorbed or sorption as a function of sorbent mass). Reports on metal biosorption studies using non-living biosorbents are also widespread in the literature. These non-living biosorbents may in fact support some microbial growth (e.g. a biofilm), but to date this has generally not been reported and, therefore, there has been no need to distinguish between the sorption capacities of the living and non-living components. Both living and non-living biomass, amongst other materials, may sequester heavy metal ions, but at different rates (Pagnanelli *et al*., 2002; Keskinkan *et al*., 2003; Montes *et al*., 2003; Ahalya *et al*., 2005; Mhavi *et al*., 2005; Ahalya *et al*., 2007; Oh and Tshabalala, 2007; Mohan and Sumitha, 2008; Gadd, 2009; Dostalek 2011). Thus it may be useful to distinguish the extent of metal ion biosorption between the components in a system combining both living and non-

living biosorbents, in order to ascertain whether such a dual system is able to biosorb more metal ions than a traditional, single biosorbent system.

In **Chapter 4** it was shown that Cu^{2+} sorption was favoured when the predominant biosorbent was pine bark (a non-living organic material), and nutrient levels were minimal. This system also had the lowest pH (final pH 4.9 - 5.4). However, sorption of both Zn^{2+} and Cd^{2+} was favoured over that of Cu^{2+} if the pine bark supported a substantial biofilm, ie a system containing a non-living organic material and a living biomass when a supplementary nutrient source was provided (final pH 7.25 – pH 8.1). The differences in pH may explain the different sorption levels of the various metals. The solution chemistry of dissolved metal ions, as well as the activity of the functional groups in the biomass, are affected by the medium pH (Kuyucak and Volesky, 1988). Therefore, the competitive behaviour of metal ions for the binding sites within biotic material is affected. Consequently, in a dual biosorbent system, the biofilm and the pine bark may contribute to overall metal sorption differently under various environmental conditions.

In **Chapter 4**, pine bark was shown to biosorb the metals under investigation and to support a living biofilm. However, the performance of this dual biosorbent system needed to be compared with a traditional, single biosorbent approach to assess whether it provided any significant advantages/disadvantages over the more conventional single sorbent systems, or conversely, displayed any noteworthy shortcomings.

To assess the individual metal ion biosorptive capacities of the biofilm and pine bark, a series of experiments was conducted using either colonised or non-colonised pine bark (biological material), or plastic bioballs (non-biological material) as BSP.

Residual metal ion concentration in the bulk liquid was determined by ICP-OES and the biofilm and BSP were analysed for metal content using ESEM coupled with EDX.

5.2 Experimental Procedure

5.2.1 Laboratory-Scale Bioreactors

The bioreactors used were similar to those described in **Chapters 3 and 4** i.e. they were constructed from a single glass, domestic aquarium. However, in this case a larger aquarium, divided into six identical bioreactors, was used. As described previously, each bioreactor comprised an inlet chamber and a BSP-containing chamber separated by a glass baffle plate which directed the liquid from the influent chamber to the bottom of a fibreglass BSP-containing chamber, the top and bottom of which were perforated to allow the liquid medium to flow vertically upwards through the container. The BSP containers were sealed against the internal walls of the bioreactor to prevent liquid by-passing the BSP. The liquid medium, therefore, flowed upwards through the interstices between the BSP particles and exited at the top of the BSP chamber where it was led, via an outflow pipe, back to the reservoir for mixing and reintroduction to the top of the inlet chamber. This constituted a closed-loop recirculation system.

Each bioreactor and reservoir combination had a total working volume of 40ℓ.

The bioreactors are shown in **Plate 5.1**.

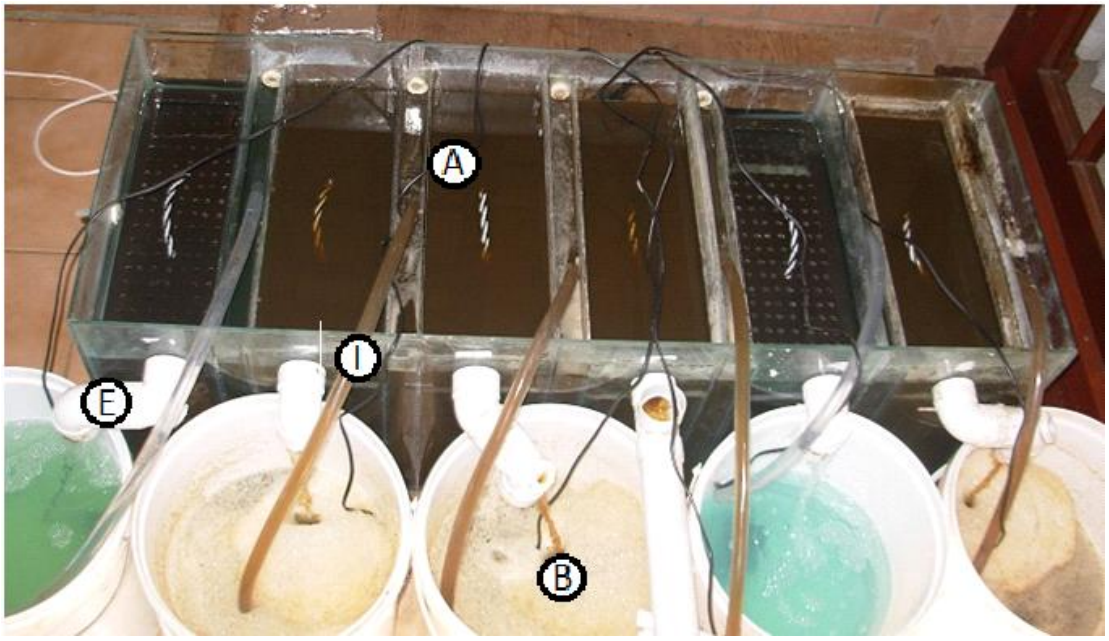

Plate 5. 1: Elevated view showing the six identical bioreactors (A), each with its own reservoir (B). Also visible are the influent (I) and effluent (E) pipes and the electrical cables to the submerged pumps in the reservoirs (B)

5.2.2 Preparation of Synthetic Wastewater

Because of the large volume (240ℓ) of liquid required to fill the six bioreactors, two separate batches of metal containing synthetic wastewaters were prepared as follows:

- One 80ℓ batch, containing metal ions only (100 mg. ℓ^{-1} Cu^{2+}, Zn^{2+} and Cd^{2+}) and no supplementary nutrients nor inoculum, was prepared for use in the control bioreactors (Bioreactors A and D).
- A separate 160ℓ batch, containing metal ions (100 mg. ℓ^{-1} Cu^{2+}, Zn^{2+} and Cd^{2+}), supplementary nutrients (0.2% Voermolas) and 10% (v/v) inoculum (sewage sludge). This was used in the experimental bioreactors (Bioreactors B, C, E and F).

The masses of metal salts used in preparation of the synthetic wastewater are shown in **Table 5.1**.

Table 5. 1: Volumes and initial heavy metal compositions of the synthetic wastewaters

	$CuCl_2.2H_2O$	$(CH_3COO)_2.Cd.2H_2O$	$(CH_3COO)_2.Zn.2H_2O$	Final volume[a]
Control	21.64g	19.04g	26.84g	80ℓ
Experimental	43.28g	38.08g	53.69g	160ℓ

a Final volume was achieved by making up with municipal tap water (**Chapter 2**)

5.2.3 Biofilm Support Particles (BSP)

5.2.3.1 Pine Bark BSP

Commercially available decorative pine bark was supplied free of charge, and ready for use as BSP, by Gromed Organics (no longer trading) in Crammond, KwaZulu-Natal. Unless otherwise stated, uncomposted pine bark of particle size 6-16mm was used. A typical example is shown in **Plate 5.2.**

Plate 5. 2: A pine bark nugget

5.2.3.2 Bioball BSP

Bioballs were used as an alternative BSP to pine bark. These are icosikaidigonal plastic structures used in domestic wet/dry and submerged aquarium filter applications. They have evenly spaced protruding fins which provide a large surface area and a protective environment suitable for biofilm attachment and growth,

The bioballs were purchased from Petwise, Hayfields Mall in Pietermaritzburg, KwaZulu-Natal. The product was unbranded and retailed at a cost of ZAR 89.99 per 1kg bag. The bioballs each had an average mass of 4.72g and a diameter of 25mm. Unless specifically stated, the bioballs were not sterilised before use. A typical bioball is shown in **Plate 5.3**.

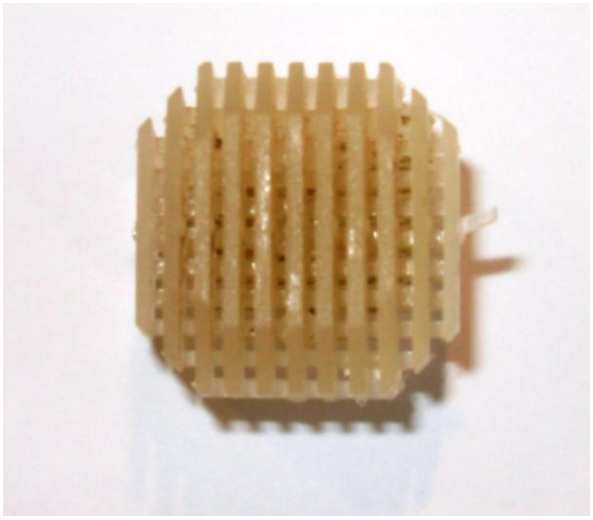

Plate 5. 3: A bioball

5.2.3.3 Comparison of Pine bark and Bioballs as BSP Materials

Some of the differences between the two BSPs are shown in **Table 5.2**.

Table 5. 2: Comparison of the properties of pine bark and bioballs pertinent to their use as BSP

	Pine Bark	**Bioballs**
Approximate surface area per matrix unit (m^2 x10^{-3}) [a]	ND[b]	7.077
Specific surface area ($m^2.m^{-3}$) [c]	ND	146
Pore volume per bioreactor (ℓ) [d]	1.14	0.051
Void volume per bioreactor (ℓ) [e]	3.96	5.54

a. The surface area of the plastic bioballs was calculated geometrically. The values given are approximations because of irregularities between the surfaces of individual units caused by the manufacturing process.

b. ND = Not determined.

c. Specific surface area was determined using the number of individual units (calculated according to mass) in each BSP chamber volume occupied by the BSP.

d. Pore volume was measured by submerging ten randomly selected, individual units of each matrix separately in distilled water for 72 hours to allow maximum absorption to occur. The saturated samples were weighed, dried in an oven at 70^{o}C for 72 hours and re-weighed. The procedure was performed in triplicate and the average difference in mass was regarded as the pore volume. The pore volume per bioreactor was calculated using the average number of individual units required to fill each BSP chamber.

e. Void volume was calculated by filling a vessel with each BSP up to a known mark (1.80ℓ); distilled water was then added until this mark was reached. The amount of water required to reach the selected mark was considered to be the void volume. The experiment was performed five times for each material, and the average volume was used to extrapolate to the volume used in the BSP chamber bioreactor (7.96ℓ).

5.2.3.4 Cost Comparison of Pine bark and Bioballs as BSP

The cost of the two BSP materials is compared in **Table 5.3**.

Table 5. 3: Comparison of purchase prices of pine bark and bioballs shown as cost per bioreactor and per m^2

	Pine Bark	**Bioballs**
Cost per bioreactor [a] (Retail, delivered, including V.A.T)	ZAR 36.72	ZAR 192.49
Cost/m^3 [b] (Wholesale, not delivered, including V.A.T)	ZAR 65.00	ZAR 8,400.00 [c]

a. Cost per bioreactor was calculated using the mass of material required to fill the BSP-chamber of a bioreactor. Although the pine bark was supplied free of charge, in order to ensure comparability between the two BSPs, the costing was based on the retail price of the delivered product.

b. Wholesale pricing for pine bark was for unpackaged, loaded at source product.

c. No wholesale supplier for bioballs could be sourced. A 30% delivery charge, and 50% mark-up were assumed in order to calculate an expected wholesale price.

5.2.4 Bioreactor Configuration

Six identical laboratory-scale bioreactors, each containing a 40ℓ volume of synthetic, metal-containing wastewater were set up and operated as described in **Table 5.4**. The BSP particles used in the experimental bioreactors (pine bark nuggets or bioballs) were not washed, nor sterilised. Conversely, in the control bioreactors, these support materials were autoclaved three times at 121^{o}C (103kPa) for 15min at 24h intervals and then placed in sterile distilled water in a sonicator bath, operated at 40kHz for 20min to remove any attached microbial biomass, before being re-autoclaved at 121^{o}C (103kPa) for 15min.

Table 5. 4: Bioreactor contents and operating conditions at startup

	Bioreactor					
	A (Control)	**B**	**C**	**D (Control)**	**E**	**F**
BSP	Sterilised Bioballs	Untreated Bioballs	Untreated Bioballs	Sterilised Bark	Untreated Bark	Untreated Bark
BSP Dry Mass (kg)[a]	4.00[c]	4.01	4.01	3.06	3.04	3.03
Biofilm presence at the start of the experiment	No	No	Yes[b]	No	No	Yes[b]
Dry Mass of biosorbent[c] (kg)	4.00[c]	4.05	4.19	3.06[c]	3.14	3.25
Hydraulic Retention Time (min:s)	04:12	04:15	04:09	04:10	04:12	04:04
Supplementary nutrients (Voermolas)	None	0.1% v/v	0.1% v/v	None	0.1% v/v	0.1% v/v
Inoculum added	None	10% (v/v)	10% (v/v)	None	10% (v/v)	10% (v/v)

a The dry mass of BSP was determined by weighing the BSP prior to the experiment.

b A biofilm was grown on the BSP of Bioreactors C and F for 14d prior to commencing the experiment. Synthetic metal-containing wastewater + 10% inoculum and 0.1% v/v Voermolas

c After the experiment, the contents of all bioreactors were air dried simultaneously until the BSP mass in Bioreactors A and D were consistent with pre-experiment values.

5.2.5 Temperature Range of the Experiment

The experiments were conducted in a modified greenhouse. Ambient daytime laboratory temperature was maintained at 24°C ±6°C for the duration of the experiment.

5.2.6 Determination of pH

The pH of the samples was monitored using a Crison micro 2002 pH meter, calibrated using pH 4.0 and pH 7.02 standards.

5.2.7 Liquid and Biofilm Sampling Procedures

5.2.7.1 Liquid Sampling Procedure

Triplicate samples of the bulk liquid were collected from the outlet pipe of each bioreactor (between the bioreactor and the reservoir) for residual metal ion concentration and pH determinations. For the first 2h of the experiment replicate samples were collected 30s apart and thereafter, 5min apart. Initial (T0) samples were taken from the top of each filled bioreactor immediately after filling but before starting the pumps.

All samples were gravity filtered through fluted Whatman 3 µm filter paper and stored at -4^{o}C. Before quantitative analysis, 10x and 100x dilutions of filtered samples were prepared using deionised water.

The averaged residual metal ion concentrations determined at each sampling time are presented in **Tables 5.5 and 5.6**. Averaged pH values for each sample set are presented in **Table 5.7**. Raw data for each of the six bioreactors are given in **Appendix D, Tables D1 to D12**.

5.2.7.2 BSP Sampling Procedure

Triplicate samples of both pine bark and bioball BSP were randomly selected from each bioreactor at T0 and after 48h, 168h and 336h for electron microscopic examination. All samples were stored in liquid medium taken from the shared reservoir and kept at 4^{o}C until analysis.

5.2.7.3 Sediment Sampling Procedure

At conclusion of the experiment, three randomly selected replicate samples of sediment were collected from each of the control bioreactors (Bioreactor A and D). Sediment samples were air dried for 48h and mounted on carbon tape for electron microscopic examination.

5.2.8 Determination of Residual Metal Ion Concentration

Residual metal ion concentrations were determined using a Varian 702 ES Inductively Coupled Plasma Optical Emission Spectrometer. Details of the procedure are described in **Section 4.2.4.4**.

5.2.9 Environmental Scanning Electron Microscopy (ESEM)

Sediment and BSP samples were viewed under high magnification in an Environmental Scanning Electron Microscope (Phillips XL30 ESEM). Individual microscope settings are indicated on the electron micrographs. This microscope allows specimens to be viewed uncoated and fully hydrated. Unless otherwise specified, specimens were mounted in carbon paste. Frozen samples were thawed before analysis.

Cross sections of both BSPs and their attached biofilm were prepared by freezing samples in liquid nitrogen and fracturing them using two methods.

Method 1 is illustrated diagrammatically in **Figure 5.1**.

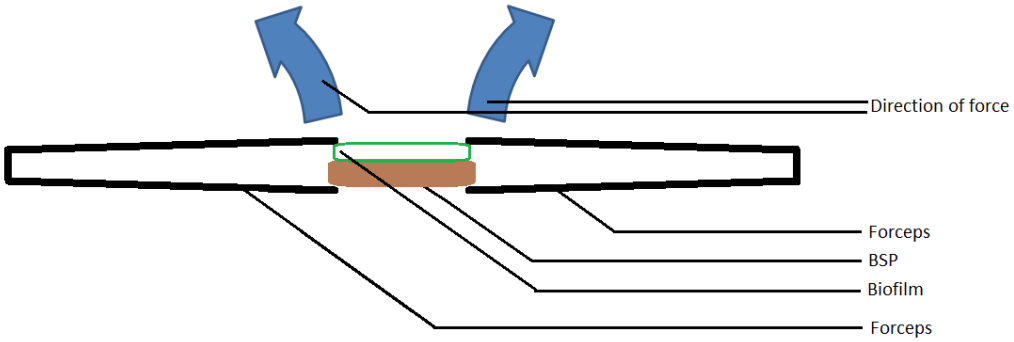

Figure 5. 1: Illustration of how BSP-biofilm samples were fractured to obtain cross sections through both the BSP and attached biofilm.

Method 2: (See **Figure 5.2**) was preferred since it produced much less distorted cross sections through the BSP and attached biofilm. In this method a twisting action was applied to the frozen samples and this prevented the biofilm from detaching from the support matrix.

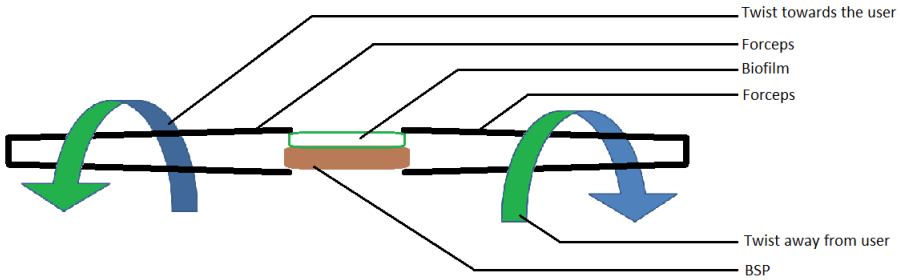

Figure 5. 2: Illustration of how BSP-biofilm samples were fractured using a twisting motion to obtain cross sections through both the BSP and biofilm without destroying the structural integrity of either.

5.2.10 Energy Dispersive X-ray Microanalysis (EDX)

EDX was used to determine the basic compositional characteristics of the sediment and BSP samples. This technique identified the elements present in the samples by their signature x-ray spectra emitted after exposing them to an electron beam. A Link eXl II EDX system attached to the Phillips XL30 ESEM with a UTW-Sapphire detector was used. The samples were orientated at a 10^{o} angle to the detector, the take off angle was 33^{o}, and the working distance was 10mm.

5.3 Results and Discussion

5.3.1 Synthetic Wastewater Analysis

The mean residual concentrations of all three metal ions in the bioreactors containing bioballs as BSP, and associated medium pH values, are shown in **Tables 5.5 and 5.7** respectively. The raw data and basic statistical analyses are presented in **Appendix D, Tables D1 – D6**. Corresponding residual metal ion concentrations and pH values for the bioreactors containing pine bark as BSP, and associated medium pH measurements, are shown in **Tables 5.6 and 5.7** respectively. The raw data and basic statistical analysis are presented in **Appendix D, Tables D7 – D12**. Sample variances are reported in **Appendix D** but they are not presented in the **Tables 5.5 – 5.7** because of space constraints.

Table 5. 5: Changes in residual metal ion concentrations (mmol) over time and total metal ion removal within the bioreactors containing plastic bioballs as BSP. Each value represents the mean of three replicate samples. Raw data and basic statistical analyses are presented in Appendix D, Tables D1 – D6

		A Control (Bioballs only)			B Experimental (Bioballs + Inoculum)			C Experimental (Bioballs + Biofilm)		
Sample Number	Time [a] (h)	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$
(Initial) 0	0	1.10	0.97	1.45	0.43	0.83	1.32	0.31	0.83	1.31
1	0.07	0.89	0.79	1.41	0.38	0.66	1.31	0.28	0.58	1.06
2	0.14	0.84	0.75	1.34	0.37	0.63	1.13	0.29	0.53	1.04
3	0.28	0.78	0.75	1.32	0.36	0.59	1.08	0.22	0.51	0.96
4	0.57	0.70	0.74	1.24	0.36	0.57	1.01	0.20	0.49	0.95
5	1	0.70	0.74	1.22	0.35	0.53	0.98	0.20	0.42	0.82
6	2	0.26	0.70	1.22	0.32	0.48	0.97	0.18	0.34	0.66
7	6	0.09	0.67	1.21	0.30	0.45	0.88	0.16	0.21	0.62
8	12	0.02	0.62	1.18	0.27	0.39	0.85	0.16	0.20	0.48
9	24	0.01	0.53	1.07	0.26	0.42	0.81	0.16	0.15	0.37
10	48	0.00	0.49	1.16	0.26	0.23	0.60	0.17	0.11	0.27
11	72	0.01	0.52	1.14	0.23	0.20	0.51	0.17	0.10	0.25
12	96	0.01	0.47	1.12	0.23	0.18	0.41	0.16	0.09	0.24
13	120	0.00	0.47	1.09	0.23	0.16	0.41	0.16	0.09	0.22
14	144	0.00	0.46	1.08	0.22	0.16	0.41	0.16	0.05	0.17
15	168	0.00	0.39	1.07	0.21	0.16	0.36	0.15	0.05	0.15
16	192	0.00	0.43	1.02	0.20	0.16	0.36	0.14	0.04	0.15
17	216	0.00	0.43	0.97	0.20	0.14	0.32	0.13	0.04	0.14
18	240	0.00	0.37	0.94	0.20	0.12	0.29	0.13	0.04	0.12
19	288	0.00	0.36	0.90	0.18	0.11	0.28	0.12	0.03	0.11
(Final) 20	336	0.00	0.36	0.72	0.13	0.10	0.24	0.11	0.04	0.12
Change in conc. (Initial - Final)		1.1	0.61	0.73	0.3	0.73	1.08	0.2	0.79	1.19
Total M^{2+} removed [b] (mmol)		44.0	24.4	29.2	12.0	29.2	43.2	8.0	31.6	47.6
Relative M^{2+} removal [c]		100%	63%	50%	70%	88%	82%	65%	95%	91%
M^{2+} removed per g sorbent ($\mu mol.g^{-1}$) [d]		11.0	6.1	7.3	3.0	7.2	10.7	1.9	7.5	11.4
M^{2+} removed per g sorbent ($mg.g^{-1}$) [e]		0.70	0.40	0.82	0.19	0.47	1.20	0.12	0.49	1.28

a Time at which the first sample of each replicated series was withdrawn

b Total M^{2+} removed = [Change in concentration] x [Total volume (40ℓ)]

c Relative M^{2+} removal = [Change in concentration] / [Initial concentration]

d/e M^{2+} removed per g sorbent = [Total M^{2+} removed] / [dry mass of sorbent (BSP or BSP + Microorganisms)]

Table 5. 6: Changes in residual metal ion concentrations (mmol) over time and total metal ion removal within the bioreactors containing pine bark as BSP. Each value represents the mean of three replicate samples. Raw data and basic statistical analyses are presented in Appendix D, Tables D7 – D12

		D Control (Bark only)			E Experimental (Bark + Inoculum)			F Experimental (Bark + Biofilm)		
Sample Number	Time [a] (h)	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Cd^{2+}]$
(Initial) 0	0	1.47	1.09	1.43	0.28	0.83	1.31	0.27	0.83	1.31
1	0.07	1.33	0.97	1.39	0.24	0.55	1.12	0.25	0.53	1.19
2	0.14	1.32	0.90	1.35	0.22	0.51	1.00	0.22	0.53	1.11
3	0.28	1.31	0.92	1.25	0.20	0.49	0.96	0.20	0.49	1.09
4	0.57	1.28	0.87	1.22	0.19	0.48	0.86	0.21	0.42	0.93
5	1	1.10	0.80	1.18	0.20	0.37	0.74	0.22	0.37	0.84
6	2	1.00	0.80	1.14	0.12	0.36	0.73	0.20	0.32	0.83
7	6	0.70	0.75	1.00	0.08	0.25	0.72	0.19	0.23	0.63
8	12	0.66	0.74	0.99	0.14	0.22	0.56	0.19	0.15	0.59
9	24	0.64	0.68	0.98	0.18	0.16	0.54	0.19	0.13	0.39
10	48	0.60	0.67	0.98	0.18	0.13	0.38	0.19	0.09	0.35
11	72	0.58	0.66	0.96	0.17	0.08	0.27	0.18	0.07	0.19
12	96	0.50	0.65	0.94	0.16	0.06	0.20	0.17	0.05	0.15
13	120	0.48	0.62	0.88	0.16	0.04	0.17	0.17	0.04	0.12
14	144	0.41	0.61	0.77	0.16	0.05	0.18	0.17	0.03	0.09
15	168	0.38	0.55	0.70	0.14	0.05	0.15	0.17	0.03	0.09
16	192	0.34	0.51	0.69	0.15	0.03	0.13	0.16	0.03	0.08
17	216	0.33	0.45	0.66	0.14	0.04	0.11	0.16	0.02	0.07
18	240	0.31	0.43	0.63	0.14	0.03	0.11	0.15	0.02	0.07
19	288	0.30	0.41	0.63	0.13	0.03	0.11	0.13	0.02	0.07
(Final) 20	336	0.29	0.41	0.61	0.11	0.02	0.09	0.10	0.02	0.06
Change in conc. (Initial - Final)		1.18	0.68	0.82	0.17	0.81	1.22	0.17	0.81	1.25
Total M^{2+} removed[b] (mmol)		47.2	27.2	32.8	6.8	32.4	48.8	6.8	32.4	50.0
Relative M^{2+} removal[c]		80%	62%	57%	61%	98%	93%	63%	98%	95%
M^{2+} removed per g sorbent ($\mu mol.g^{-1}$)[d]		15.4	8.9	10.7	2.2	10.3	15.5	2.1	10.0	15.4
M^{2+} removed per g sorbent ($mg.g^{-1}$)[e]		0.98	0.58	1.20	0.14	0.67	1.74	0.13	0.65	1.73

a Time at which the first sample of each replicated series was withdrawn

b Total M^{2+} removed = [Change in concentration] x [Total volume (40ℓ)]

c Relative M^{2+} removal = [Change in concentration] / [Initial concentration]

d/e M^{2+} removed per g sorbent = [Total M^{2+} removed] / [dry mass of sorbent (BSP or BSP + Microorganisms)]

Table 5. 7: Change in Bioreactor pH over time. Each value represents the mean of three replicate samples. Raw data and basic statistical analyses are presented in Appendix D, Tables D1 – D12

	Mean Sample pH					
Time (h)	A Control (Bioballs only)	B Experimental (Bioballs + Inoculum)	C Experimental (Bioballs + Biofilm)	D Control (Bark only)	E Experimental (Bark + Inoculum)	F Experimental (Bark + Biofilm)
0	5.8	5.8	6.8	6.0	6.8	6.8
0.07	5.8	5.8	6.9	5.8	6.9	6.9
0.14	5.8	5.8	7.2	5.8	6.9	6.9
0.28	5.8	5.8	7.1	5.8	7.1	7.1
0.57	5.9	5.9	7.0	5.6	7.2	7.2
1	5.9	5.9	7.2	5.5	7.3	7.3
2	6.4	6.0	7.3	5.1	7.3	7.3
6	6.2	6.2	7.6	4.9	7.5	7.5
12	6.4	6.4	7.5	4.7	7.5	7.5
24	6.4	6.4	7.5	4.7	7.6	7.6
48	6.5	6.5	7.7	4.7	7.7	7.7
72	6.5	6.5	7.9	4.7	7.9	7.9
96	6.5	6.5	8.0	4.6	8.0	8.0
120	6.5	6.5	8.3	4.5	8.1	8.1
144	6.8	6.6	8.5	4.5	8.1	8.1
168	6.6	6.6	8.5	4.4	8.3	8.3
192	6.6	6.6	8.1	4.4	8.3	8.3
216	6.8	6.8	8.3	4.6	8.4	8.4
240	6.8	6.8	8.2	4.6	8.5	8.5
288	6.8	6.9	8.3	4.6	8.5	8.5
336	7.0	7.0	8.5	4.4	8.3	8.3

Over the duration of the experiment, increases in pH were recorded for all bioreactors except for Bioreactor D (which contained non-colonised pine bark i.e. no living biomass) in which the pH decreased from pH 6.0 at the start of the experiment to pH 4.4 at the conclusion.

The initial metal ion concentrations differed not only between the controls and the experimental bioreactors, always being higher in the former, (**Tables 5.5 and 5.6**), but, in the case of copper, the initial concentration in Bioreactor B (0.43mmol) was higher than in the other experimental bioreactors (0.31 – 0.27mmol). These fluctuations in initial metal ion concentrations were possibly due to the increased pH in the experimental bioreactors (pH 5.8 – pH 6.8) (**Table 5.7**), compared with the control bioreactors (pH 5.8 – pH 6.0), causing precipitation of some of the metals (Collins and Stotzky, 1989). Armenante (1997) reported that minimum solubility of Cu (<0.001 mg/ℓ), Cd (<0.01 mg/ℓ) and Zn (<0.01 mg/ℓ) occurred at pH 8.1, pH 11.0 and pH 10.1 respectively. The solubility of the metal hydroxides decreases as the environmental pH approaches these values. This is supported by the results obtained since, of the experimental bioreactors, Bioreactor B which had the lowest initial pH (pH 5.8), also had the highest initial copper concentration (0.43mmol). In contrast, initial concentrations of the other two metals were comparable across all the experimental bioreactors.

It is also possible that the organic compounds present in the Voermolas and inoculum bonded with the ionic species to form organometallic compounds. Omae (1998) and Macek and Mackova (2011) state that some organometallic compounds tend to be insoluble in aqueous solution. The tendency of Cu^{2+} to outcompete Zn^{2+} and Cd^{2+} for binding sites on biotic matter has already been discussed in **Section 4.3.1**. The potential formation of water-insoluble organometallic compounds in the experimental bioreactors may also account for the differences in initial metal ion concentrations, particularly in the case of copper.

The increasing pH in Bioreactors B, C, E and F could potentially be attributed to the presence and influence of actively metabolising microorganisms. Microorganisms are known to alter the pH of their environments as a consequence of their growth (Brock and Madigan, 1991). Microbial growth, however, cannot explain the pH changes in the control bioreactors (i.e. Bioreactors A and D). The decrease in medium pH which occurred in Bioreactor D was not surprising as similar findings were reported in **Chapter 4**. There the medium pH in the (unsterilised) pine bark control bioreactors changed from and pH 5.9 – pH 4.9 over 384h in Experiment 1 and from pH 6.1 – pH 5.4 over 216h in Experiment 2. The change in medium pH was attributed to the innate acidity of the pine bark, which can influence medium pH to varying extents, depending on the state of decomposition of the bark (Tucker, 2005; Krewer and Ruter, 2009). Tucker (2005) and Krewer and Ruter (2009) report the pH of pine bark to be pH 3.4 – pH 4.5 and pH 4.0 – pH 5.0 respectively. It is likely that the pine bark itself caused the pH of the medium to become increasingly acidic as was observed in the control bioreactor. The decrease in pH in Bioreactor D (**Table 5.7**) was more dramatic than reported earlier (**Chapter 4**). In contrast to the situation here, the control bioreactors in **Chapter 4** contained unsterilised pine bark, on which a biofilm subsequently developed. The pH effects of microbial growth as observed in **Chapter 4** likely counteracted the pH effects of the pine bark. Therefore, the more dramatic medium pH changes reported in **Table 5.7** (pH 6 – pH 4.4) are likely to be entirely due to the pine bark matrix and physicochemical interactions between the metals and the organic matrix.

Bioreactor A contained a sterilised, apparently inert plastic matrix and no known biosorbents, yet the pH in this bioreactor increased from pH 5.8 to neutral pH over the course of the experiment. Furthermore, residual concentrations of Cu, Zn and Cd in this bioreactor were reduced by 100%, 63% and 50% respectively, over the course

of the experiment. As Bioreactor A contained an abiotic BSP and no known sorbents, it is likely that a combination of metal adsorption to the bioballs and metal precipitation, as a result of the increasing pH of the wastewater, was responsible for the observed decrease in residual metal ion concentration in this bioreactor. Increased pH tends to promote the formation of insoluble heavy metal species (Hahne and Kroontje, 1973; Said and Lewis, 1991). However, the specific pH at which metal hydroxides become insoluble varies between individual metals (discussed on **Page 129**). According to Sheremata and Kuyucak, (1996) Cu began to precipitate at pH > 4. Balintova and Petrilakova (2012) found that, in their experiments, 92.3% precipitation of Cu occurred at pH 6, and 84% of Zn precipitated in the range pH 5.5 to 7. Reports in the literature support the present experimental findings insofar as complete Cu removal had occurred at pH 6.5 after 48h. Removal of Zn and Cd was slower, with concentrations reducing throughout the 336h experimental period. Minimum solubility of Cd and Zn hydroxides is reported to occur at pH 11.0 and pH 10, respectively, viz. higher pH values than for Cu (pH 8.1) (Armenante, 1997). Precipitation due to increasing pH offers a plausible explanation for the removal of the metal ions from solution. It also explains why Cu was removed to the greatest extent and Cd removal was least favourable. It is possible that changes in the ionic composition of the medium resulting from metal precipitation led to the continued increase in pH.

The initial concentrations of Cu^{2+} varied considerably between the control and experimental bioreactors making comparisons difficult. The problem was exacerbated by the different behaviour of Cu^{2+} in those bioreactors where biosorption was suspected, compared to that of Zn^{2+} and Cd^{2+}. The results did suggest, however, that the different metal ions were removed from the wastewater through different mechanisms. In every bioreactor, copper biosorption was consistently favoured over

that of zinc, which in turn was favoured over that of cadmium. This was likely due to the higher affinity for biotic matter shown by Cu^{2+} than by Zn^{2+} and Cd^{2+} (Garnham, 1997; Shi *et al*., 2003). Furthermore, inter-ion competition for surface binding sites may explain the differences in behaviour of the three metals studied. For example, Holan and Volesky (1995) found that the presence of copper and zinc ions in solution had a significant negative effect on cadmium biosorption by dried *A. nodosum*. Mongar and Wassermann (1949) and Haug (1959) found that algal biomass had higher selectivity coefficient values for copper ions [*L. hyperborea* (K=340) and *L. digitata* (K=230)] than for other metals, thus making Cu^{2+} a strong competitor for surface binding sites on biotic materials. Also, the sorbent surface may display selective binding for different metal ions. This has been linked to the polysaccharide composition of the cellular surface structures, as well as the ratios between them (Huang *et al*., 1967; Williams *et al*., 1997). The different behaviours of the metal ions within the bioreactors were consistent with the earlier findings reported in **Chapter 4.**

Tables 5.5 and 5.6 show that the total Cu^{2+} (mmol) removed from solution in the bioreactors was highest in Bioreactor D (47.2mmol), containing the unsupplemented, uninoculated pine bark, followed by Bioreactor A (44.0mmol) (the bioball control – no biological/organic material). Removal of Zn and Cd was similar, but seemed to follow a different pattern to Cu, with greatest removal efficiency occurring in the pine bark reactor with an already established biofilm (Bioreactor F), followed by the pine bark reactor with only inoculum at the start of the experiment (Bioreactor E). Unsurprisingly the bioball control (Bioreactor A – no biological material) removed the least amounts of Zn^{2+} and Cd^{2+}.

The relative M^{2+} (%) removed from solution in each bioreactor followed a similar trend to that observed for total M^{2+} (mmol) removal except in the case of Cu^{2+}, and to

a lesser extent Zn^{2+}. In the bioball control (Bioreactor A – no biological material) 100% of the copper present was removed after only 48h. Zinc removal was also marginally greater in this bioreactor (63%) than in the other control, Bioreactor D (62%), despite the latter containing pine bark, a known sorbent of heavy metal ions (de Vasconcelos and Beca, 1992; Tshabalala *et al*, 2004; Oh and Tshabalala, 2007). Furthermore, the pH in Bioreactor D decreased progressively over time (pH 6.0 – pH 4.4). This would favour solubilisation of the metals and, therefore, increase their availability for surface bonding to the biomass (Roane *et al*., 2005; Macek and Mackova, 2011).

The trends observed for total M^{2+} (mmol) and relative M^{2+} (%) removal from solution in each experimental bioreactor showed that in those containing biological material, Cu^{2+} removal was inversely proportional to the amount of such material present; viz. 12.0mmol Cu removed in Bioreactor B, followed by 8.0, 6.8, and 6.8 mmol Cu in Bioreactors C, E and F respectively. By contrast Zn^{2+} and Cd^{2+} removal was most efficient in the systems containing dual biosorbents, e.g. 32.4 and 32.4 mmol Zn and 48.8 and 50.0 mmol Cd were removed in Bioreactors E and F respectively. Marginally less efficient were the systems containing bioballs and microorganisms, viz. 31.6 and 29.2 mmol Zn and 47.6 and 43.2 mmol Cd removed in Bioreactors C and B respectively. These findings were consistent with those reported in **Chapter 4**, which showed that an increase in living biomass favoured the removal of Zn^{2+} and Cd^{2+} but, surprisingly, appeared to reduce Cu^{2+} removal. Crist *et al. (*1990) found that Cu biosorption by *Nitzschia palea* was limited to cell walls and extracellular substances, indicating that metabolism- independent biosorption mechanisms were responsible. Garnham (1997) found that Cu^{2+} has a higher affinity for dead biotic matter than both Zn^{2+} and Cd^{2+}. Mongar and Wassermann (1949) and Haug (1959) found that dead algal biomass had higher selectivity coefficient values for copper

ions [*L. hyperborea* (K=340) and *L. digitata* (K=230)] than for other metals. This made Cu^{2+} a stronger competitor for surface binding sites on dead biotic materials. Tobin (1988) found that, in general, cadmium competed poorly for biosorption sites, uptake decreasing by as much as 70% in the presence of other divalent cations.

Furthermore, the nature of the surface of the biomass itself may display selective binding for various metal ions. Ion selectivity by biological sorbents has been linked to the polysaccharide composition of the cellular surface structures, as well as the ratios between them (Huang *et al*., 1967; Williams *et al*., 1997). As mentioned previously, cadmium biosorption occurs mainly due to weak electrostatic interactions between the metal cation and sulphate ester groups, forming complexes with cysteine-rich proteins (Kagi and Vallee, 1961). Although the surface biochemistry of neither the pine bark nor biofilms was investigated, it is possible that a paucity of sulphate ester groups on the pine bark surface, compared with the surface of a growing biofilm and its associated EPS, may also have contributed to the reduced biosorption of Cd in the control bioreactor (A) compared with the experimental bioreactors. In addition, metabolism-dependant mechanisms for the internalisation of both Cd^{2+} and Zn^{2+} are known. Belliveau *et al. (*1987) state that absorption of Cd^{2+} and Zn^{2+} is via the Mn^{2+} transport system. All three are like-charged, similarly sized ions which are quickly and efficiently accumulated by the CorA Mn uptake system present in many microorganisms. *Escherichia coli* has been shown to have inducible Cd^{2+} binding proteins (Gadd, 1988; 1990b). Additionally, polyphosphate granules have been shown to sequester intracellular metal ions, including Zn^{2+} (Andrade *et al*., 2004), and dedicated microbial systems for the uptake of Zn^{2+} have also been studied (Hudek *et al*., 2009). Chipman *et al*. (1958) demonstrated that living *Nitzchia closterium* cells readily accumulated Zn to levels 80x in excess of the metabolic needs of the organism.

5.3.2 Electron Microscopic Analysis of the Sorbent Materials

A combination of ICP-OES and ESEM with EDX microanalysis were used to investigate the distribution of heavy metals between the BSP surface, the biofilm and the sediment.

5.3.2.1 Electron Microscopic Analysis of the Control Bioreactor BSPs

Before setting up the experiment both BSPs were analysed using EDX to determine their inherent (T0) heavy metal contents. Pine bark nuggets and bioballs were also taken randomly from the control bioreactors (A and D) after 48h, 168h and 336h to determine whether metal adsorption and/or microbial colonisation had taken place. The results of the EDX analyses are shown in **Table 5.8**. Corresponding electron micrographs are shown in **Plates 5.4 - 5.9**. The EDX spectra and their associated compositional data are presented in **Appendix F, Figures F1 – F8**.

Both the bioball and pine bark T0 samples showed traces of all three metals before exposure to the synthetic wastewater. Heavy metals are common in plastic materials, historically used as inexpensive stabilisers to retard their degradation (Yergeau and Dillon, 2007). Dimitrakakis *et al*. (2009) detected heavy metal concentrations of up to 5.7mg.kg^{-1} for Cd and median values of 100mg.kg^{-1} for Cu and Zn in the plastics of consumer electrical and electronic goods. Likewise, elevated levels of heavy metals are not uncommon in forest products. Gyozo *et al*. (2011) detected Cu, Zn and Cd in the leaves and stems of forest plants at levels up to 4,323mg.kg^{-1}, 285mg.kg^{-1} and 4.63mg.kg^{-1} respectively.

Both BSP materials were stored for several weeks in a multiuser wastewater laboratory. Therefore, it is possible, although unlikely, that both materials could have, been inadvertently contaminated by metal containing solutions, metal salts or other metal containing substances prior to use.

Table 5. 8: Cu, Zn and Cd content (%) of the surface of randomly selected BSP samples taken from the control bioreactors (A and D) before exposure to the synthetic wastewater and at various time intervals during the experiment. EDX spectra are shown in Appendix F, Figures F1 – F8

	Bioreactor A (Bioball Control)				Bioreactor D (Pine Bark Control)			
Element	**0h (Before Exposure)**	**48h**	**168h**	**336h**	**0h (Before Exposure**	**48h**	**168h**	**336h**
C	92.03	86.42	52.06	62.57	65.96	65.38	78.27	70.53
O	7.13	10.79	26.05	21.96	32.85	31.17	17.42	24.14
Cu	0.19	2.05	20.01	3.99	0.41	1.02	1.49	2.73
Zn	0.16	0.17	ND	4.64	0.66	0.85	0.99	1.63
Cd	0.17	0.18	ND	2.86	0.13	0.39	0.59	0.97
Al	ND	ND	ND	0.83	ND	1.03	0.39	ND
Ca	0.18	0.40	ND	0.80	ND	ND	ND	ND
Cl	ND	ND	1.88	2.35	ND	ND	ND	ND
Si	0.09	ND	ND	ND	ND	0.16	0.85	ND
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

ND = Not Detected

The liquid samples (discussed earlier) showed that over the course of the experiment the concentration of each metal remaining in solution in Bioreactor A decreased as the pH increased from pH 5.8 to pH 7.0. Copper was completely removed in under 48h, while the residual concentrations of Zn^{2+} and Cd^{2+} had decreased by 63% and 50% respectively, over the 336h experimental period. The EDX results (**Table 5.8**) show a continuously increasing proportion of metal on the bioball surfaces at 48h,

168h and 336h compared with the T0 sample, particularly in the case of Cu^{2+}. This confirms that the decrease in metal ion concentration in the bulk liquid could be partly attributed to adsorption to the bioball surfaces.

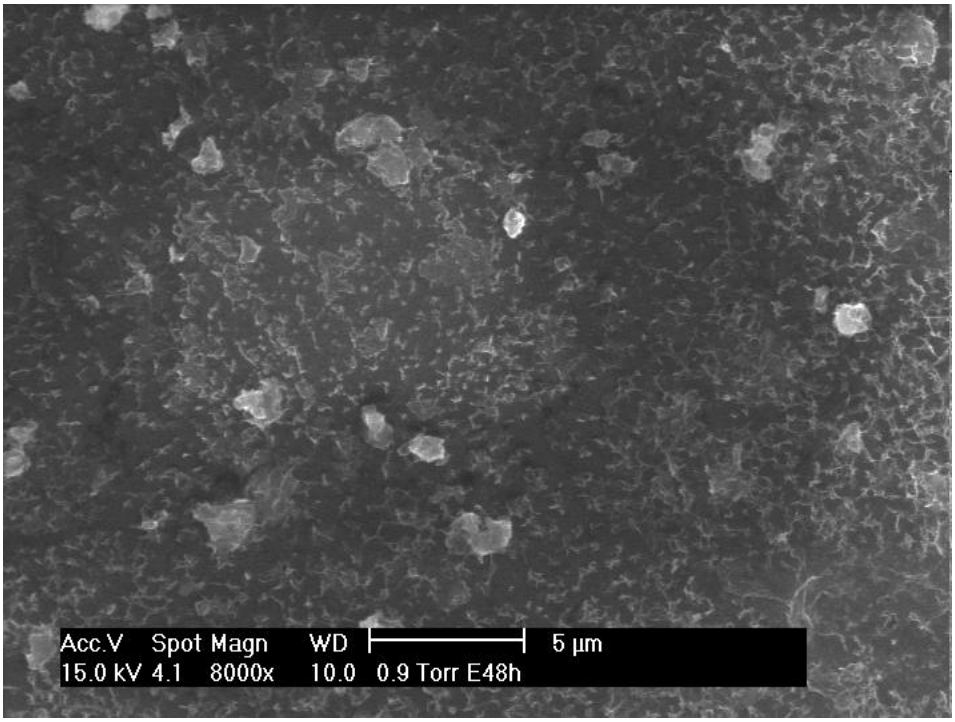

Plate 5. 4: Electron micrograph showing the absence of a biofilm on the surface of a bioball taken from Bioreactor A after 48h

Plate 5.4 shows that after 48h microbial colonisation of the bioball surface had not occurred, indicating that sterilisation had been effective and confirming that the decrease in Cu concentration was not due to biosorption but rather to surface adsorption, or precipitation. This contention was supported by the EDX results (**Table 5.8**) which show that the amount of Cu present on the bioball surface had increased from 0.19% at T0 to 2.05% after 48h.

Marginal increases in Zn and Cd (+0.01%) content were also observed. The amount of Zn and Cd on the bioball surface had not increased as substantially as that of Cu

relative to the T0 sample. This suggests that the observed decrease in residual, dissolved Zn^{2+} and Cd^{2+} concentration at this point was likely due to mechanisms other than adsorption to the bioball surface, possibly precipitation. The implications of the medium pH on the solubility of the metals have already been discussed (**Section 5.3.2**).

Plate 5.5 shows crystals on the surface of a bioball removed from Bioreactor A after 168h, which EDX spectroscopy identified as Cu. Neither Zn nor Cd were detected in the crystals (**Table 5.8**).There was also a complete absence of microorganisms (**Plate 5.5**).

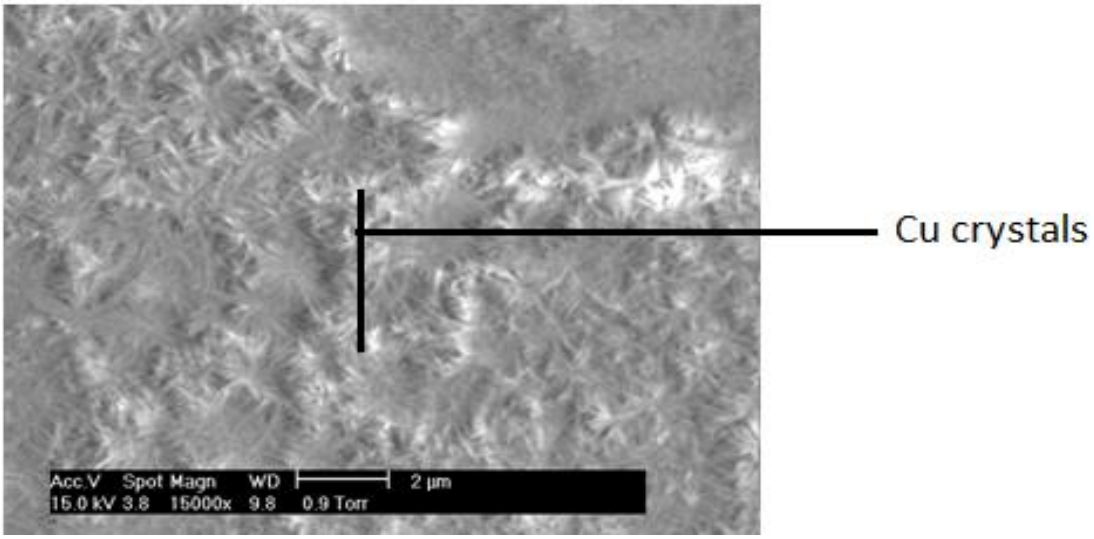

Plate 5. 5: Crystals on the surface of a randomly selected bioball taken from Bioreactor A after 168h. Note the absence of colonising microorganisms

The deposition of abundant Cu crystals on the bioball surface at 168h, potentially accounted for complete removal of Cu from the synthetic wastewater. The absence of Cd or Zn crystals indicated that adsorption to the bioballs' surface was likely not the main cause for removal of these two metals from solution.

After 336h the surfaces of most bioballs removed from Bioreactor A were similar to that shown in **Plate 5.5** (168h), i.e. a covering of Cu crystals and a lack of contiguous biofilm. However, at a single point on one of the samples some microbial growth was observed (**Plate 5.6**). The levels of all three metals were higher in the microbial biomass than on the surface of the non-colonised bioball samples examined after 168h. This indicated that some of the metal removal in this bioreactor could be attributed to biosorption by contaminant microorganisms. Because the bioreactor was not operated aseptically some microbial colonisation of the BSP by competent cells was eventually inevitable. However, as this was the only instance of microbial growth detected in this bioreactor which, together with its contents, had been rigorously sterilised prior to commencement of the experiment, it is considered unlikely that biosorption accounted for any significant metal removal.

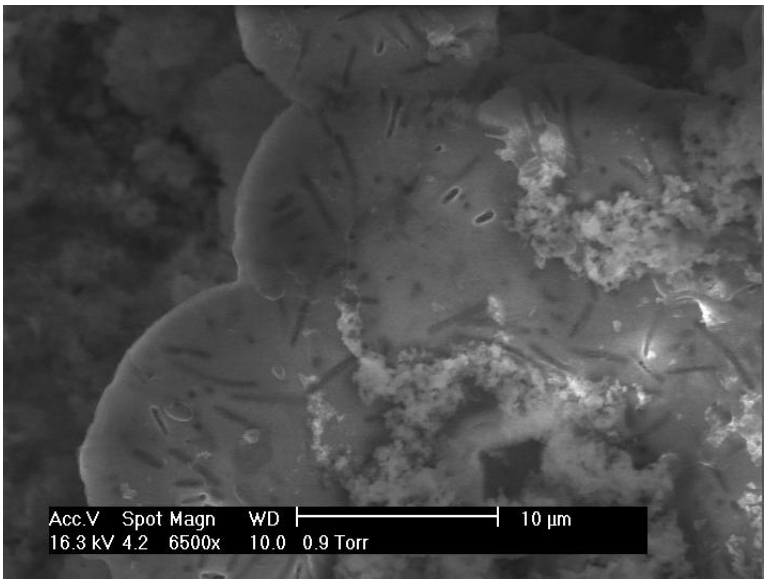

Plate 5. 6: Electron micrograph showing patches of microbial growth on the surface of a randomly selected bioball removed from Bioreactor A after 336h

Plate 5.7 shows that no microbial colonisation of the pine bark surface had occurred in 48h, confirming that the BSP remained sterile. Thus, changes in residual metal ion concentration could not be attributed to microbial biosorption. Furthermore, the proportions of all three metals present on the pine bark surface after 48h (Cu - 1.02%; Zn - 0.85%, Cd - 0.39%) (**Table 5.8**), were greater than those at T0 (Cu - 0.41%; Zn - 0.66%, Cd - 0.13%), confirming that adsorption to the pine bark surface had taken place during the initial 48h period.

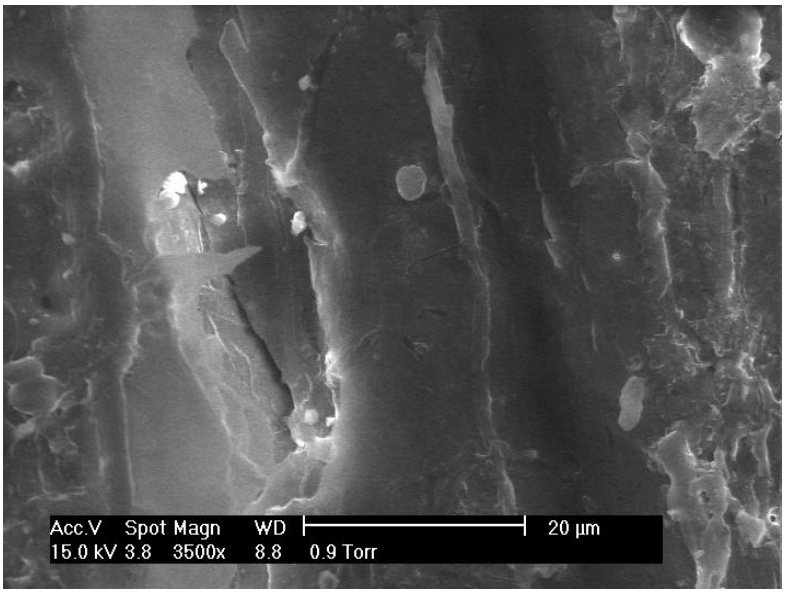

Plate 5. 7: Electron micrograph showing the absence of a biofilm on the surface of a pine bark nugget taken from Bioreactor D after 48h

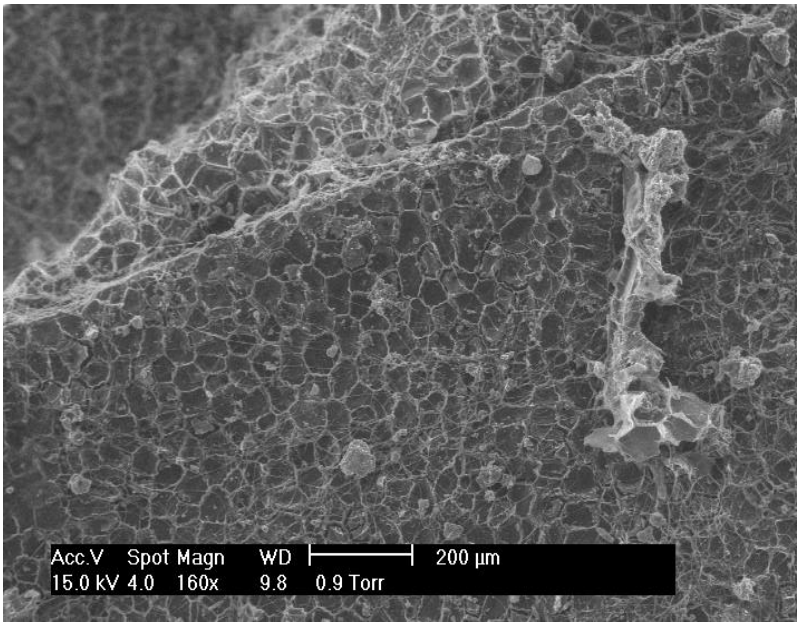

Plate 5. 8: Electron micrograph of a pine bark nugget taken randomly from Bioreactor D after 168h. Note absence of microorganisms

Plate 5.8 shows the surface of a randomly selected pine bark nugget removed from Bioreactor D after 168h. No microbial growth was detected on any of the nuggets viewed at this time, suggesting that the pine bark control was not microbially

contaminated at this point. The EDX spectroscopy results (**Table 5.8**) confirmed that the amounts of Cu, Zn and Cd on the biofilm surface had increased from 1.02%, 0.85% and 0.39% respectively, at 48h to 1.49%, 0.99% and 0.59% respectively after 168h.

After 336h, EDX analysis of the surface of the sample pine bark nugget once again showed a substantially increased proportion of all three metals, compared with the values obtained after 168h, indicating that biosorption to the pine bark had continued for the duration of the experiment.

Plate 5.9 shows that after 336h some microbial cells were present on the pine bark surfaces in Bioreactor D, but no extensive biofilm had developed. Some microbial colonisation of the sterilised pine bark was inevitable because the bioreactor could not be operated aseptically. Because of the small number of cells present it is unlikely that microbial biosorption contributed significantly to the observed decrease in residual metal ion concentration in the bulk liquid.

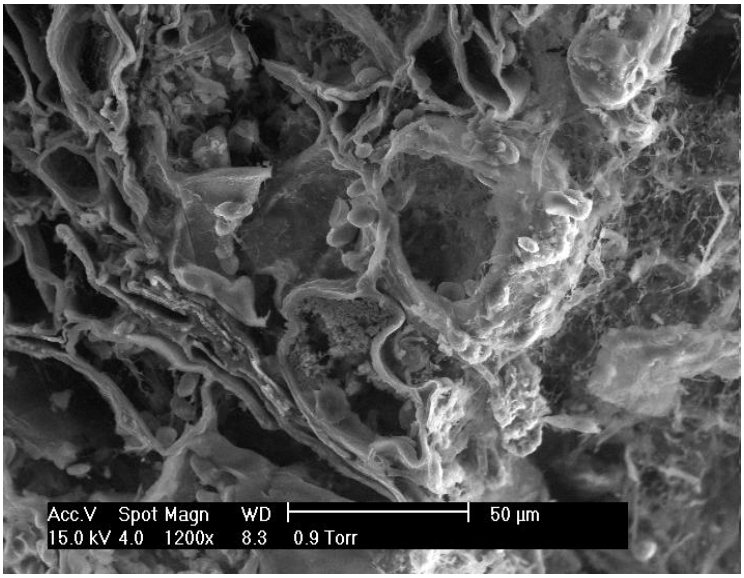

Plate 5. 9: Electron micrograph of a partly-colonised pine bark nugget taken randomly from Bioreactor D after 336h.

The EDX analyses (**Table 5.8**) show that, over the course of the experiment, the amounts of all three metals on the matrix surface in Bioreactor D (pine bark control) showed a steady increase, [viz. T0 (Cu - 0.41%, Zn - 0.66%, Cd - 0.13%); 48h (Cu - 1.02%, Zn-0.85%, Cd - 0.39%); 168h (Cu - 1.49%, Zn - 0.99%, Cd - 0.59%); 336h (Cu - 2.73%, Zn - 1.63%, Cd - 0.97%)]. This indicates that all three metals were biosorbed by the pine bark over the course of the experiment. This was made possible since Bioreactor D was the only one in which the pH decreased (from pH 6.0 at T0 to pH 4.4 at 336h) over the duration of the experiment (**Table 5.7**), and, as previously discussed, the more acidic medium conditions were likely to favour metal solubility. Thus the primary mechanism for metal removal in this bioreactor was most probably biosorption to the pine bark.

When the residual metal ion concentrations in the bulk fluid (**Table 5.6**) and the EDX results (**Table 5.8**) are considered together, it is clear that the uncolonised pine bark preferentially binds Cu^{2+} over Zn^{2+} and Cd^{2+}. Naja and Volesky (2011) report that some biosorbent materials are specific with regard to metal ion binding, with binding selectivity generally favouring heavier metals. Also, because biosorption appears to involve ion exchange to a high degree, the charge of the target sorbate is an important factor to consider in biosorption selectivity. All three metals investigated here are of like charge, Cu^{2+} and Zn^{2+} are of similar atomic weight while Cd^{2+} is heavier. Notwithstanding this, the latter showed the lowest level of binding. However, Cu^{2+} has been reported to have a higher affinity for organic matter than Cd^{2+} (Garnham, 1997) and Zn^{2+} (Garnham, 1997; Shi *et al.*, 2003; Martin-Dupont *et al*., 2006). Hydrated Cu^{2+} has a smaller ionic radius than either Zn^{2+} or Cd^{2+} (a comparison of the hydrated ionic radii of the metal ions in solution is presented in **Chapter 4**, **Table 4.6**). Ions with smaller hydrated ionic radii are held more strongly than larger, similarly charged ions (Boyd *et al*., 1947; Kressman and Kitchner, 1949).

Therefore, it is expected that Cu^{2+} should consistently form stronger electrostatic bonds with the biosorbent materials than the other metal cations investigated. This may explain why Cu behaved differently from the Zn or Cd, and why biosorption values for Zn and Cd were consistently similar.

5.3.2.2 Electron Microscopic Analysis of the Control Bioreactor Sediments

Because no appreciable amounts of Zn or Cd were detected on the bioball surfaces in Bioreactor A, random, triplicate samples of the sediment were collected and analysed using EDX spectroscopy. Triplicate samples of the sediment from Bioreactor D were also collected at random for comparison. The results presented in **Table 5.9** indicate the presence of large amounts of all three metals, particularly Zn (27.87%) in the sediment from the bioball control (Bioreactor A). As already discussed, increasing pH may lead to the precipitation of insoluble multiple-hydroxylated metal species (Hahne and Kroontje, 1973; Said and Lewis, 1991). It is possible that the change from acidic to neutral pH in this bioreactor caused desolubilisation of all three metals, suggesting that the primary mechanism for metal removal was probably precipitation. By contrast, in the pine bark control bioreactor (Bioreactor D) in which the medium became more acidic over time, the amounts of all three metals in the sediment were much lower, especially that of Zn, than in Bioreactor A. This suggested that much of the metal was adsorbed to the pine bark. It has already been noted that copper crystallised on the bioball surfaces (**Plate 5.5**).

Table 5. 9: Cu, Zn and Cd contents (%) of the sediment removed from the control bioreactors after 336h. EDX spectra are shown in Appendix F, Figures F9 and F10

Element	Bioreactor A (bioball control)	Bioreactor D (pine bark control)
C	32.36	65.71
O	24.99	25.79
Cu	9.14	2.47
Zn	27.87	1.39
Cd	3.77	0.90
Ca	0.92	1.41
P	0	0.61
Si	0.96	1.72
Total	**100**	**100**

The effects of pH on the solubility of the metals have already been discussed, and provide a logical explanation for the observed differences in the compositions of the sediments of the two control bioreactors.

5.3.2.3 Electron Microscopic Analysis of the Experimental Bioreactor BSPs

A study of the control bioreactors provided an understanding of how the two BSP's behave in the presence of metal ions and in the absence of a biofilm. However, it provided no insight into the distribution of the metal ions in a bioreactor containing both a nonliving biological BSP and attached living biofilm. This became possible when the results from the experimental bioreactors were compared with those from the control bioreactors. In this way the roles played by the individual components in a system containing both living and nonliving biosorbents can be elucidated.

Because crystallisation and precipitation removed 100% of the Cu^{2+} in the bioball control bioreactor, meaningful comparison between the results obtained in this bioreactor and those from the corresponding experimental bioreactors was difficult. Clearly evident however, was that in the inoculated bioreactors (B and C) significantly lower levels of Cu^{2+} were removed and no copper crystals were detected. Organic materials are reactive towards metals and their presence may result in the production of soluble organometallic complexes (Macek and Mackova, 2011). Thus the presence of living microbial cells may have prevented metal precipitation making biosorption the dominant mechanism for metal removal in these bioreactors. In order to verify this speculation, ESEM and EDX were used to investigate the elemental compositions of the BSPs and biofilms at various sample points.

Preparing cross sections through two congruent materials with different physical characteristics without compromising the integrity of either, through cross contamination or distortion, was difficult. Simply slicing through samples proved unsatisfactory because of structural damage caused by the blade. To overcome this problem, samples were frozen in liquid nitrogen and sheared in a controlled manner as shown in **Figure 5.1** (Method A) and **Figure 5.2** (Method B). Although Method A did not cause serious distortion at the pine bark-biofilm interface, it did cause them to become separated (**Plate 5.10**), making cross sectional analysis impossible. Method B, however, provided undistorted and uncontaminated cross sections allowing adjacent regions of both biosorbents to be clearly viewed (**Plates 5.11 and 5.12**), and satisfactorily analysed by EDX spectroscopy (**Table 5.10**).

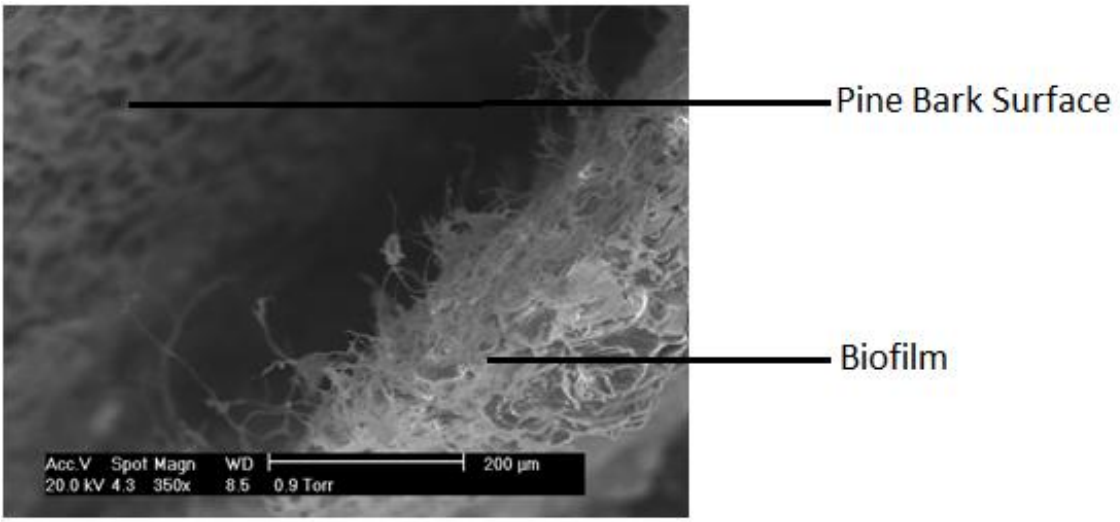

Plate 5. 10: Separation of the biofilm from the pine bark support matrix (Bioreactor E after 336h) following application of method A

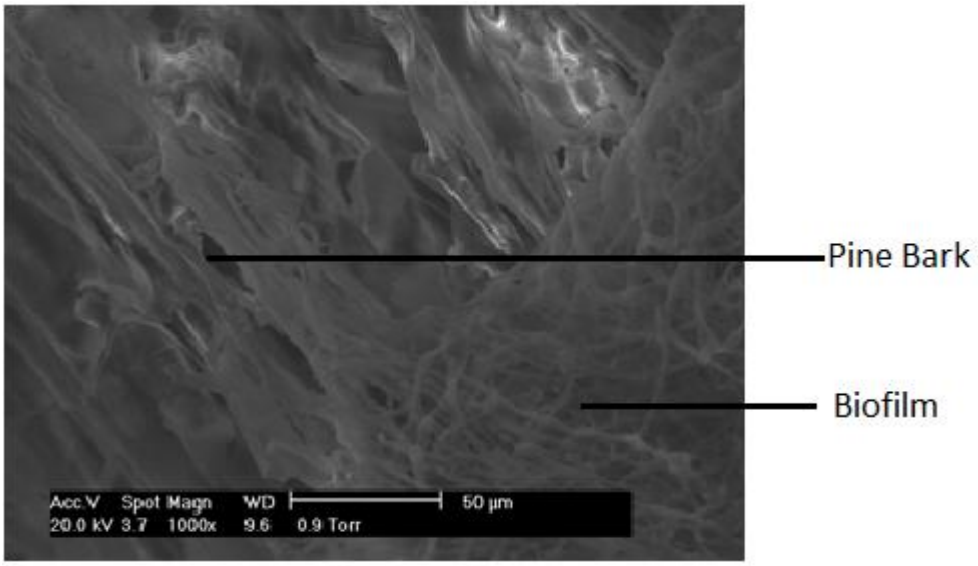

Plate 5. 11: Cross section though a pine bark nugget and attached biofilm taken from Bioreactor E after 336h

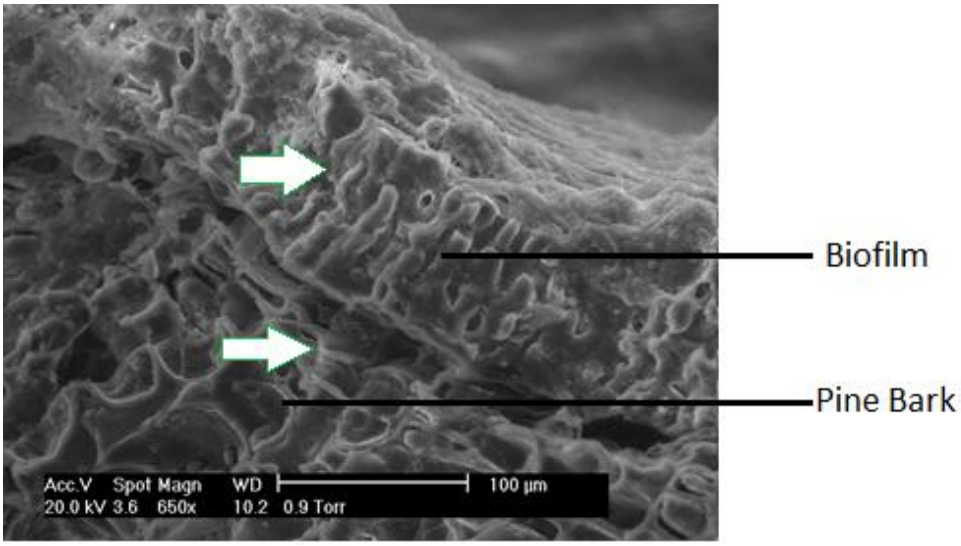

Plate 5. 12: Cross section though a pine bark nugget and attached biofilm taken from Bioreactor F after 336h. The exact locations of the EDX analyses are indicated by the arrows

Plates 5.11 and 5.12 show cross sections of pine bark BSP with attached biofilm, taken from Bioreactors E and F respectively. **Table 5.10** shows the amount of metals present in the biofilm and BSP respectively, at the locations shown in **Plate 5.12**.

Table 5. 10: Cu, Zn and Cd content (%) of the pine bark and biofilm components in Bioreactor F after 336h

Element	Biofilm component	Pine bark component
C	54.66	67.51
O	24.95	29.54
Cu	11.29	1.28
Zn	3.56	1.18
Cd	1.07	0.48
Al	0.57	0
Cl	1.72	0
K	1.44	0
Si	0.75	0
Total	**100**	**100**

All three metals were present in significantly greater amounts in the biofilm than within the pine bark matrix (i.e. Cu 11.29% vs. 1.28%; Zn 3.56% vs. 1.18%; Cd 1.07% vs. 0.48%). Because the cross sectional area scanned by the EDX spectrometer included some of the subsurface structures of the pine bark, and because Bioreactor F had a pre-established biofilm on the pine bark surface. Any dissolved metals reaching the internal structures of the pine bark would have passed through the biofilm and the surface layers of the pine bark nugget. Therefore, even though the amounts of metals detected within the pine bark nuggets were substantially lower than those in the biofilm, the BSP, nevertheless still acted as a valuable additional sorbent.

The proportions of all three metals in the bark in Bioreactor F at 336h were lower than those in the corresponding sample from the pine bark control bioreactor (**Table 5.8**), especially in the case of Cu. A key difference between the samples was that the area of pine bark sampled from Bioreactor F at 336h and shown in **Plate 5.12** was a subsurface area of the cross section, whereas the data presented in **Table 5.8** were measured at the pine bark surface. The differences in elemental composition between the matrix surface and subsurface tissue indicates that the elevated metal ion levels detected on the surface of the control pine bark specimen occurred at some point during the physical handling of the bark, rather than as a consequence of tree growth. The comparatively high proportions of metal ions in the biofilm, compared with the pine bark (**Table 5.10**), indicate that the biofilm effectively biosorbed a high proportion of the metal cations during passage of the synthetic wastewater across the biofim-BSP interface.

It proved impossible to obtain a cross section through a bioball and the attached biofilm as the two elements were easily separated. However, this is probably immaterial to this investigation since it is unlikely that metal ions would penetrate the plastic bioball surface. Therefore, contiguous areas of biofilm-covered and non-colonised bioball surface (**Plate 5.13**) were compared using EDX. The areas marked on the micrograph show the exact locations of the analysed materials. **Table 5.11** shows the compositional data for the respective locations analysed. **Appendix F, Figures F11 and F12** show the EDX spectra obtained.

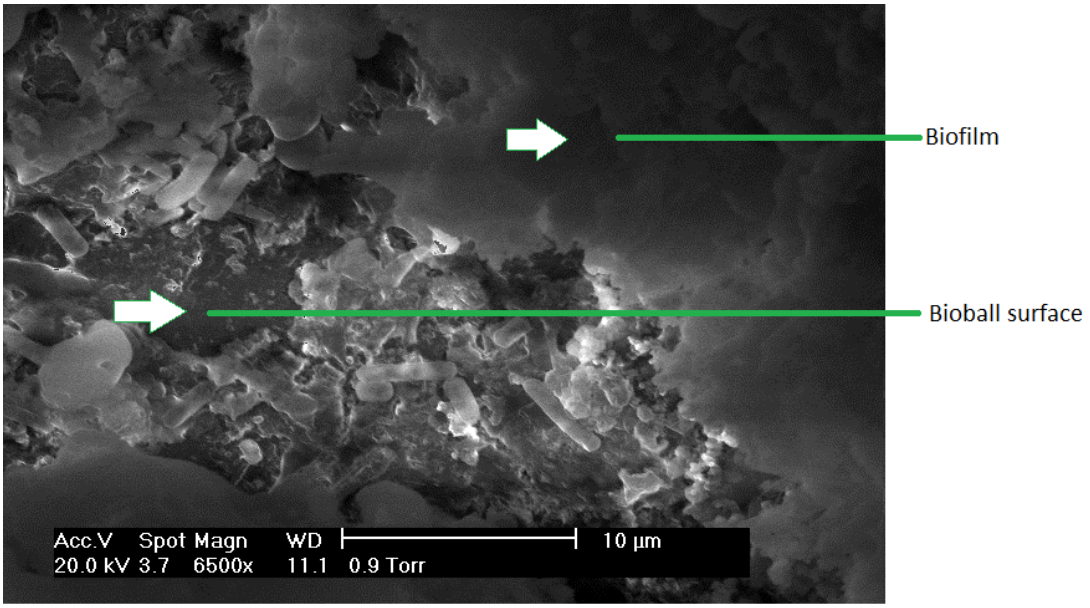

Plate 5. 13: Surface view of a randomly selected bioball from Bioreactor B after 336h showing colonised and non-colonised areas. The exact locations of the areas analysed by EDX are shown by the arrows.

Table 5. 11: Cu, Zn and Cd content (%) of colonised and non-colonised areas on the surface of a randomly selected bioball from Bioreactor B after 336h (shown in Plate 5.13)

Element	Colonised Bioball Surface	Non-Colonised Bioball Surface
C	63.33	91.15
O	15.42	7.10
Cu	5.62	0.66
Zn	7.1	0.66
Cd	3.41	0.25
Al	1.45	0
Ca	0	0.1
Si	3.68	0.8
Total	**100**	**100**

Table 5.11 shows that although the amounts of all three metals present at the BSP surface in Bioreactor B increased with time, compared with that in the control bioreactor (**Table 5.8**), the values (except for Cd) were still below those of the bioball control 48h sample. Also, the amounts of all three metals associated with the non-colonised areas of the BSP surface in Bioreactor B after 336h were substantially lower than those found in the adjacent biofilm-covered areas (initially, Bioreactor B had only inoculum added so the biofilm became established only later). This was not surprising because, as previously discussed, living microbial biomass is a known biosorbent of heavy metal cations, whereas plastic is not.

In the pine bark-based bioreactors (as with the bioball-based bioreactors), Cu^{2+} removal was more effective in the control (47.2mmol Cu^{2+} removed) than in either of the corresponding experimental bioreactors, viz. only 6.8mmol Cu^{2+} removed in both

Bioreactors E and F. But, because the starting concentration of Cu^{2+} was substantially higher in Bioreactor D, these data are difficult to compare meaningfully. Separate synthetic wastewaters were prepared for the control and experimental bioreactors. Although the same amounts of the three metal salts were used in the preparation of the medium, the T0 metal concentrations differed markedly between the control and the experimental bioreactors.

The data for Zn^{2+} and Cd^{2+} in the pine bark-based bioreactors are more comparable than those for Cu^{2+}. Bioreactors E and F delivered very similar final results even though their start-up conditions differed. Bioreactors E (pine bark and inoculum) and F (pine bark and biofilm) both removed an additional 5.2mmol Zn^{2+} and an additional 16.0mmol and 17.2mmol Cd^{2+} respectively, compared with Bioreactor D (pine bark control). All three pine bark bioreactors contained a biosorbent at start-up. Because the control did not contain a living biosorbent, the surface area available for metal biosorption in this reactor remained constant throughout the experiment. By contrast, the experimental pine bark bioreactors contained a living biosorbent, in the form of microbial cells, at start-up and, hence, the mass of material available for biosorption increased over the course of the experiment. Erikson (2004) states that the rate of reaction is usually directly proportional to the concentration of microbial mass within the bioreactor; therefore, it is generally desirable to increase the amount of biomass available within the reactor as much as possible. Thus, the additional biomass in Bioreactors E and F, comprising either planktonic inoculum or an attached biofilm, was possibly responsible for the additional metal removal recorded. This supposition is supported by a comparison between Bioreactor C and Bioreactor F. Although both these bioreactors had fully established biofilms at start-up, the pine bark reactor contained additional, albeit dead, biomass, resulting in more Zn^{2+} and Cd^{2+} being removed than in the equivalent bioball-containing bioreactors.

The results presented in **Table 5.10** indicate that the metal-containing liquid could diffuse into the pine bark BSP, thereby coming into contact with additional surface area where more biosorption could occur. Such metabolism-independent biosorption is a rapid process (Duncan and Brady, 1992; Hutchins *et al*., 1986; Cobbett, 2000; Ahalya *et al*., 2003; Ahalya *et al*., 2007; Naja and Volesky, 2010b), with equilibrium often achieved in 1 - 24h (Tunali *et al*., 2006; Nakajima *et al*., 2001; Ozdemir, 2009). Therefore, the overall amount of metal biosorption in the pine bark bioreactors (up to 336h) was likely limited by the rate of diffusion into the support matrix. This was further slowed by passage through the biofilm and therefore resembled metabolism-dependent biosorption. This would explain the comparatively slow biosorption rate observed here, compared with those of other authors cited in **Tables 1.5 - 1.7**.

In addition to the comparatively rapid sorption times shown in **Tables 1.5 - 1.7**, some authors also reported achieving significantly higher sorption capacities than those presented here, e.g. Oh and Tshabalala (2007) reported estimated metal sorption values of 0.89mmol.g^{-1}, 0.43mmol.g^{-1} and 0.73mmol.g^{-1} for Cu, Zn and Cd respectively. However, these authors used physically and chemically modified pine bark. Some of the physical modifications reported in the literature include: grinding or milling the sorbents in order to reduce particle size and increase surface area, [viz. >0.6mm (Mohan and Sumitha, 2008); 3.28mm x 11.60mm (Oh and Tshabalala, 2007); <3mm (Shin *et al*., 2007); 0.25mm – 0.5mm (Oboh and Aluyor, 2008)]. Such modifications overcome any need for the metal-bearing bulk liquid to diffuse into the pine bark or other biological material. Chemical modification included washing with formaldehyde to alter the material's surface chemical characteristics (Vázques *et al.*, 2002; Haussard *et al.,* 2003; Dostelek, 2011). All these modifications significantly improve biosorption capacities. For example, Mun *et al*. (2009) reported copper

sorption capacities of 0.5mmol.g^{-1} and 1.8mmol.g^{-1} for unmodified and chemically modified bark, respectively.

Although physico-chemical pretreatment and mechanical modification (such as grinding or milling) may minimise the problems associated with diffusion into large sorbent particles, such manipulations inevitably add cost and complexity to the system (Gadd, 2009) and may be a barrier to scale-up.

5.3.3 Comparison between Bioballs and Pine Bark for Industrial/Commercial use in Forced-Upflow Bioreactors

In order to statistically compare unprocessed pine bark nuggets with bioballs as potential BSP materials for larger scale operations, the data from the three pine bark- and three bioball-based bioreactor experiments were standardised as residual ion percentages. The metal content in all bioreactors was taken as 100% at T0 and declined over time. These data are shown in **Appendix E**.

5.3.3.1 Chi Squared Tests

Figure 5.3 shows that the total residual metal concentrations decreased in all bioreactors over time.

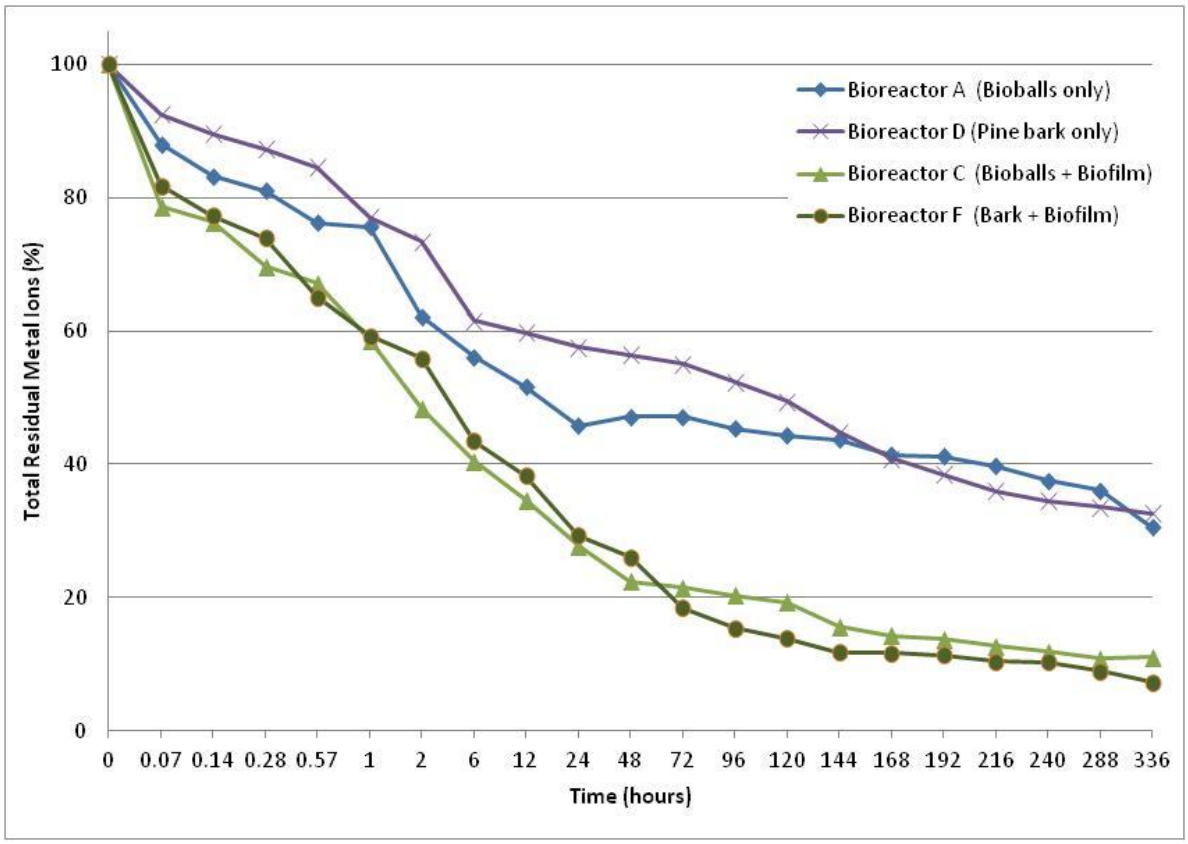

Figure 5. 3: Change in total residual metal ion concentration with time. Note that the control bioreactors showed similar trends, as did the bioreactors containing biofilms

From the curves in **Figure 5.3**, the changes in residual metal ion concentrations in the control bioreactors appear similar. The same applies for Bioreactors C and F. However, the residual metal ion concentrations in the experimental bioreactors were always lower than in the corresponding control bioreactors, regardless of the matrix used. In order to assess whether there was any statistically significant difference between the results obtained in the various bioreactors, the chi squared statistical test was applied because it compares the "Goodness of Fit" of two curves, using all data points (except T0).

The settings for the chi squared tests were as follows:

- The curves in question all have 20 data points (ignoring T0), therefore, the chi squared degrees of freedom was set to 19 (20 observed time periods less 1).
- 95% confidence limit was used, therefore, the chi squared alpha was 0.05, and the chi squared critical value was 30.1.

Four chi squared tests were performed:

Test 1: Null hypothesis: Pine bark and bioballs had the same impact on metal ion removal from synthetic wastewaters in the absence of microorganisms.

- Bioreactor A was the expected sorption pattern while Bioreactor D was the observed.
- Chi squared statistic calculated as 15.0.

Since the calculated chi squared value was less than the chi squared critical value the null hypothesis cannot be rejected.

Test 2: Null hypothesis: Pine bark and bioballs had the same impact on metal ion removal when a biofilm was present on both support matrices.

- Bioreactor C was the expected sorption pattern while Bioreactor F was the observed.
- Chi squared statistic calculated as 10.1.

As the calculated chi squared value was less than the chi squared critical value the null hypothesis cannot be rejected.

Test 3: Null hypothesis: The presence of a biofilm had no impact on biosorption in the bioreactors with bioballs as BSP.

- Bioreactor A was the expected sorption pattern while Bioreactor C was the observed.
- Chi Squared statistic calculated as 203.3.

The calculated chi Squared value was greater than the chi squared critical value so the null hypothesis is rejected. It was concluded, therefore, that the presence of a biofilm significantly impacted on the biosorption capacity of a system.

Test 4: Null hypothesis: The presence of a biofilm on pine bark BSP had no impact on biosorption in the bioreactors with pine bark BSP.

- Bioreactor D was the control or expected sorption pattern while Bioreactor F was the observed.
- Chi squared statistic calculated as 274.2.

The calculated chi squared value was greater than the chi squared critical value so the null hypothesis is rejected. Therefore, it was concluded that the presence of a biofilm significantly impacted on the biosorption capacity of the system.

From **Tests 1 and 2** it can be inferred that pine bark and bioballs have no significantly different effect on metal sorption when used as BSP. The decision on which BSP to use would, therefore, be made on other factors such as; purchase price transportation costs and availability. From **Tests 3 and 4** it is apparent that addition of a biofilm has a significant positive impact on metal biosorption, regardless of BSP.

5.3.3.2 Comparison of Confidence Intervals

In addition to the chi squared tests, other basic statistical analyses were conducted. All data points and a 90% confidence interval for these points are shown in **Figure 5.4**. All points fell within the 90% confidence interval.

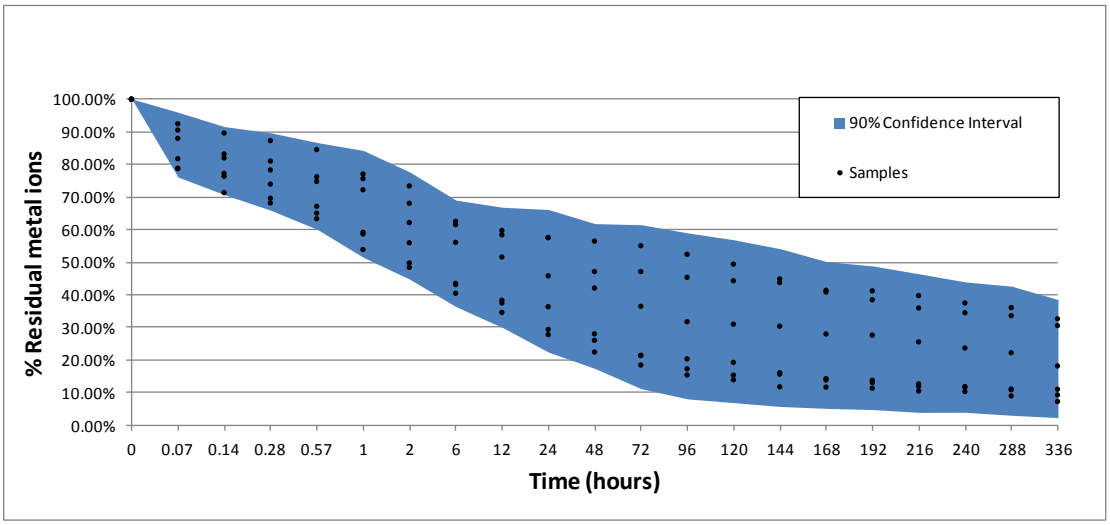

Figure 5. 4: Standardised residual metal ion concentrations for each bioreactor at each sampling time plotted against a 90% confidence interval determined across all data

Although the individual experiments may suggest that metal ion removal in a forced-upflow bioreactor is favoured, in systems containing pine bark BSP, rather than an inert material, the data should be properly analysed to check whether the former, indeed provides any statistically significant benefit over the latter. Thus, all the data from the bioreactors containing bioball BSP and from those containing pine bark BSP were compared by plotting against 90% confidence intervals. The results of these analyses are shown in **Figures 5.5 and 5.6** respectively.

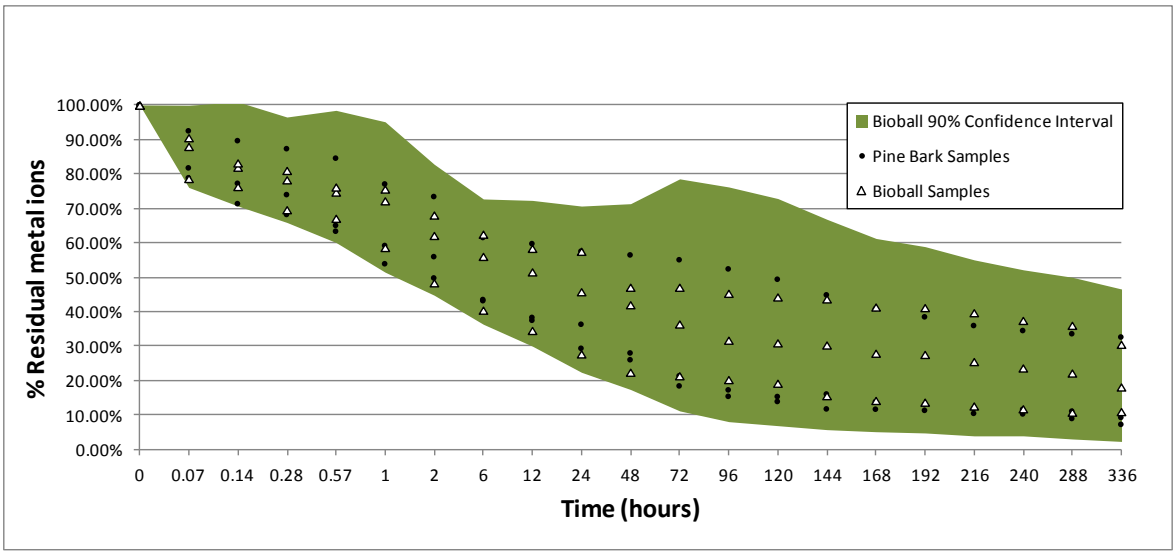

Figure 5. 5: Standardised residual metal ion concentrations for each bioreactor at each sampling time plotted against a 90% confidence interval determined across all bioreactors containing bioball BSP

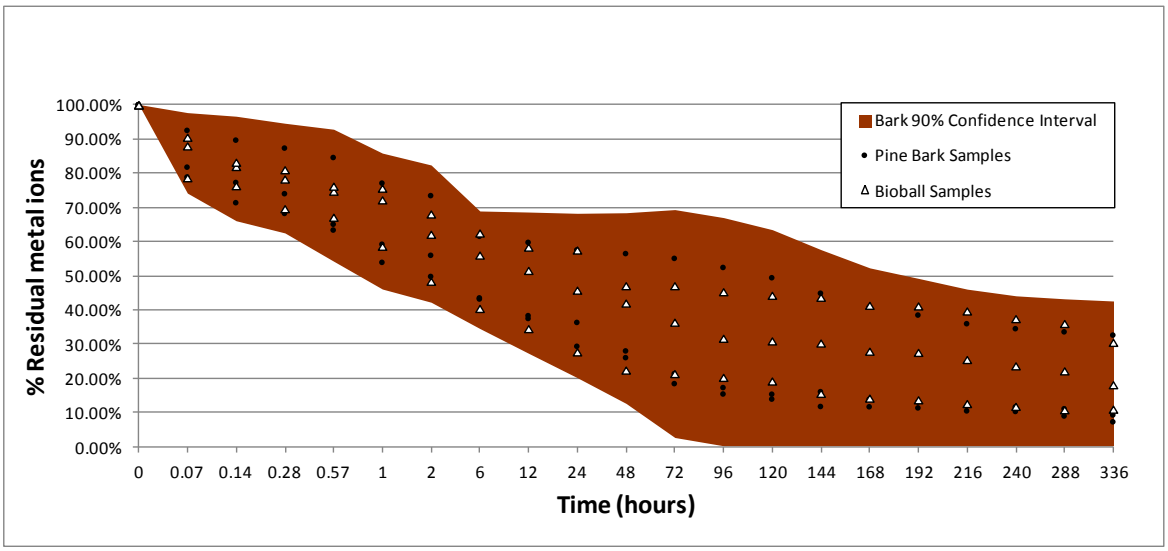

Figure 5. 6: Standardised residual metal ion concentrations for each bioreactor at each sampling time plotted against a 90% confidence interval determined across all bioreactors containing pine bark BSP

Figures 5.5 and 5.6 show that all data points lay within the 90% confidence intervals determined independently for both bioball BSP bioreactors and pine bark BSP bioreactors. Therefore, pine bark and bioballs do not differ significantly in their performance as BSP in forced-upflow bioreactors for the treatment of heavy metal ion containing wastewaters.

5.3.3.3 Cost Comparison of Pine Bark and Bioballs

Sections 5.3.3.1 and 5.3.3.2 show that there was no significant difference in the performance of forced-upflow bioreactors containing either bioballs or pine bark as BSP during treatment of synthetic wastewaters. Both matrices were capable of supporting growth of a biofilm, which is the critical factor in optimising the system for metal sorption. Therefore, the decision on which matrix to use in any industrial application is likely to be based on cost, availability and other considerations such as durability.

Table 5.3 shows a cost comparison between pine bark and bioball BSPs. Some assumptions had to be made regarding the wholesale price and bulk delivery costs of the bioballs.

Retail costs per bioreactor volume were ZAR 192.49 and ZAR 36.72 for the bioballs and pine bark respectively. Thus, including delivery charges, pine bark was more than 5x cheaper than bioballs. However, it is unlikely that retail pricing and postal delivery would be used in commercial applications. Although the pine bark used in the experiments was donated, actual wholesale pricing (ZAR 65.00 per m^3) was used. This figure had to be estimated for the bioballs (ZAR 8,400.00 per m^3). The wholesale pricing estimate places the cost of the bioballs well over 1000x more expensive to use than pine bark. It is also worthwhile noting that 4kg of pine bark were required to fill each bioreactor compared with only 3kg for bioballs (**Table 5.4**). On an industrial scale the difference in weight may affect transport costs and effect engineering considerations.

5.4 Conclusions

- Pine bark nuggets and bioballs do not differ significantly in their performance as BSP in forced-upflow bioreactors for the treatment of metal ion containing wastewaters.
- Systems combining living and nonliving biosorbents were more effective at removing metal ions from solution than corresponding systems containing only a single sorbent.
- A simple forced-upflow bioreactor system combining unmodified pine bark and a biofilm may not be as effective at removing heavy metal ions from solution as one containing highly engineered sorbents.
- Pine bark releases organic molecules when steeped in aqueous solutions. Although undesirable, these organics did not appear to hinder biofilm development. However, their presence may necessitate secondary treatment.
- Bioballs are substantially more expensive to purchase and transport than equivalent quantities of pine bark. The latter is, therefore, more likely to be considered for commercial applications, especially in poorer, technologically less developed countries.
- Cu^{2+} ions displayed different sorption characteristics to Zn^{2+} and Cd^{2+} during treatment of multi-metal-containing solutions. Therefore, forced-upflow bioreactor systems combining pine bark and a biofilm might be more appropriately used for treating wastewaters containing single metals.
- The novel laboratory-scale forced-upflow bioreactor system, combining both pine bark and a biofilm, as used in this investigation, removed copper, zinc and cadmium from an artificial wastewater. Because of this successful operation, it was decided to test a bark plus biofilm system of similar design at pilot-scale.

Chapter 6

Treatment of Single Metal Ion Solutions in Pilot-Scale Bioreactors under Different Mixing Conditions

6.1 Introduction

In **Chapters 4 and 5** it was reported that a forced-upflow bioreactor containing a biofilm supported by a pine bark BSP was potentially an effective method for treating heavy metal contaminated wastewaters. However, in these experiments laboratory-scale equipment was used and some pretreatment of the BSP (i.e. washing to remove bark fragments <5mm) was undertaken. Vijayaraghavan and Yun, (2008) state that although biosorption is a proven technique for the removal of heavy metal ions from water (at laboratory-scale), its usefulness under large-scale operating conditions is of concern. Currently the results of only a handful of pilot-scale studies have been published (Bargar *et al*., 2008; Macek *et al*., 2008), including work focussing on dairy wastes (Cannon *et al*., 2000) and landfill leachate (Jou and Huang, 2003; Bilgili *et al*., 2008). Despite the dramatic increases in published research on biosorption, there has been little or no exploitation of this technology in an industrial context (Gadd, 2009, 2010).

In most of the literature consulted, biological materials were employed which required substantial modification before use, viz. physical grinding and/or chemical treatment, usually with acid or alkali, before being useful as heavy metal ion sorbents. Although such materials may be cheap initially, the additional cost and time-consuming efforts associated with pretreatment could be undesirable in an industrial application (Naja

and Volesky, 2005; Macek and Mackova, 2011). Furthermore, unused chemicals from the pretreatment of the sorbent may necessitate further, expensive waste treatment (Lesmana *et al*., 2009) (this may also be of concern for unconsumed Voermolas and organics leached from the pine bark). Therefore, a pilot-scale bioreactor system with the potential to be commissioned for industrial application, using unmodified and untreated pine bark as biosorbent was investigated. Although supplementary nutrition was not investigated here, this parameter would require optimisation for any particular commercial application, in order to limit the amounts required and to minimise the COD/BOD of the effluent.

Two pilot-scale forced-upflow reactors were constructed and operated in batch mode to investigate the feasibility of using pine bark as the biofilm support matrix for the large-scale biosorption of heavy metals. Appropriate operating parameters, such as retention times and mixing conditions, also had to be established. Contact time between metal ions and sorption surfaces is of paramount importance when considering biological systems because shortening residence times can significantly reduce metal removal rates (Winkler, 1983; Zhou and Kiff, 1991). Conversely, extended operating time can impact negatively on the economic viability of a system (Naja and Volesky, 2005). Kinetic characterisation of metal biosorption in dual biosorbent systems is important to understand when designing systems for industrial application and, therefore, should be explored at pilot-scale. Batch kinetics of metal biosorption are typically characterised by an initial major decrease in ion concentration, followed by a second, minor diminution in the residual metal concentration – the latter occurring at a considerably slower rate than the former (Pagnanelli, 2011). These trends were observed in the mixed-metal solutions used in earlier experiments (**Chapters 4 and 5**) in which biosorption kinetics were

considered. Unfortunately, no directly comparable literature on pilot-scale dual biosorbent systems, using large-size BSP, could be sourced.

Since successful microbiological wastewater treatment requires physical contact between the target contaminant and the desired organism/s for an appropriate period of time (Winkler 1983), the effect of mixing rates on metal removal within the bioreactors was investigated. Enhanced mixing was achieved by adjusting the flow rate through the bioreactor and by introducing supplementary aeration. The latter agitates the bulk liquid causing turbulence within the bioreactor chambers, resulting in improved mixing and reduced channelling (Hall, 1992).

The synthetic wastewaters used in the laboratory-scale experiments (**Chapters 4 and 5**) contained a mixture of Cu^{2+}, Zn^{2+} and Cd^{2+}. These investigations found that Cd^{2+} and Zn^{2+} responded similarly while Cu^{2+} sorption patterns were markedly different; potentially due to competition or interferences between the different metal ions present (Vijayaraghavan and Yun, 2008; Andres and Gerente, 2011). However, a more plausible explanation was that the pH of the bulk liquid affected the different metal species differently. At the pH ranges tested, Cu was the least soluble, followed in order by Zn and Cd. Because mixed-metal solutions have already been investigated in the previous chapters and the findings suggested that inter-ion competition may have occurred, synthetic wastewaters containing only one metal at-a-time were used here in order to limit such interferences and characterise the behaviour of Cu^{2+}, Zn^{2+} and Cd^{2+} ions individually in a dual sorbent (pine bark and biofilm) pilot-scale bioreactor.

6.2 Experimental Procedure

6.2.1 Construction of the Pilot-Scale Bioreactors

Two identical five-chambered forced-upflow bioreactors were constructed from fibreglass. Each bioreactor was connected to a 100ℓ reservoir giving a total working volume of 1000ℓ. The increased volume of these pilot-scale reactors necessitated slight modifications in design in order to provide comparable conditions to those in these bioreactors. To maintain largely aerobic conditions and achieve frequent mixing of the bulk liquid, the bioreactors were designed as recommended by Cannon *et al*. (2000). This approach had the additional benefit of reducing the amount of manual handling.

The five chambers were linked in series such that when the synthetic wastewater exited the first chamber it was fed into the bottom of the second chamber. This procedure was repeated for the remaining chambers. The configuration of the system is shown in **Figure 6.1**.

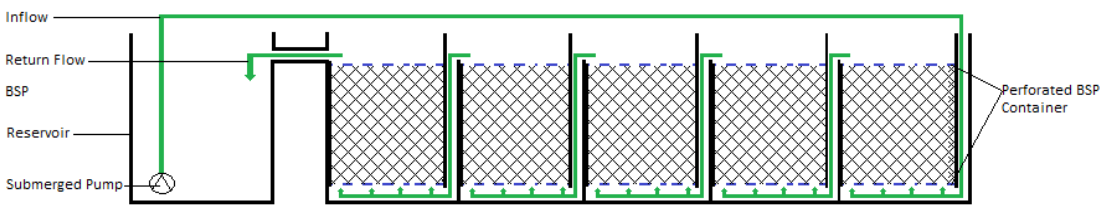

Figure 6. 1: The five-chambered bioreactor used in the pilot-scale experiments; the chambers are linked in series.

The effluent from the last chamber returned to the reservoir to ensure a continuously mixed, closed loop system.

To ensure that the upward velocities of the bulk liquid were similar to those in the laboratory-scale bioreactors, viz. 8ℓ.min^{-1}, a flow-through rate of 500 (±10) ℓ.h^{-1} was applied to Bioreactor A. This rate was calculated from the BSP surface area in the pilot-scale reactors, viz. 0.25 m^2, which is 4x larger than that of the laboratory-scale reactors. Therefore, a 4x greater flow-through rate was required to maintain upflow velocities of 8ℓ.min^{-1}.

In order to determine whether enhanced mixing in the pilot-scale bioreactors contributed to improved biosorption, Bioreactor B was always operated at a flow-through rate of 1000 (±15) ℓ.h^{-1}, with further mixing provided by evenly and simultaneously aerating all five BSP-containing bioreactor chambers with unfiltered air, at a rate of 0.2m^3.m^{-3}.min^{-1}. Each compartment had a separate sparging system comprising two 300mm lengths of PVC pipe (15mm diameter), each with ten 1mm holes evenly spaced along its length.

6.2.2 Selection of BSP Type and Size

In **Chapter 5** it was shown that there were no significantly detrimental consequences to using pine bark as BSP rather than commercially available bioballs. Furthermore, because pine bark was approximately 1000x cheaper than bioballs, both bioreactors were filled with 170Kg of unsieved, uncomposted pine bark nuggets with standard particle size < 65mm as BSP.

6.2.3 Preparation of the Synthetic Wastewater

Three separate experiments were conducted. In each experiment the synthetic wastewater contained only a single metal. The synthetic wastewaters were prepared as follows:

- Experiment 1: 2ℓ Voermolas and 536.5g $CuCl_2.2H_2O$ in 1998ℓ tap water. i.e. 0.1% (v/v) final Voermolas concentration and 100mg.ℓ^{-1} Cu^{2+}.
- Experiment 2: 2ℓ Voermolas and 671.5g $Zn(CH_3COO)_2.2H_2O$ in 1998ℓ tap water. i.e. 0.1% (v/v) final Voermolas concentration and 100mg.ℓ^{-1} Zn^{2+}.
- Experiment 3: 2ℓ Voermolas and 474.2g $Cd(CH_3COO)_2.2H_2O$ in 1998ℓ tap water. i.e. 0.1% (v/v) final Voermolas concentration and 100mg.ℓ^{-1} Cd^{2+}.

Each experiment's synthetic wastewater solution was prepared in a 2000ℓ mixing tank and distributed to both bioreactors simultaneously in order to ensure that the liquid medium in both bioreactors was identical at the start of each experiment.

6.2.4 Bioreactor Operating Conditions

The three experiments each comprised two parts:

1. The bioreactors were each filled with 898ℓ (±10ℓ) tap water, 100ℓ inoculum and 2ℓ Voermolas. The bioreactors were operated for 14 days as described in **Section 6.2.1** in order to establish a biofilm on the pine bark BSP.

2. After this time, the bioreactors were drained and subsequently refilled with synthetic wastewater containing either Cu^{2+}, Cd^{2+} or Zn^{2+}. The bioreactors were then operated as described in **Section 6.2.1** for a further seven days. Liquid samples were collected at 0, 2, 4, 6, 12, 24, 48, 72, 120 and 168h to determine any change in residual metal ion concentration. T0 samples were collected in the mixing tank and therefore apply to both bioreactors.

Between each experiment the bioreactors and all associated plumbing were cleaned and the BSP replaced to ensure that no contamination from the previous experiment occurred.

6.2.5 Temperature Range for Both Experiments

As before, the experiments were conducted in a modified greenhouse. Ambient daytime temperature was maintained at 25°C ±6°C for the duration of the experiment.

6.2.6 pH Determinations

The pH of the samples was monitored using a Crison micro 2002 pH meter calibrated using pH 4.0 and pH 7.02 standards.

6.2.7 Liquid Sampling and Sample Preparation

Samples (40mℓ) for analysis, were drawn from the return flow pipes of each bioreactor, between the final chamber and the reservoir. The samples were gravity filtered through fluted Whatman 3 µm filter paper and stored at -4^{o}C. Before quantitative analysis using atomic absorption spectroscopy, 10x and 100x dilutions of filtered samples were prepared using deionised water. Because of the low variance in metal ion concentration between replicate samples (**Chapter 5**), (the standard deviation was typically less than 0.007, but did not exceed 0.035) single samples were collected at each time interval.

6.2.8 Atomic Absorption Spectroscopy (AAS)

A Varian Spectr AA-200 series AAS, equipped with a Varian SPS-S autosampler, was used to quantify the single metal ion solutions. Samples were aspirated into a flame and atomised. A light beam of appropriate wavelength was passed through the atomised samples into a monochromator and detector (**Table 6.1**). The amount of absorbed energy at the particular wavelengths was proportional to the concentration of the elements analysed in the sample.

Standard solutions of 0, 0.5, 1, 2.5, 5, 10 and 15mg.ℓ^{-1} Cu^{2+}, Zn^{2+} and Cd^{2+} were used separately to calibrate the spectrometer. Samples were read in triplicate with the mean value reported.

Table 6. 1: Conditions used for each metal analysis by AAS

Element	Wavelength (nm)	Lamp Current (mA)	Slit Width (nm)	Flame
Cu^{2+}	324.8	4	0.5	air-acetylene
Zn^{2+}	213.9	5	1.0	air-acetylene
Cd^{2+}	228.8	4	0.5	air-acetylene

6.2.9 Biofilm Analysis

Triplicate samples of colonised pine bark were selected randomly from **Chamber 5** of both bioreactors, after each experiment. These biofilm samples were analysed using EDX spectroscopy to ascertain whether or not the observed decrease in residual metal ion concentration had occurred as a result of biosorption. EDX was not used for quantitative analysis of residual metal ion concentration.

6.2.10 Biosorption Capacity

Equilibrium Biosorption Capacity (q_e) [$mg.g^{-1}$] was calculated by the following equation (Feng *et al.,* 2009):

$$q_e = \frac{(\rho_0 - \rho_e)V}{m}$$

(**Equation 6.1**)

Where ρ_0 and ρ_e are the initial and equilibrium M^{2+} ion concentrations respectively ($mg.\ell^{-1}$); V is the volume of the solutions (ℓ) and m is the amount of sorbent used (g).

In order to better understand the biosorption processes occurring in the pine bark-biofilm bioreactors, the sorption kinetics of the three metals investigated were determined.

6.2.11 Biosorption Kinetics

6.2.11.1 Determination of Pseudo-First-Order Kinetics

Lagergren's first-order rate equation (**Equation 6.2**) describes adsorption rate based on adsorption capacity (Pagnanelli, 2011). In order to distinguish kinetic equations based on concentrations in solution from kinetic equations pertaining to solids, Larergren's first order rate equation is referred to as pseudo-first order.

$$\frac{dq}{dt} = k_1(C_e\text{-}C)$$

(**Equation 6.2**)

Where C is the metal concentration at time t, C_e is the equilibrium metal concentration and k_1 is the first-order rate constant.

Integration of **Equation 6.2** (boundary conditions: t=0, q=0 and t=t, q=q) gives

$$q - q_e [1 - \exp(-k_1 t)]$$

(**Equation 6.3**)

Equation 6.3 stated in linear form gives

$$\ln (q_e - q) = \ln qe - k_1 t$$

(**Equation 6.4**)

q_e and $k_1$were determined using plots of $\ln (q_e - q)$ versus t.

6.2.11.2 Determination of Pseudo-Second-Order Kinetics

For a second-order reaction, the rate of reaction is directly proportional to the square of the concentration of one of the reactants. Where concentration of one of the reactants remains constant (e.g. the biomass), its concentration can be grouped with the rate constant, obtaining a pseudo constant. The pseudo-second-order model is represented by **Equation 6.5** which may be linearised as shown in **Equations 6.6 and 6.7**.

$$\frac{dq}{dt} = k_2(q_e-q)^2$$

(**Equation 6.5**)

Where q is the metal concentration in solid phase at time t, q_e is the equilibrium metal concentration and k_2 is the second-order rate constant.

Integration of **Equation 6.5** (boundary conditions: t=0, q=0 and t=t, q=q) gives

$$q = q_e \left(1- \frac{1}{1+k_2t}\right)$$

(**Equation 6.6**)

Equation 6.5 stated in linear form gives

$$\frac{t}{q} = \frac{t}{q_e} + \frac{1}{k_2q_e^2}$$

(**Equation 6.7**)

The pseudo-second-order parameters (q_e and k_2) were determined by plotting t/q against t.

6.3 Results and Discussion

6.3.1 Determination of Metal Presence in the Biofilm using EDX Analysis

EDX spectroscopy was used to confirm whether or not metal biosorption by the biofilm had occurred. The results are shown in **Table 6.2**.

Table 6. 2: Presence of Cu, Zn and Cd (expressed as compositional %) in biofilm samples from each bioreactor after 168 hours, as determined by EDX Spectroscopy

	Experiment 1 (Cu^{2+})		Experiment 2 (Zn^{2+})		Experiment 3 (Cd^{2+})	
	Bioreactor A	Bioreactor B	Bioreactor A	Bioreactor B	Bioreactor A	Bioreactor B
Element	Flow-through rate 500ℓ.h^{-1}	Flow-through rate 1000ℓ.h^{-1}	Flow-through rate 500ℓ.h^{-1}	Flow-through rate 1000ℓ.h^{-1}	Flow-through rate 500ℓ.h^{-1}	Flow-through rate 1000ℓ.h^{-1}
C	54.66	56.74	56.34	55.83	56.81	55.06
O	25.95	25.58	27.32	27.12	27.53	26.05
Cu	11.59	11.36	1.21	1.00	1.11	1.19
Zn	0.26	0.21	10.79	8.8	-	-
Cd	0.07	0.13	0.25	0.21	9.07	8.41
Other elements*	7.47	5.98	4.09	7.04	5.48	9.29
Total	100%	100%	100%	100%	100%	100%

* Al, Ca, Cl and Si

The elevated proportions of the target metal ions relative to other elements detected by EDX spectroscopy indicated that, in all cases, the decrease in residual metal ion concentration had occurred as a result of biosorption.

6.3.2 Quantification of Biosorption

Residual metal ion concentrations were determined using AAS, as described in **Section 6.2.4.4**. Because single metal ion solutions were used in these experiments, stoichiometry was unlikely to be of concern, therefore the results are reported in mg.ℓ^{-1}.

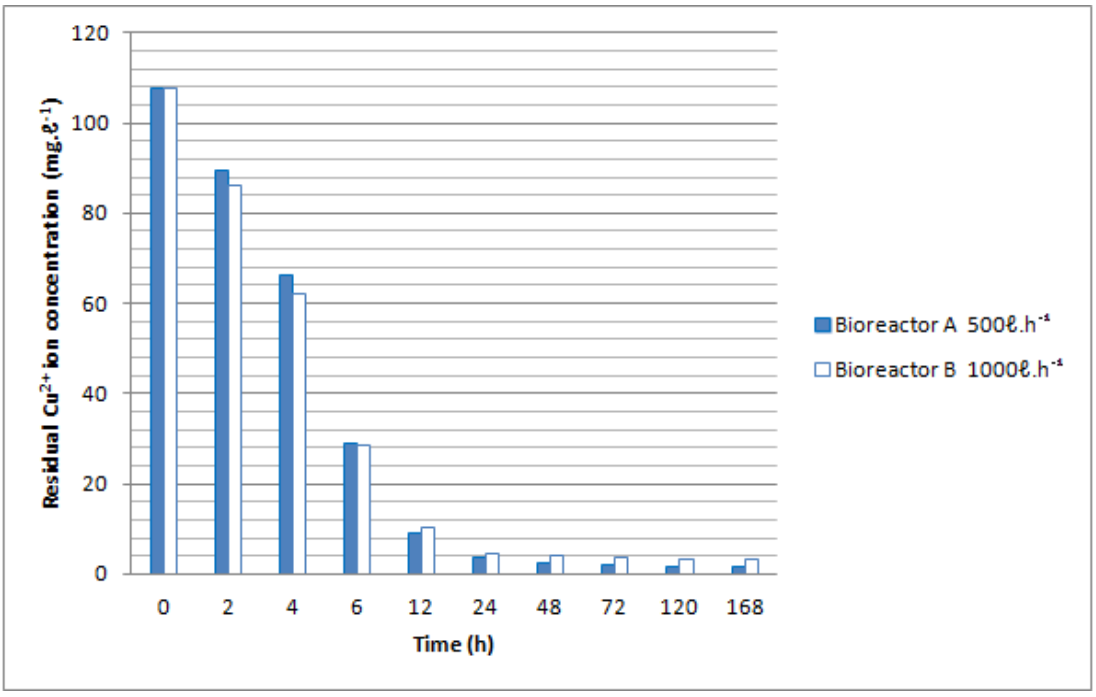

Figure 6. 2: Residual Cu^{2+} concentration in both bioreactors at the indicated sampling intervals.

The initial Cu^{2+} concentration in both bioreactors was 107.8mg.ℓ^{-1}, which decreased to 1.7mg.ℓ^{-1} in Bioreactor A (500ℓ.h^{-1}) and 3.3mg.ℓ^{-1} in Bioreactor B (1000ℓ.h^{-1} + aeration) after 168h. Therefore, the pine bark and biofilm in Bioreactor A together sorbed 98.4% of the Cu^{2+} (achieved biosorption capacity 0.62mg.g^{-1}), whilst in Bioreactor B 96.9% of the Cu^{2+} was sorbed (achieved biosorption capacity 0.61mg.g^{-1}).

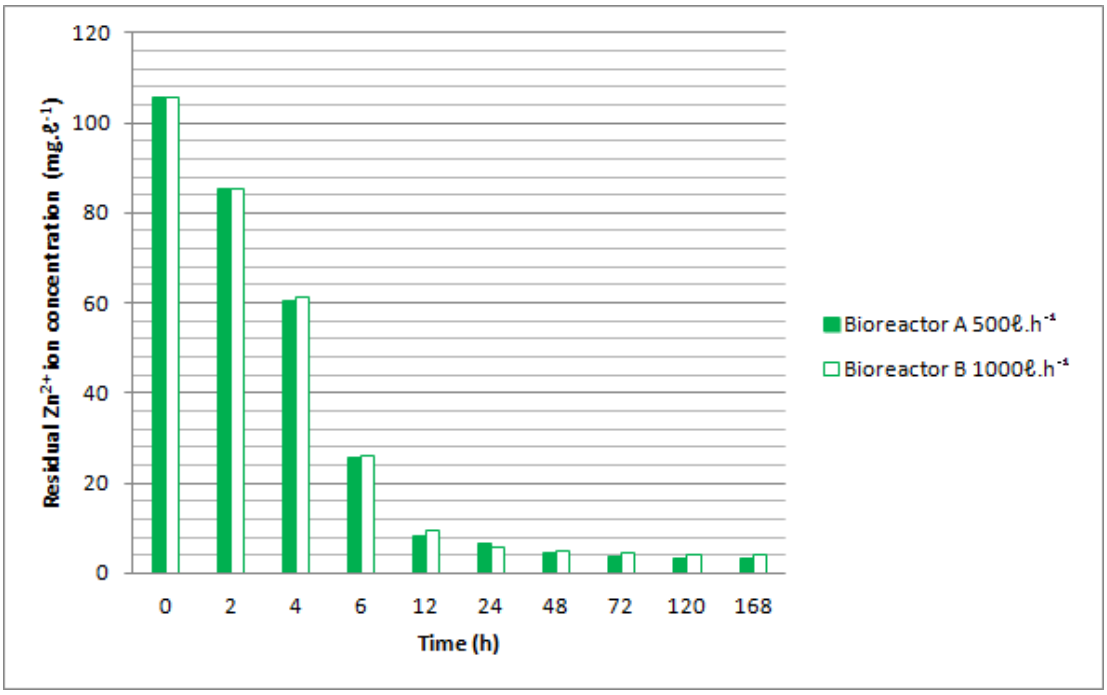

Figure 6. 3: Residual Zn^{2+} concentration in both bioreactors at the indicated sampling intervals.

The initial Zn^{2+} concentration in both bioreactors was 105.6mg.ℓ^{-1}, which decreased to 3.4mg.ℓ^{-1} in Bioreactor A (500ℓ.h^{-1}) and 4.2mg.ℓ^{-1} in Bioreactor B (1000ℓ.h^{-1} + aeration). Therefore, the pine bark and biofilm in Bioreactor A together sorbed 96.8 % of the Zn^{2+} (achieved biosorption capacity of 0.60mg.g^{-1}), whilst in Bioreactor B 96.0% of the Zn^{2+} were sorbed (achieved biosorption capacity of 0.60mg.g^{-1}).

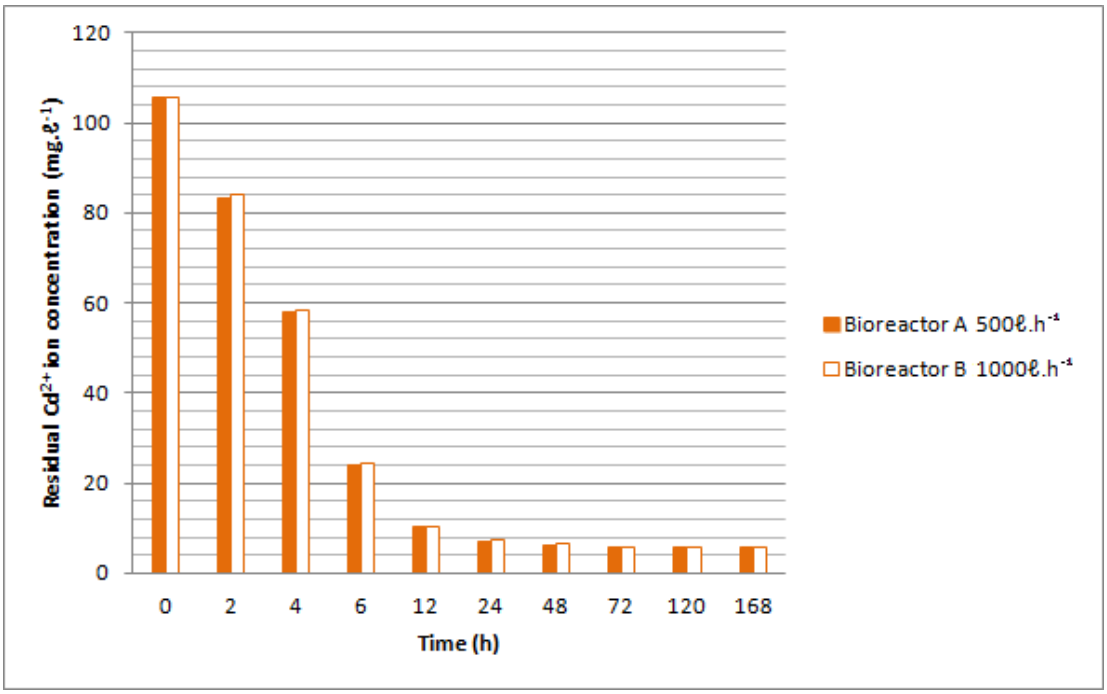

Figure 6. 4: Residual Cd^{2+} concentration in both bioreactors at the indicated sampling intervals.

The initial Cd^{2+} concentration in both bioreactors was 105.7mg.ℓ^{-1}, which decreased to 5.6mg.ℓ^{-1} in Bioreactor A (500ℓ.h^{-1}) and 5.7mg.ℓ^{-1} in Bioreactor B (1000ℓ.h^{-1} + aeration). Therefore, the pine bark and biofilm in Bioreactor A together sorbed 94.7% of the Cd^{2+} (achieved biosorption capacity of 0.59mg.g^{-1}), whilst in Bioreactor B 94.5% of the Cd^{2+} was sorbed (achieved biosorption capacity of 0.59mg.g^{-1}).

Figures 6.2, 6.3 and 6.4 show that under both sets of mixing conditions, Cu^{2+}, Cd^{2+} and Zn^{2+} behaved similarly (the raw data are presented in **Appendix G**, **Tables G1 – G3)**. The behaviour of all three metal ions was characterised by rapid initial sorption (0-12h), followed by a secondary slower decrease in residual concentration (12-168h). This is a typical pattern in biosorption experiments (Pagnanelli, 2011).

Decreased agitation appeared to result in marginally increased biosorption overall, but especially in the case of Cu^{2+}. However, during the first four hours, the Cu^{2+} ions

appeared to be sorbed at 1.08mg.h^{-1} faster rate in the bioreactor with the higher mixing rate.

The biosorption capacity of the sorbent is often used as an indication of the effectiveness of a bioreactor system for sequestering metals and may be expressed as $mg_{(sorbate)}.g^{-1}_{(sorbent)}$ (Mhavi *et al*., 2008; Feng *et al.,* 2009; Pagnanelli, 2011). The results shown in **Figures 6.2, 6.3 and 6.4** were used to determine the biosorption capacity of the pine bark-biofilm system for each tested metal. These are summarised in **Table 6.3**. Of the three metals investigated, Cu^{2+} was most efficiently sorbed, followed by Zn^{2+} and Cd^{2+}, but the differences were marginal.

Table 6. 3: Experimentally determined equilibrium biosorption capacities calculated in each experiment

	Bioreactor A	**Bioreactor B**
	500ℓ.h^{-1}	**1000ℓ.h^{-1}**
Cu^{2+}	0.62mg.g^{-1}	0.61mg.g^{-1}
Zn^{2+}	0.60mg.g^{-1}	0.60mg.g^{-1}
Cd^{2+}	0.59mg.g^{-1}	0.59mg.g^{-1}

Table 6.3 shows almost identical biosorption capacities for all three experiments regardless of the metal used or the prevailing mixing conditions. The results suggest that bulk transport of metal ions in the solution phase was not rate limiting. Furthermore, pine bark appears to display non-specific metal binding tendencies, a characteristic commonly associated with plant materials (Naja and Volesky, 2011). It is becoming increasingly accepted that biosorption involves a high degree of cation exchange (Naja and Volesky, 2011), therefore, in the absence of competing metal

ions, similar levels of sorption could be expected for each of the metals investigated. This is because operating conditions were the same, and similar numbers of sorption sites were available, in each experiment.

Biosorption efficiencies of 98.4%, 96.8% and 94.7% were achieved for Cu^{2+}, Zn^{2+} and Cd^{2+} respectively, at a mixing rate of 500ℓ.h^{-1}, and 96.9%, 96.0% and 94.5% for the removal of Cu^{2+}, Zn^{2+} and Cd^{2+} respectively, at a mixing rate of 1000ℓ.h^{-1}. These values compared favourably with results reported in the literature, for metal biosorption by agricultural waste products. For example, 77.6% Cu^{2+} removal and 56.4% Zn^{2+} removal by sour sop seeds (Oboh and Alouyor, 2008) and 85% Cd^{2+} removal by *Ulmus* leaves and their ash (Mhavi *et al*., 2008). However, the biosorption capacities achieved here, viz. 0.62mg.g^{-1}, 0.60mg.g^{-1} and 0.59mg.g^{-1} for Cu^{2+}, Zn^{2+} and Cd^{2+} respectively, at a mixing rate of 500ℓ.h^{-1} and 0.61 mg.g^{-1}, 0.60mg.g^{-1} and 0.59mg.g^{-1} for the removal of Cu^{2+}, Zn^{2+} and Cd^{2+} respectively, at a mixing rate of 1000ℓ.h^{-1} (**Appendix G, Tables G1, G2 and G3**), were considerably lower than most reported values (**Chapter 1, Tables 1.4, 1.5 and 1.6**), i.e. 6.6 - 198.5mg.g^{-1} for Cu^{2+}, 6.9 – 133.0mg.g^{-1} for Zn^{2+} and 8.0 - 278.0mg.g^{-1} for Cd^{2+}.

However, the values reported in **Tables 1.4, 1.5 and 1.6** were obtained from laboratory-scale experiments using physically and/or chemically modified sorbents with greatly increased surface area and/or number of surface receptor sites. Thus these values are not directly comparable with those obtained in the present investigation. Furthermore, other experimental parameters such as biomass dosage and pH, which have strong bearing on biosorption activity (Daniels and Wright, 1988; El-Sayed *et al*., 2010), vary across the cited literature and differ from the conditions used here. pH values (varying between pH4.0 and pH 7.0 for Cu, pH 5.5 and pH 7.5 for Zn, pH5.0 and pH 8.0 for Cd) are reported in the literature **Chapter 1, Tables 1.4,**

1.5 and 1.6. Although these pH values are not dissimilar from those reported in this study, only discrete values (as opposed to ranges) were reported by each author in the literature, thus limiting comparability. Furthermore, these authors applied biomass dosage rates typically below 3g.ℓ^{-1}, whereas the biomass dosage rates applied in the pilot-scale experiments reported here were 170g.ℓ^{-1}.

The comparatively high biomass dosage rates, combined with the large particle size of the BSP/sorbent used in the present experiment, i.e. max. nugget size ±65mm, compared with ground/milled sorbents as used and reported in the literature; typically <3mm [e.g. <3mm (Shin and Rowell, 2005); <250µm (Pandey *et al*., 2007); <0.45mm (Feng *et al*., 2009); 400-600µm (El-Sayed *et al*., 2010)] may account for the poor correlation between the experimental biosorption data (mg.g^{-1}) obtained here and those reported in the literature. This is supported by the findings of Daniels and Wright (1988) who reported that the cation exchange capacity (CEC) of pine bark decreased significantly with an increase in particle size. These researchers reported Ba/Mg CEC values of 24.7meq.100g^{-1} for particle sizes of <0.05mm; 16.2meq.100g^{-1} for particle sizes of 0.05mm – 1.19mm, and 4.7meq.100g^{-1} for particle sizes of 2.38mm – 6.35mm. The larger particle size (±65mm) of the pine bark used in the pilot-scale bioreactors overcame the engineering challenges experienced with using finely divided particles, such as composted pine bark, (**Chapter 3**). However, with larger particles the surface area, and hence the number of surface sites available for sorption, is limited. Access to subsurface binding sites in larger particles is limited by diffusion into the biofilm and BSP, thus limiting biosorbent efficiency. Pagnanelli (2011) states that intra-particle diffusion is often regarded as the rate limiting step in such biosorption scenarios.

Furthermore, the absence of literature reports suggests that the use of finely ground/milled materials in industrial /pilot-scale applications has not been previously investigated. This is likely due to engineering problems resulting from their physical characteristics, such as low density and small particle size, which makes separation of the biomass from the liquid-phase difficult (Iqbal and Edvean, 2004; 2005). These problems were experienced when composted pine bark was used as BSP (**Chapter 3**).

Figures 6.2, 6.3 and 6.4 show that under both sets of mixing conditions Cu^{2+}, Zn^{2+} and Cd^{2+} all behaved similarly, viz. rapid sorption over the initial 12h period, with a decrease in biosorption rate thereafter (12-168h). This is characteristic of biosorption systems (Pagnanelli, 2011). This pattern can be explained by considering the initial abundance of surface binding sites on the biomass which become increasingly occupied, thereby reducing the biosorption rate. However, mass transfer effects due to penetration into the biomass should also be considered. Pagnanelli (2011), describes the following steps relating to the overall rate of biosorption.

- Bulk transport of metal ions in solution
- Diffusion of metals through a hydrodynamic boundary layer around the biosorbent surface
- Intra-particle diffusion through the gel phase of the biomass
- Chemical binding with active sites

Because chemical reaction is too fast to govern overall reaction speed, it is likely that the rate determining step is either external film diffusion or intra-particle diffusion (Pagnanelli, 2011).

The most notable difference between the three experiments was the behaviour of the Cu^{2+} ions during the first six hours of the experiment. Between 0h and 6h, the Cu^{2+} ions were sorbed at a slightly higher rate (13.2 $mg.\ell^{-1}.h^{-1}$) in the bioreactor with increased mixing (1000$\ell.h^{-1}$ + aeration) than in the bioreactor operating at the lower mixing rate (500$\ell.h^{-1}$) (13.0 $mg.\ell^{-1}.h^{-1}$). After 6h the trend inverted and the biosorption rate was higher in the 500$\ell.h^{-1}$ bioreactor (3.3 $mg.\ell^{-1}.h^{-1}$ between 6h and 12h) than in the bioreactor with increased mixing, i.e. 1000$\ell.h^{-1}$ + aeration (3.1 $mg.\ell^{-1}.h^{-1}$) over this period. This suggests that during the first 6 hours bulk transport of metal ions in the solution phase could have been rate limiting, therefore increased mixing favoured biosorption of Cu ions. However, this was not the case for the Zn^{2+} (**Figure 6.3**) or Cd^{2+} (**Figure 6.4**) ions. This is consistent with the earlier findings (**Chapter 3**) which showed that Cu^{2+} adsorbed more readily to biological material than did Zn^{2+} and Cd^{2+}. Similar results have been reported by Garnham (1997) and Shi *et al*. (2003). Therefore, it is likely that the increased incidents of contact between the sorbate and sorbent, arising as a consequence of the increased mixing rate, increased biosorption rates in the short term.

6.3.3 Biosorption Kinetics

The trends shown by the modelled data for Experiments 1, 2 and 3 (**Table 6.4**) did not fit well with the experimental data, and the correlation coefficients were substantially lower than those obtained for the pseudo-second-order equation. The graphical representations of the data obtained for Cu^{2+}, Zn^{2+} and Cd^{2+} are shown in **Appendix G**, **Figures G1- G3 and Figures G4 – G6** respectively.

Table 6. 4: Predicted biosorption capacity and rate constants in each experiment

		Pseudo-1st-order		Pseudo-2nd-order		
		k_1 [a] $[kg.(mg.h)^{-1}]$	R^2	k_2 [b] $[kg.(mg.h)^{-1}]$	R^2	Biosorption capacity(q_e) [c] $[mg.kg^{-1}]$
Cu^{2+}	$500\ell.h^{-1}$	1.001	0.941	0.481	0.997	0.641
	$1000\ell.h^{-1}$	0.957	0.956	0.593	0.998	0.628
Zn^{2+}	$500\ell.h^{-1}$	0.968	0.968	0.593	0.998	0.615
	$1000\ell.h^{-1}$	0.937	0.965	0.608	0.998	0.610
Cd^{2+}	$500\ell.h^{-1}$	1.001	0.971	0.722	0.999	0.600
	$1000\ell.h^{-1}$	0.942	0.961	0.709	0.999	0.599

a. k_1 = - (gradient)
b. $k_2 = 1/q_e^2$ * intercept
c. q_e = 1/gradient

The results were not unexpected because although Gupta and Rastogi (2008), when applying this equation to Ni^{2+} uptake by unmodified biomass, showed a good correlation to exist up to 150min in their experiment, Pandey *et al*. (2007) showed poor linear correlation using the same metal ions and a similar biosorbent over 180min. Furthermore, Vijayaraghavan and Yun (2008) and Febrianto *et al*. (2009) state that the pseudo-first-order model is only applicable to the initial period in a sorption process, and therefore does not fit well over the entire metal/sorbent contact time. Therefore, because the experiments took place over 14 days, the pseudo-first-

order equation was discounted as a potential model for predicting biosorption activity in pine bark-biofilm bioreactors.

A pseudo-second-order model is generally considered to be more appropriate than a pseudo-first-order model to represent kinetic data of metal biosorption in batch reactors, because the pseudo-second-order model assumes a second-order dependence of the sorption rate on available binding sites (Pagnanelli, 2011).

For the pseudo-second-order model, all six graphs had R^2 values > 0.997 indicating a good fit between the experimental data and the pseudo-second-order model. The biosorption capacity (q_e) of the pine bark for each tested metal as predicted by this model is shown in **Table 6.4**. The q_e value was calculated at approximately 1000x lower than the experimentally determined biosorption capacity. Furthermore, the predicted rate constants did not agree with experimental observations. Therefore, although pseudo-second-order models have proved effective in predicting biosorption capacities and rate constants in laboratory-scale experiments using finely divided sorbents (Gupta and Rastogi 2009; Feng *et al*., 2009), this model did not fit well for the pilot-scale forced-upflow bioreactors with large sized composted pine bark nuggets as BSP, despite the high correlation coefficients obtained.

6.4 Conclusions

- In all the pilot-scale experiments, biosorption was largely responsible for the observed decreases in Cu^{2+}, Zn^{2+} and Cd^{2+} ion concentration.
- There is no apparent improvement in biosorption by colonised pine bark through enhanced mixing, brought about by increasing the flow-through rate beyond $500\ell.h^{-1}$ or providing supplementary aeration. Because both bioreactors were of identical construction and both operated as closed loop systems, contact time was deemed to be 168h in all cases.
- The biosorption capacities achieved for all three metals by the pine bark-biofilm sorbents compared poorly with values reported in the literature. This was not surprising because the latter were obtained from single sorbent, laboratory-scale systems, generally using physically and chemically modified sorbents. By contrast, the system investigated here comprised dual sorbents, using physically and chemically unmodified biosorbents at pilot-scale. Furthermore, sorbent particle sizes reported in the literature were significantly smaller than those utilised in this study.
- Although pseudo-second-order plots of the biosorption kinetics of all three metals tested showed good linear correlation, predicted biosorption capacities and pseudo-second-order rate constants did not reflect the experimental findings.

Chapter 7

Overall Summary and General Conclusions

Fresh water is one of Earth's most precious resources and is becoming an increasingly scarce commodity in many countries. It is widely used in industrial applications and often becomes polluted as a consequence. Worldwide there is increasing awareness of its importance and legislation controlling water use has thus become progressively stricter. This has placed pressure on industries to limit water use and to substantially detoxify any wastewater that they produce. Heavy metal ions are a common contaminant of industrial wastewater, but may be effectively and cheaply removed from liquid solutions through biosorption to either living biomass or nonliving materials such as microbial or agricultural waste products. Biosorption of heavy metals to these materials under controlled laboratory conditions is widely understood and has been extensively documented.

Notwithstanding this, there is still only limited information available on the use of biomass, either living or dead, for pilot-scale operations and no information was found in the literature on approaches combining living and dead biomass in a single system to remove metals from industrial wastewater on a large-scale. Furthermore, where biological materials have been used to sequester heavy metal ions these were typically highly modified (chemically and/or physically) to increase the number of surface receptor sites available for cation binding. Although highly effective, this may be undesirable in industrial applications because of the increased costs and time expended in modifying the sorbent. The present challenge was to develop a novel biotechnology combining living microbial biomass immobilised on a cheap

unmodified, dead plant material to effectively remove copper, zinc and cadmium ions from solution, ultimately at pilot-scale.

Various types of bioreactors have been designed to optimise contact between a sorbent and sorbate, usually some form of biomass and metal dissolved in a bulk liquid (e.g. wastewater). Forced-upflow bioreactors were chosen for use in this study because they offer the opportunity to combine living biomass, in the form of a biofilm, with nonliving material serving as support matrix, in a simple, easy to produce and easy to operate system. Pine bark was selected as BSP because it is an agricultural waste product of the timber industry and is abundant in KwaZulu-Natal where it is available at low cost.

Initially, there were concerns about pine bark's apparent lack of physical strength and potential to release harmful organics into the system. Thus composted and uncomposted pine barks were tested in laboratory-scale forced-upflow bioreactors for their effect on the growth and maintenance of a biofilm. Although the former proved excellent as a support matrix for developing a biofilm its lack of physical strength, combined with small particle size, resulted in significant biomass washout. Also the small interstitial spaces, a consequence of the small particle size, were quickly occluded by the developing biofilm, thereby causing through-flow problems. By contrast, uncomposted pine bark was sufficiently robust for use in the bioreactors and, although it did release dissolved organics into the system, it did serve successfully as a support for developing and maintaining a biofilm. Therefore uncomposted pine bark with particle size 6mm-16mm was selected for use in the laboratory-scale bioreactors, whereas uncomposted pine bark with particle size <65mm was used in the physically more demanding pilot-scale bioreactors.

The effect of supplementary nutrition on the living biomass component, and its performance in metal ion biosorption, was investigated by adding Voermolas to the bioreactor system. In those instances where a biofilm was not present at the start of the experiment, Cu^{2+} was most effectively sorbed in the absence of supplementary nutrition. By contrast, Zn^{2+} and Cd^{2+} were most effectively sorbed after addition of 0.2% (v/v) Voermolas. Conversely, where a biofilm was present at the start of the experiment, the best results, for all three metals, were achieved when 0.1% (v/v) Voermolas was added. Therefore, all further experiments were conducted using 0.1% v/v Voermolas as a supplementary nutrient source. Although Voermolas at this concentration proved to be an effective and cheap nutrient source for potential industrial applications, it is recommended that further research be conducted to investigate alternative nutrient sources which may be more cost effective or result in higher metal binding capacities in the system.

Although pine bark is known to be an effective metal sorbent and has been shown to be a suitable BSP in both laboratory and pilot-scale bioreactors, it was felt that its performance, in these respects, needed to be compared with that of other available materials. Plastic bioballs were selected as a commercially available, bespoke BSP to compare with the pine bark. Experimental results revealed that pine bark and bioballs did not differ significantly in their performance as BSP in the forced-upflow bioreactors used. However, because of low cost and ready availability, pine bark was considered the more favourable.

Because both the pine bark and biofilm components of the bioreactors sorbed heavy metals, environmental scanning electron microscopy (ESEM) and energy dispersive X-ray (EDX) microanalysis were used to analyse cross sections through the biofilm-pine bark interface to apportion the amounts of metal ions adsorbed and absorbed by the two sorbents. A procedure entailing freezing in liquid nitrogen followed by a

twisting action, was developed to prevent or minimise cross contamination and distortion of the interface. With practice, clean cross sections through the pine bark and biofilm were obtained, permitting adjacent sections of both sorbents to be analysed separately. All three metals were detected in the interfacial regions, but were always present in significantly greater quantities in the biofilm than within the pine bark matrix. Because of the structural features of the region scanned by the EDX spectrometer any metal-containing water reaching the deeper layers of the pine bark would have had to pass through the biofilm and the surface layers of the pine bark nugget before reaching these internal structures. Therefore, even though the quantities of metals found within the pine bark nuggets were substantially lower than those in the biofilm, the BSP was a valuable adjunct sorbent even when overlayed by a biofilm.

The present investigation showed that a simple forced-upflow bioreactor system combining large-size uncomposted pine bark nuggets and a microbial biofilm was effective at removing copper, zinc and cadmium, from an artificial wastewater at pilot-scale. Admittedly the present system, combining chemically and physically unmodified living and dead biosorbents, was not as effective at removing metals from solution as are the highly engineered sorbents reported in the literature. Furthermore, the biosorption kinetics involved in pilot-scale bioreactors using sorbents with large particle size, compare unfavourably with those containing highly modified, small particle sorbents described in the literature. Although pseudo-second-order plots of the biosorption kinetics of all three metals tested, showed good linear correlation, predicted biosorption capacities and pseudo-second-order rate constants did not reflect the experimental findings.

This study showed that an attached microbial association supported by a biological matrix, housed in a forced-upflow bioreactor, offers an effective biotechnology for the

treatment of metal contaminated liquid wastes. A major concern was the paucity of data from comparable studies: e.g. studies using pilot-scale equipment; studies using sorbents with diameters greater than 5mm; and studies of systems combining living and dead biomass in a single bioreactor. If a set up such as the one investigated here is to find commercial application, further investigation into the real world capital and operational costs of such a system compared with conventional treatment methods, or wastewater disposal charges, would need to be undertaken.

The original aims and objectives of this investigation have been successfully met since the experiments conducted showed that biosorption offers a technologically simple and effective strategy for treating metal contaminated wastewaters, and that the presence of dual biosorbents improves the overall performance of the system. Due to the non-specific nature of most microbial sorbents and the resilience of biofilms to stressful conditions, this technology may also find application in the treatment of industrial liquid waste streams containing other inorganic substances. However, until field trials to test and optimise such systems, or tailor the technology to specific applications, are conducted and published, industries are unlikely to invest in them for in-house wastewater treatment operations.

Such largely biological systems, with their low-key technology, may find acceptance in third world countries, which lack both the financial and engineering resources required to successfully operate many of the sophisticated wastewater treatment plants found in first world countries.

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Appendix A

Table A. 1: Masses of metal salts (Analar grade) used to produce the synthetic wastewater

Metal	Chemical Form	Molecular Weight	Mass used	Theoretical concentration in bioreactor	Theoretical molar concentration in Bioreactor
Cu^{2+}	$CuCl_2.2H_2O$	170.48g	84.0g	84mg.ℓ^{-1}	1.33mmol
Zn^{2+}	$ZnCl_2$	136.28g	77.1g	100mg.ℓ^{-1}	1.53mmol
Cd^{2+}	$(CH_3COO)_2$ $Cd.2H_2O$	266.52g	88.1g	100mg.ℓ^{-1}	0.89mmol

Appendix B

Table B. 1: Effect of nutrient levels on residual and final metal ion concentrations expressed in millimoles (Experiment 1)

		A			B			C			D			E		
Sample Number	**Time (h)**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**
(Initial) 0	0	0.68	1.09	0.83	0.74	1.21	0.91	0.76	1.15	0.87	0.77	1.19	0.90	0.77	1.24	0.94
1	1	0.61	1.04	0.85	0.60	1.09	0.85	0.50	1.16	0.85	0.60	1.06	0.79	0.69	0.90	0.68
2	2	0.61	1.13	0.87	0.55	1.07	0.82	0.44	1.18	0.89	0.58	0.99	0.78	0.68	0.93	0.69
3	6	0.55	1.06	0.80	0.50	1.16	0.90	0.46	1.19	0.77	0.52	0.98	0.79	0.66	1.02	0.82
4	12	0.41	0.92	0.72	0.52	1.04	0.82	0.52	0.96	0.71	0.44	1.22	0.74	0.50	1.01	0.78
5	24	0.36	0.90	0.73	0.38	0.99	0.77	0.33	0.95	0.70	0.46	0.93	0.75	0.44	0.92	0.69
6	36	0.28	0.92	0.77	0.35	1.01	0.79	0.27	0.83	0.65	0.36	0.93	0.73	0.41	0.90	0.70
7	48	0.20	0.78	0.60	0.28	0.92	0.72	0.27	0.81	0.62	0.27	0.76	0.60	0.28	0.90	0.69
8	120	0.19	0.84	0.64	0.24	0.84	0.65	0.20	0.96	0.70	0.24	0.78	0.60	0.31	0.81	0.60
9	168	0.16	0.75	0.57	0.16	0.57	0.46	0.22	0.80	0.60	0.30	0.83	0.65	0.27	0.87	0.68
10	216	0.13	0.84	0.68	0.25	0.92	0.73	0.20	0.73	0.62	0.25	0.70	0.55	0.27	0.78	0.63
(Final) 11	384	0.11	0.82	0.66	0.19	0.74	0.60	0.20	0.72	0.61	0.26	0.73	0.57	0.26	0.76	0.62
Change in conc. (Initial - Final)		0.57	0.27	0.17	0.55	0.47	0.31	0.56	0.43	0.26	0.51	0.46	0.33	0.51	0.48	0.32
Total M^{2+} sorbed[a] (mmol)		21.1	9.99	6.29	20.4	17.4	11.5	20.7	15.9	9.62	18.9	17.0	12.2	18.9	17.8	11.8
Relative M^{2+} sorption[b]		84%	25%	20%	74%	39%	34%	74%	37%	30%	66%	39%	37%	66%	39%	34%
M^{2+} sorbed per g Pine Bark (µmol)[c]		6.94	3.29	2.07	6.85	5.86	3.86	6.93	5.32	3.22	6.27	5.65	4.06	6.31	5.94	3.96
M^{2+} sorbed per g Pine Bark (mg)[d]		0.44	0.21	0.23	0.44	0.38	0.43	0.44	0.35	0.36	0.40	0.37	0.46	0.40	0.39	0.45

a **Total M^{2+} sorbed = [Change in concentration] x [Total volume (37ℓ)]**

b **Relative M^{2+} sorption = [Change in concentration] / [Initial concentration]**

c **M^{2+} sorbed per g Pine Bark = [Total mmol M^{2+} sorbed] / [Dry Mass of BSP**

d **M^{2+} sorbed per g Pine Bark = [Total mg M^{2+} sorbed] / [Dry Mass of BSP]**

Table B. 2: Sample pH measured during Experiment 1

Sample Number	Time (h)	pH				
		A Control	B 0.1% (v/v) Voermolas	C 0.1% (v/v) Voermolas	D 0.2% (v/v) Voermolas	E 0.2% (v/v) Voermolas
0	0	5.9	6.9	7.1	7.1	7.0
1	1	5.9	6.9	6.9	7.0	7.3
2	2	5.7	7.0	7.0	7.0	7.2
3	6	5.2	7.1	7.2	7.2	7.4
4	12	4.9	7.1	7.4	7.2	7.6
5	24	4.8	6.9	7.2	7.4	7.7
6	36	4.7	7.0	7.4	7.5	7.6
7	48	4.6	7.1	7.5	7.7	7.9
8	120	4.5	7.3	7.5	7.8	7.9
9	168	4.7	7.3	7.7	7.9	8.0
10	216	4.8	7.4	7.6	8.0	8.1
11	384	4.9	7.4	7.3	8.1	8.0

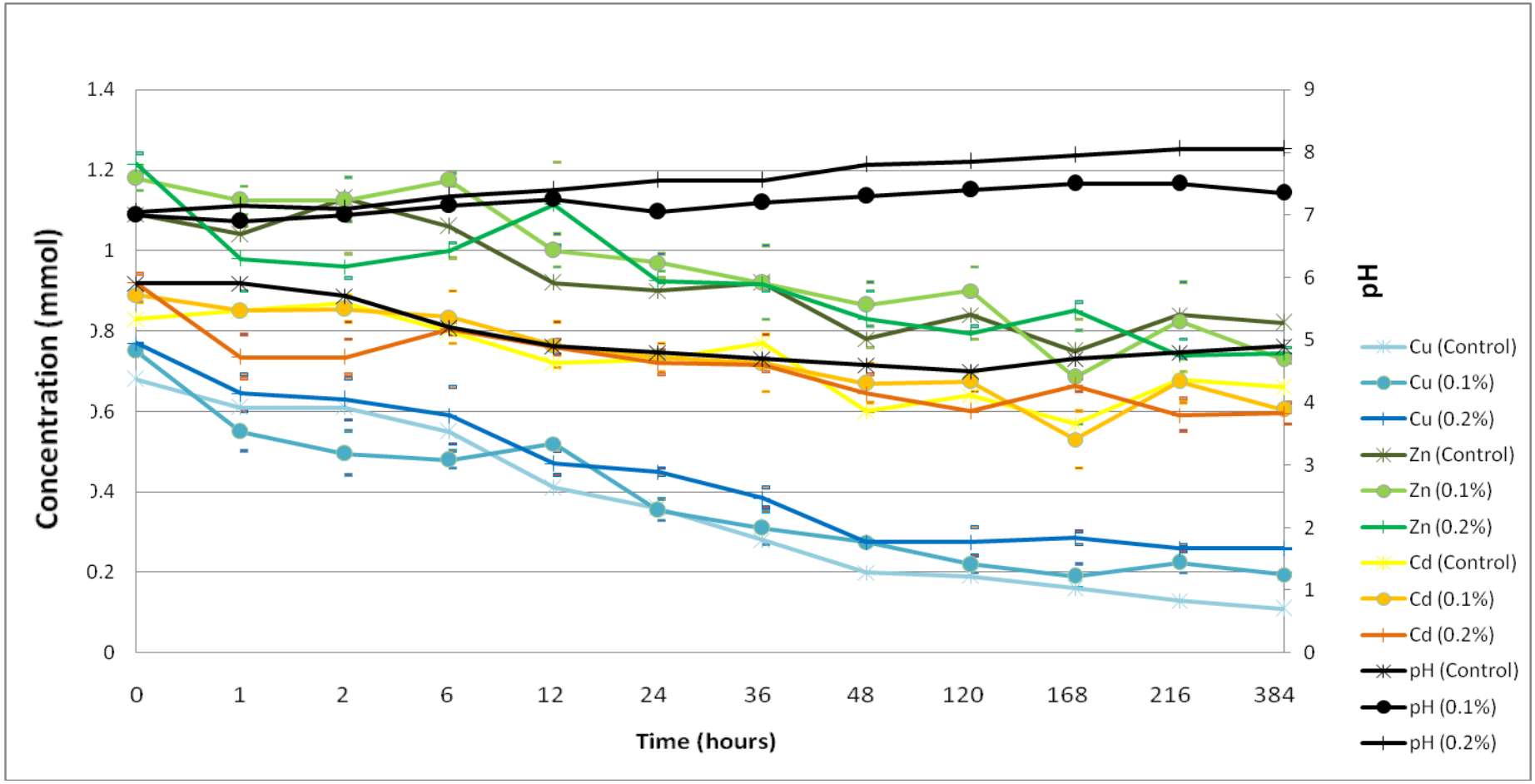

Figure B. 1: Changes in residual metal ion concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 1). Average values and control values are plotted as lines with variances indicated as colour coordinated points

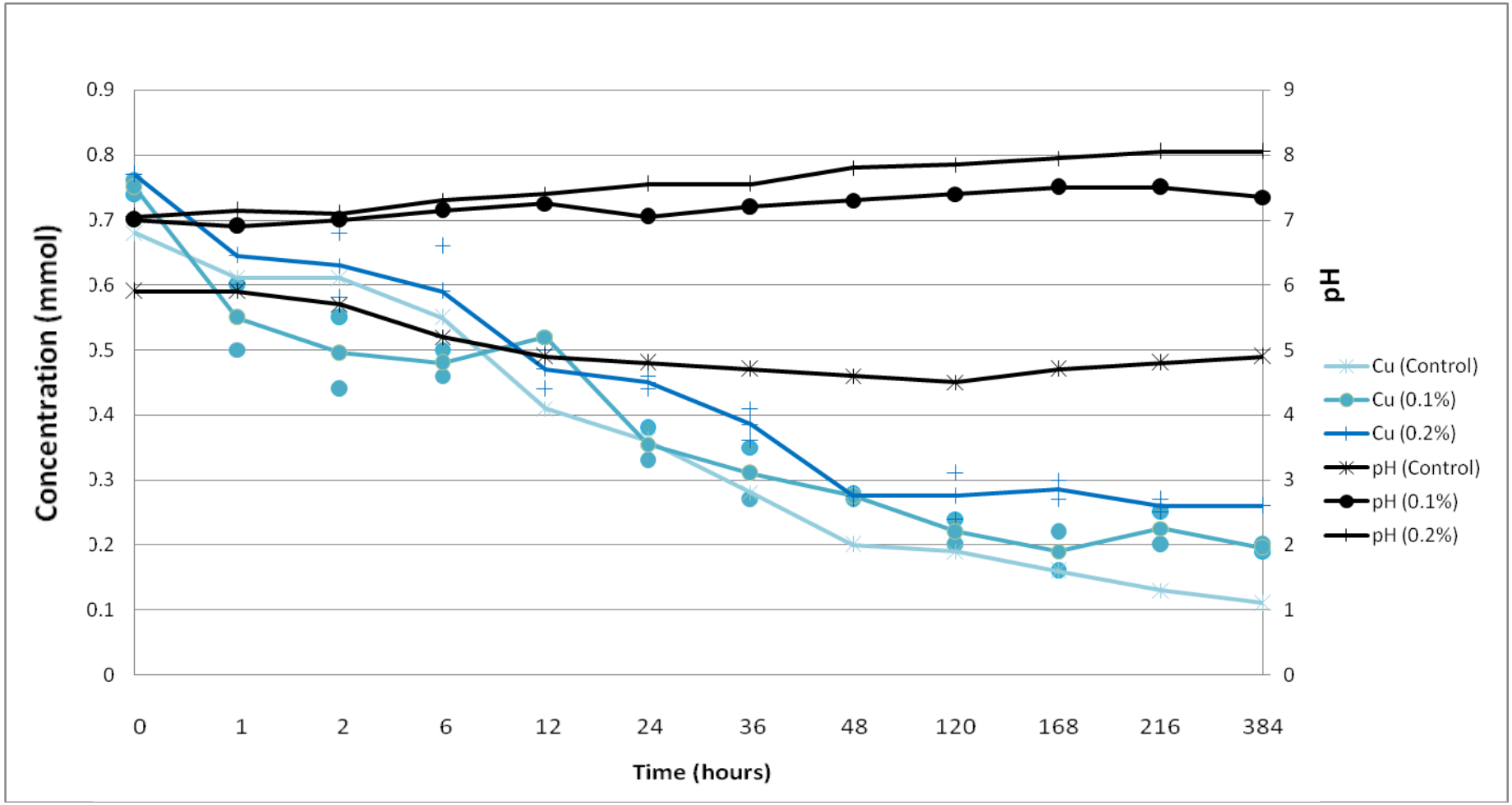

Figure B. 2: Changes in residual copper concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 1). Average values and control values are plotted as lines with variances indicated as colour coordinated points

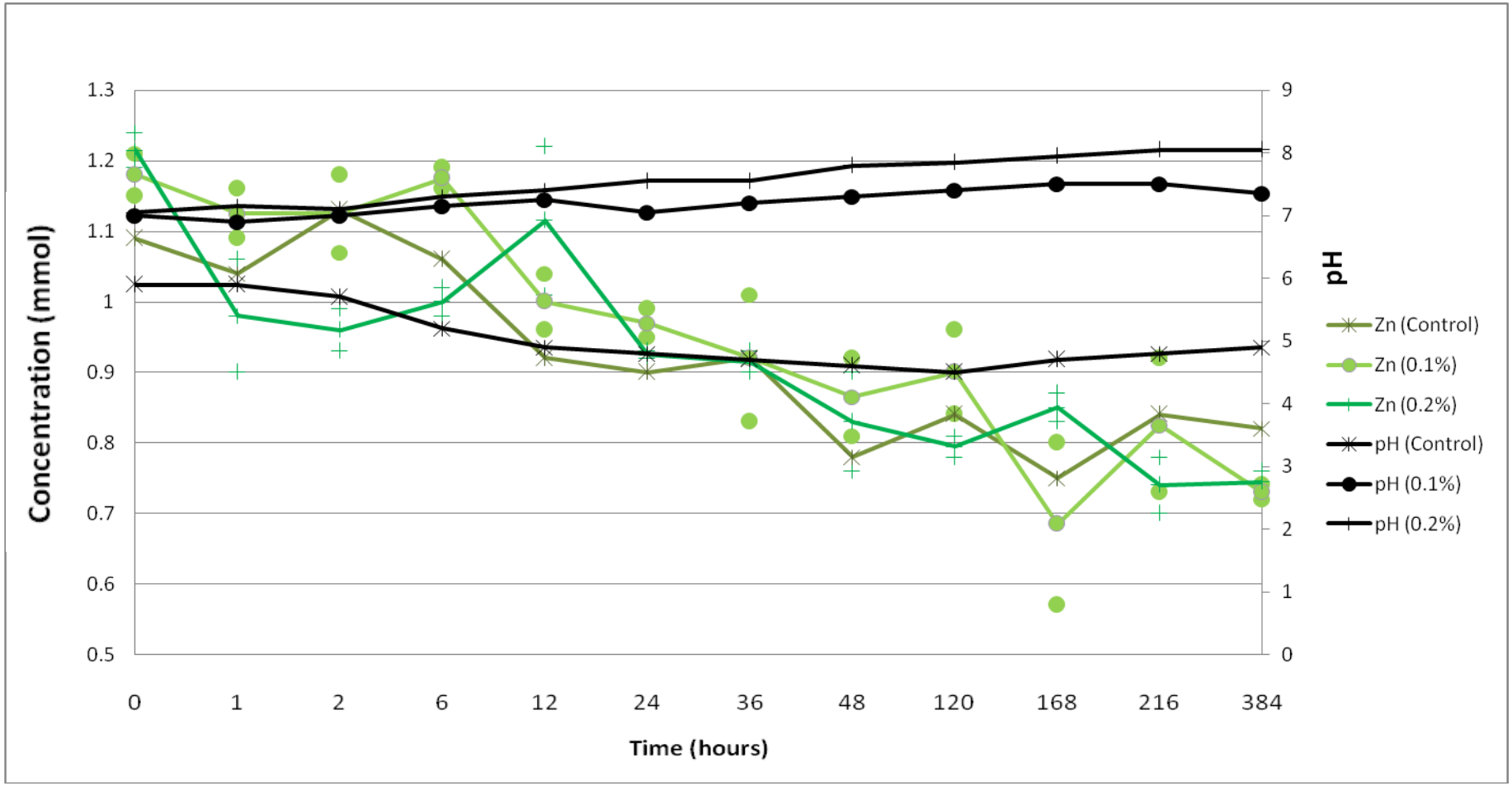

Figure B. 3: Changes in residual zinc concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 1). Average values and control values are plotted as lines with variances indicated as colour coordinated points

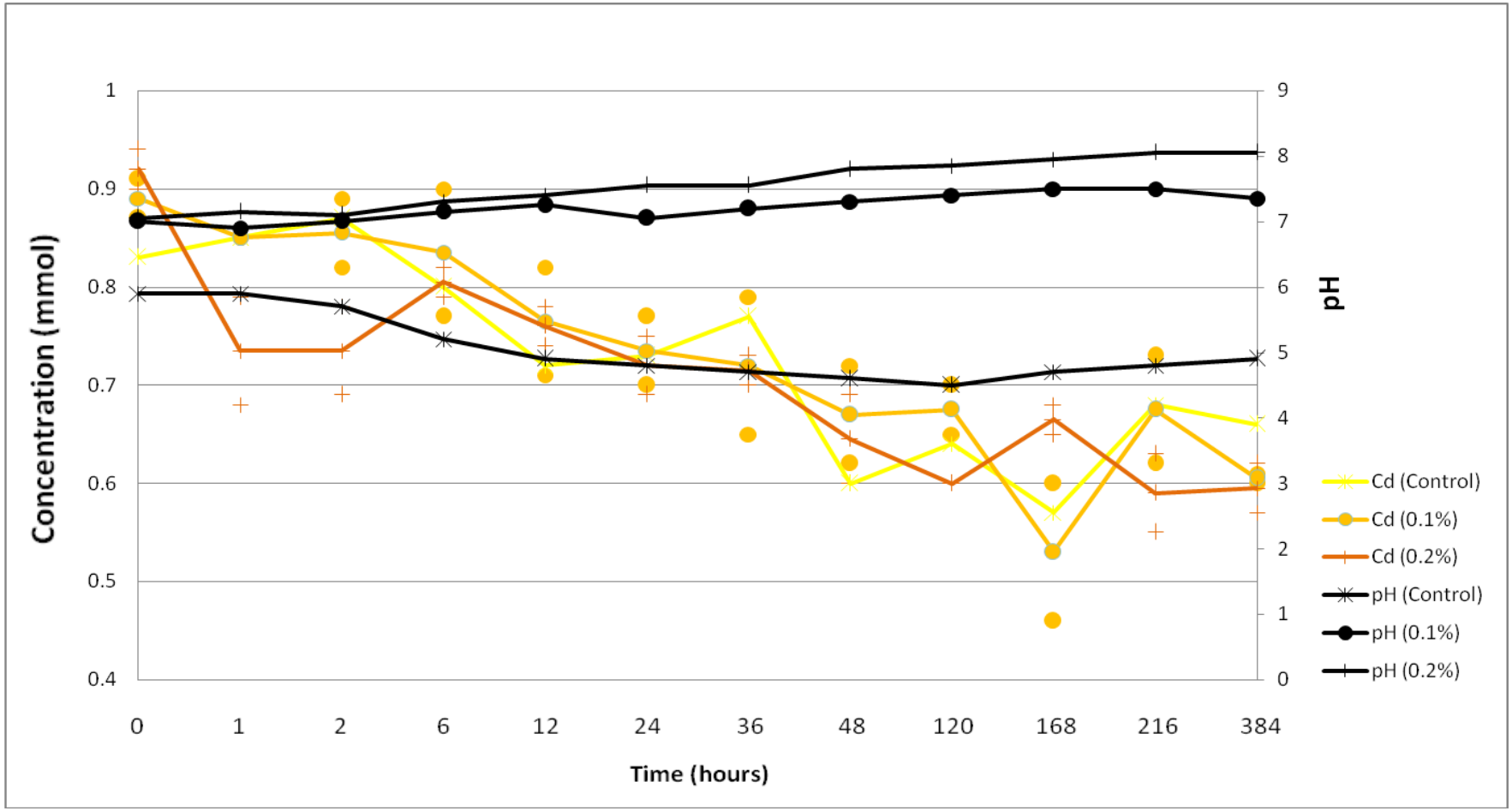

Figure B. 4: Changes in residual cadmium concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 1). Average values and control values are plotted as lines with variances indicated as colour coordinated points

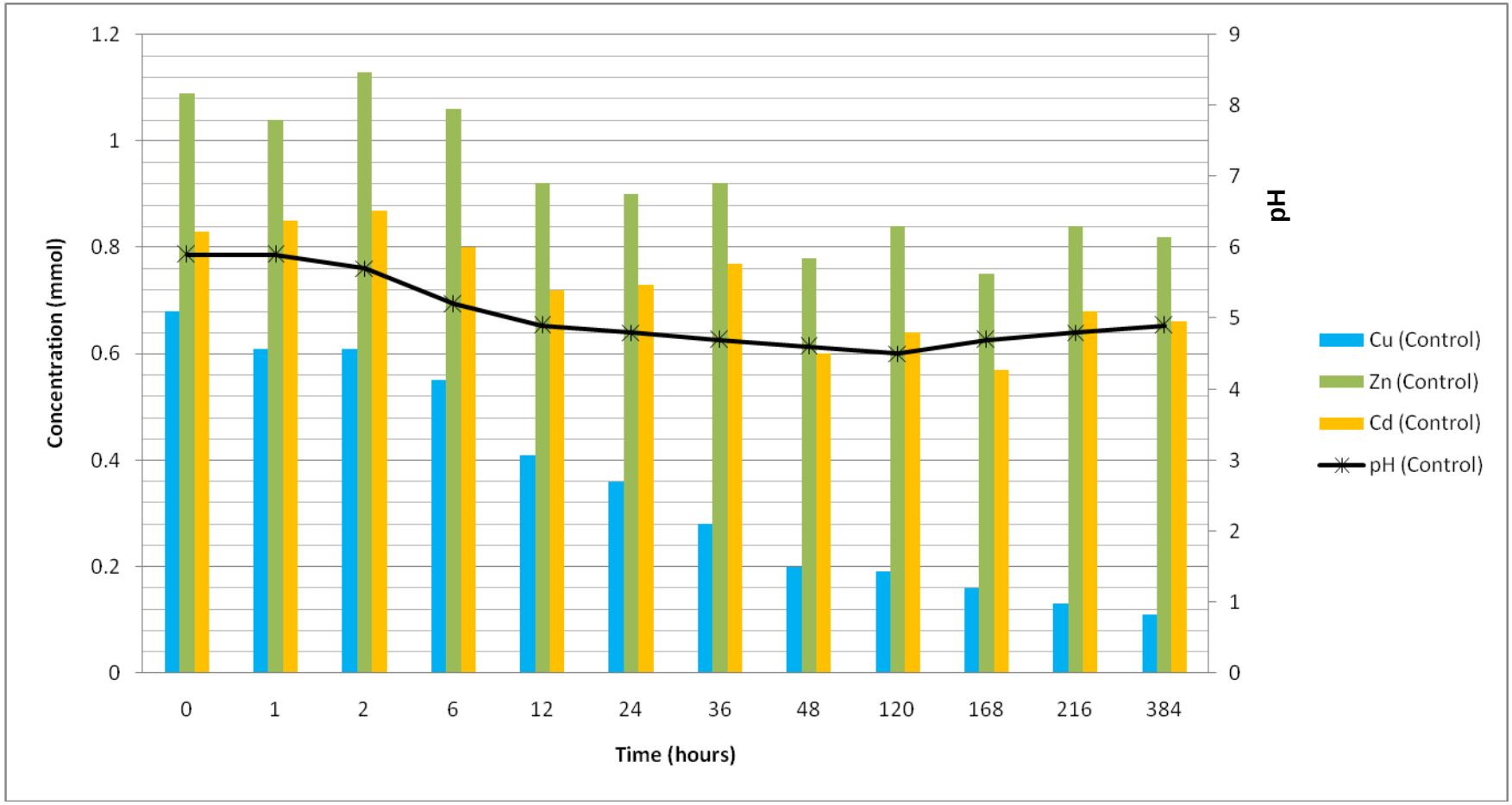

Figure B. 5: Average residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactor A – Control (Experiment 1)

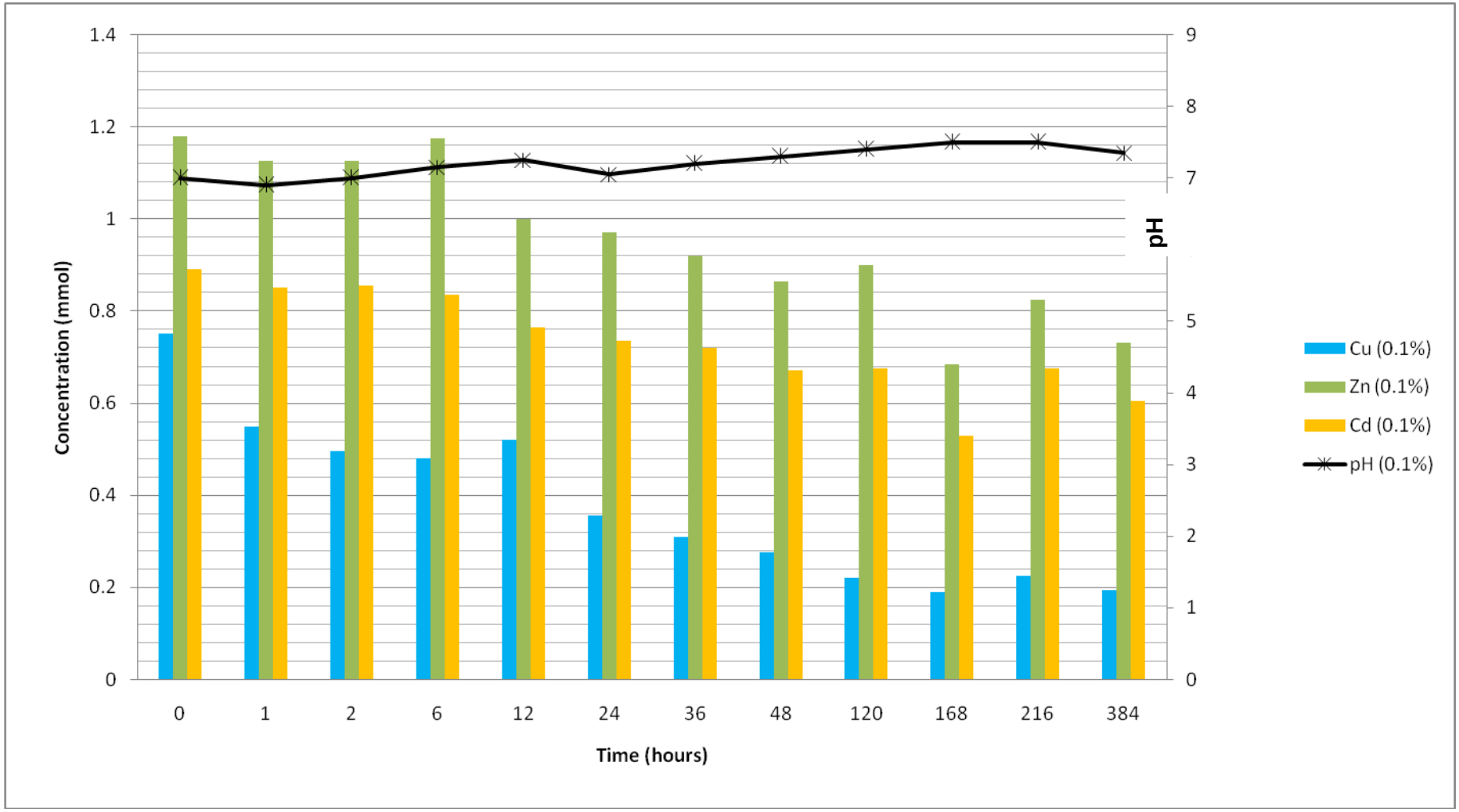

Figure B. 6: Average residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactors containing 0.1% (v/v) Voermolas (Experiment 1)

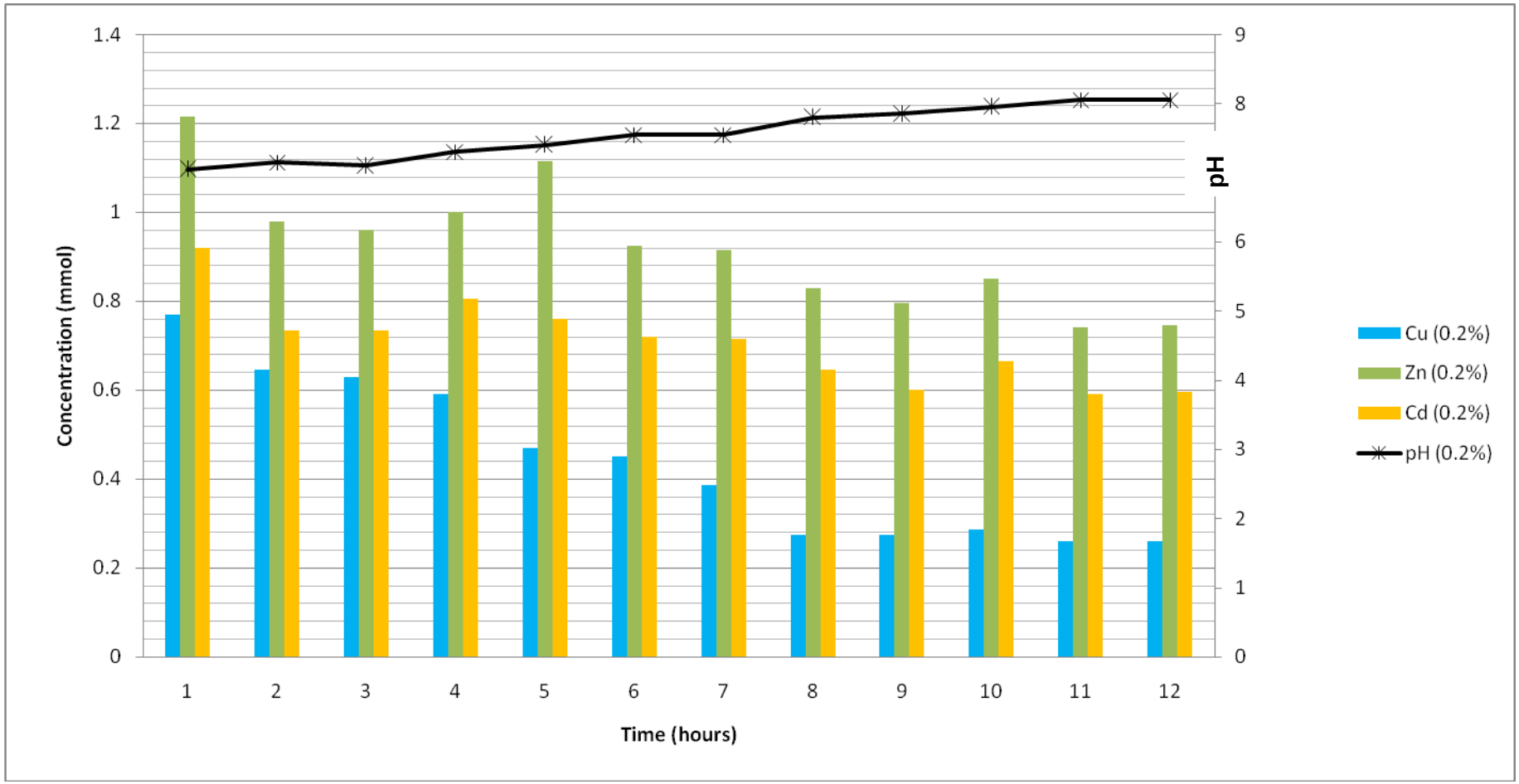

Figure B. 7: Average residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactors containing 0.2% (v/v) Voermolas (Experiment 1)

Appendix C

Table C. 1: Effect of nutrient levels on residual and final metal ion concentrations expressed in millimoles (Experiment 2)

		A			B			C			D			E		
Sample Number	**Time (h)**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Cd^{2+}**
(Initial) 0	0	0.88	1.48	0.91	0.88	1.48	0.91	0.88	1.48	0.91	0.88	1.48	0.91	0.88	1.48	0.91
1	1	0.76	1.30	0.80	0.87	1.27	0.77	0.72	1.36	0.82	0.79	1.10	0.68	0.81	1.38	0.82
2	2	0.71	1.22	0.77	0.68	0.99	0.61	0.84	1.09	0.66	0.71	1.04	0.64	0.80	1.03	0.62
3	6	0.69	1.19	0.76	0.58	0.84	0.52	0.72	0.95	0.58	0.68	1.01	0.63	0.65	0.84	0.52
4	12	0.56	0.98	0.61	0.61	0.90	0.58	0.65	0.91	0.62	0.65	1.18	0.74	0.94	1.38	0.82
5	24	0.58	1.15	0.73	0.68	1.22	0.76	0.60	0.93	0.58	0.49	0.96	0.60	0.60	0.99	0.61
6	36	0.57	1.19	0.75	0.59	1.13	0.70	0.52	0.86	0.53	0.44	0.91	0.57	0.55	0.96	0.60
7	48	0.55	1.33	0.84	0.36	0.89	0.56	0.30	0.66	0.41	0.30	0.80	0.51	0.44	0.90	0.58
8	120	0.30	1.22	0.77	0.28	0.66	0.42	0.28	0.61	0.40	0.39	1.02	0.66	0.49	0.96	0.63
9	168	0.26	0.81	0.53	0.30	0.80	0.52	0.30	0.61	0.40	0.20	0.73	0.48	0.38	0.89	0.60
(Final) 10	216	0.26	0.82	0.52	0.24	0.82	0.51	0.25	0.59	0.40	0.20	0.74	0.45	0.38	0.88	0.61
Change in conc. (Initial - Final)		0.62	0.66	0.39	0.64	0.66	0.40	0.63	0.89	0.51	0.68	0.74	0.46	0.50	0.60	0.30
Total M^{2+} sorbed[a] (mmol)		22.9	24.4	14.4	23.7	24.4	14.8	23.3	32.9	18.9	25.2	27.4	17	18.5	22.2	11.1
Relative M^{2+} sorption[b]		70%	45%	43%	73%	45%	44%	72%	60%	56%	77%	50%	51%	57%	41%	33%
M^{2+} sorbed per g Pine Bark (µmol)[c]		7.55	8.03	4.75	7.97	8.22	4.98	7.80	11.01	6.31	8.36	9.10	5.65	6.19	7.42	3.71
M^{2+} sorbed per g Pine Bark (mg)[d]		0.48	0.52	0.53	0.51	0.53	0.56	0.50	0.72	0.71	0.53	0.59	0.64	0.39	0.48	0.42

a Total M^{2+} sorbed = [Change in concentration] x [Total volume (37ℓ)]

b Relative M^{2+} sorption = [Change in concentration] / [Initial concentration]

c M^{2+} sorbed per g Pine Bark = [Total mmol M^{2+} sorbed] / [Dry Mass of BSP)]

d M^{2+} sorbed per g Pine Bark = [Total mg M^{2+} sorbed] / [Dry Mass of BSP]

Table C. 2: Sample pH measured during Experiment 2

Sample Number	Time (h)	pH				
		A Control	B 0.1% (v/v) Voermolas	C 0.1% (v/v) Voermolas	D 0.2% (v/v) Voermolas	E 0.2% (v/v) Voermolas
0	0	6.1	6.1	6.1	6.1	6.1
1	1	5.9	6.9	7.0	7.0	7.2
2	2	5.8	6.9	7.0	7.0	7.2
3	6	5.7	6.9	7.1	7.0	7.2
4	12	5.5	6.9	7.1	7.2	7.4
5	24	5.4	7.0	7.2	7.4	7.6
6	36	5.3	7.0	7.4	7.4	7.6
7	48	5.3	7.1	7.4	7.7	7.6
8	120	5.2	7.0	7.5	7.6	7.7
9	168	5.4	7.1	7.4	7.7	7.9
10	216	5.4	7.1	7.4	8.1	8.1

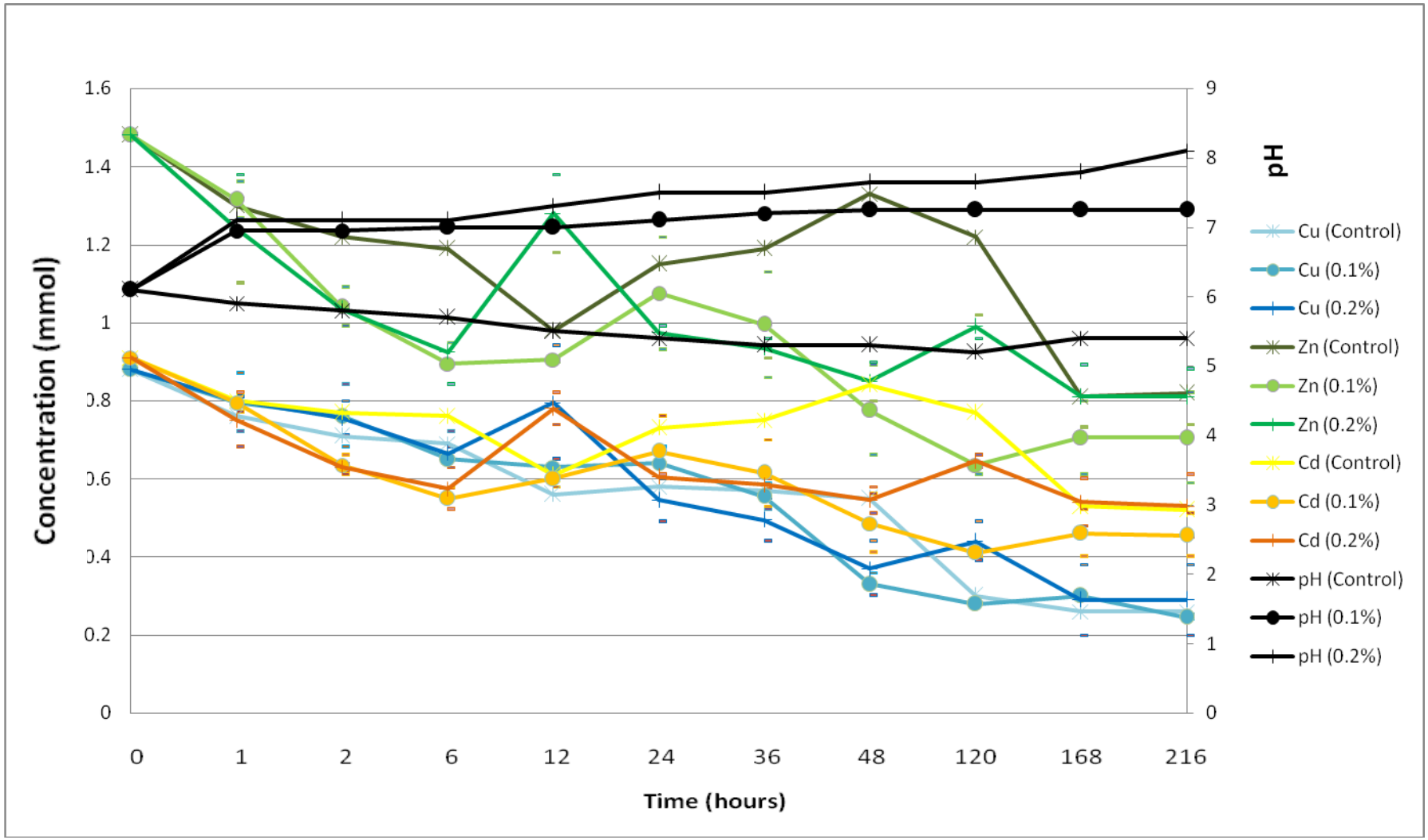

Figure C. 1: Changes in residual metal ion concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 2). Average values and control values are plotted as lines with variances indicated as colour coordinated points

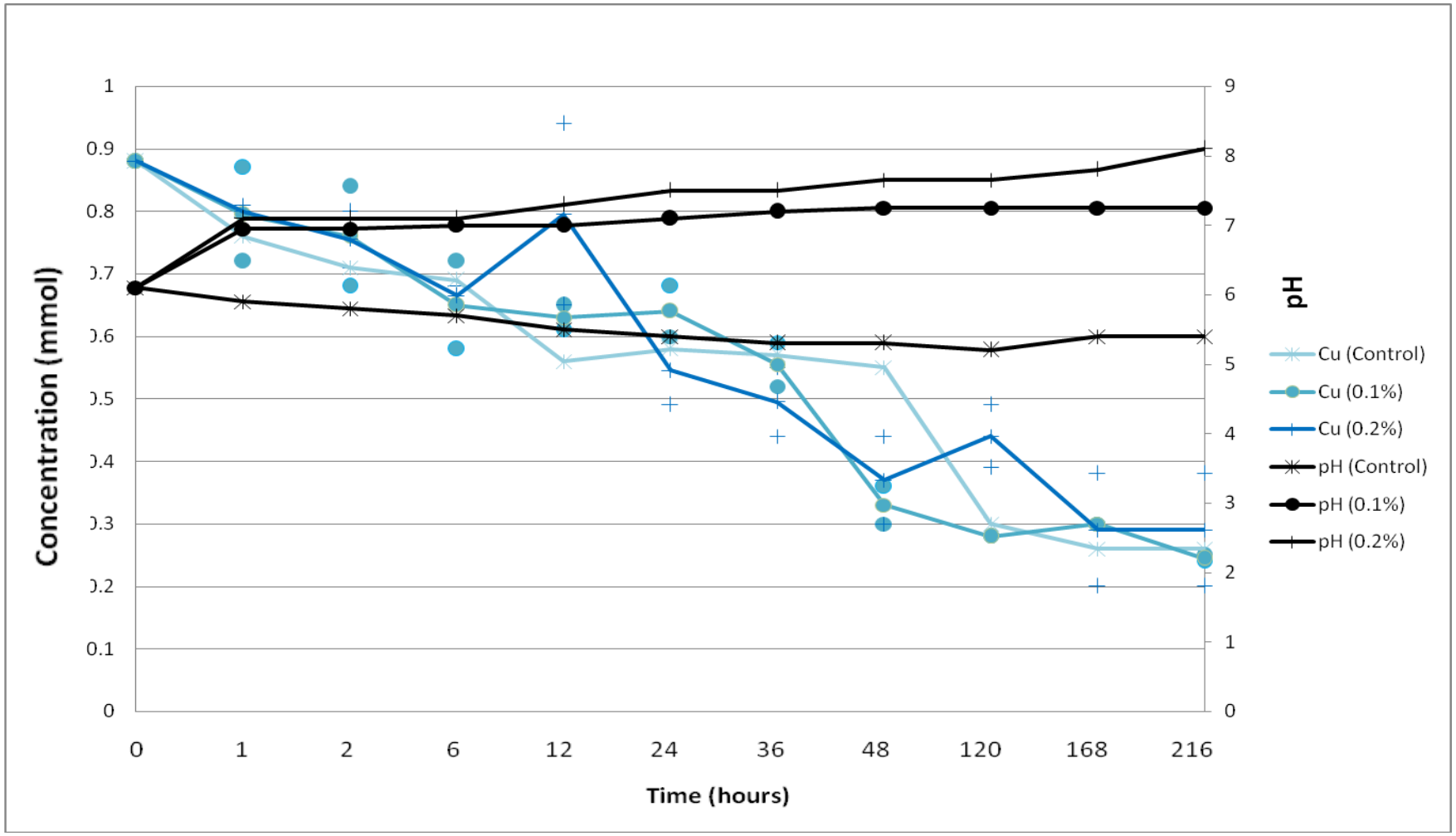

Figure C. 2: Changes in residual copper concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 2). Average values and control values are plotted as lines with variances indicated as colour coordinated points

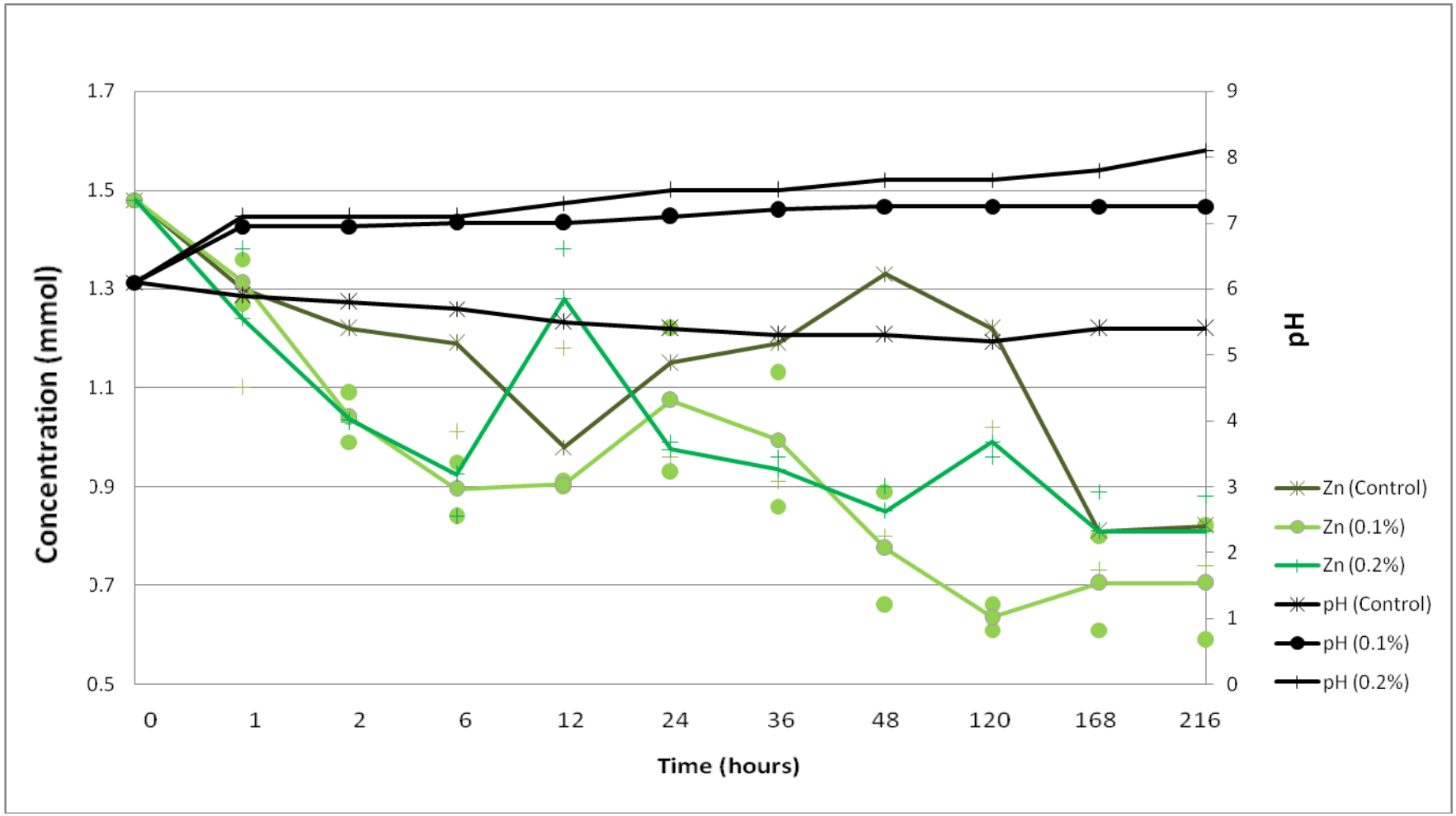

Figure C. 3: Changes in residual zinc concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 2). Average values and control values are plotted as lines with variances indicated as colour coordinated points

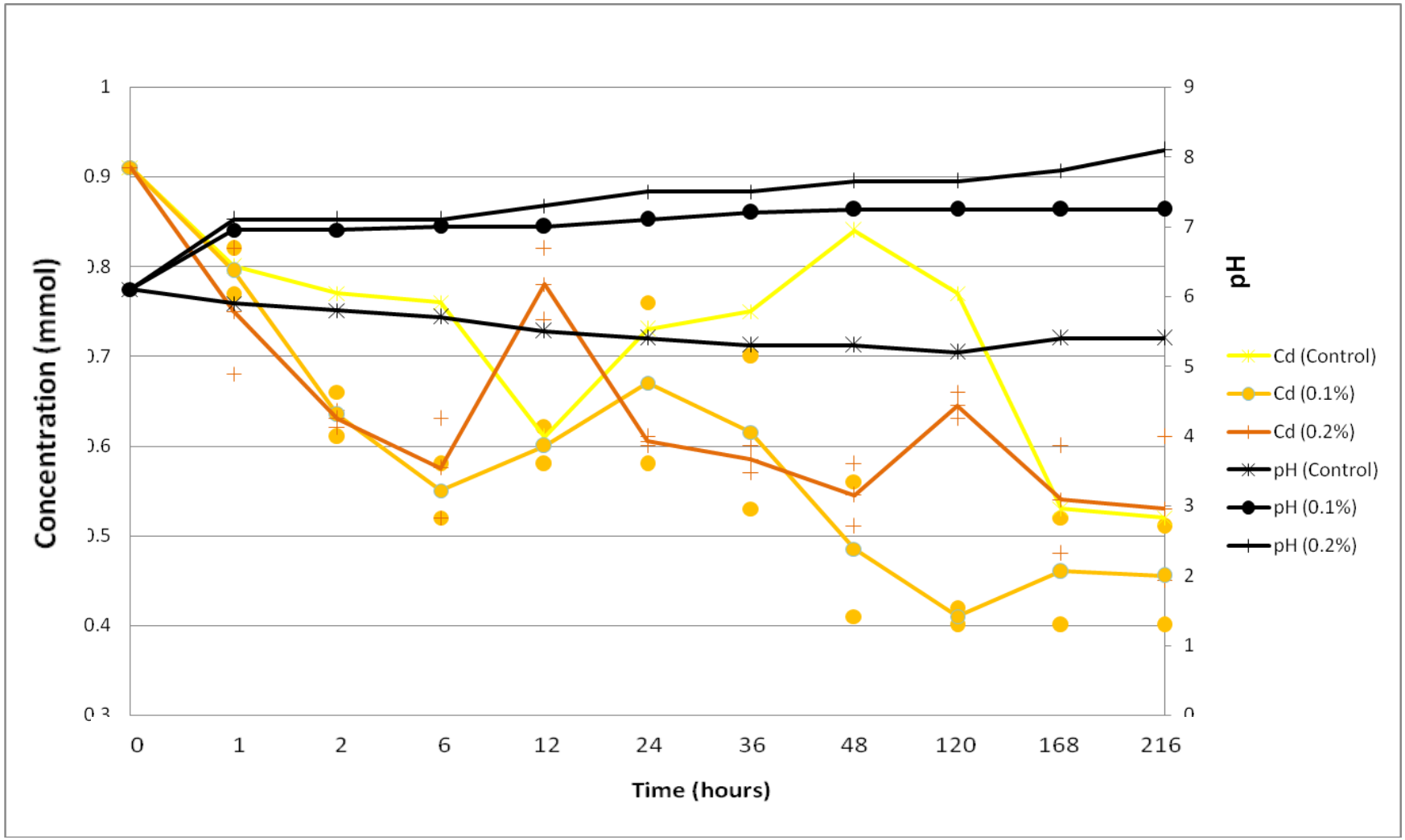

Figure C. 4: Changes in residual cadmium concentrations with time expressed in millimoles and corresponding pH values in all bioreactors (Experiment 2). Average values and control values are plotted as lines with variances indicated as colour coordinated points

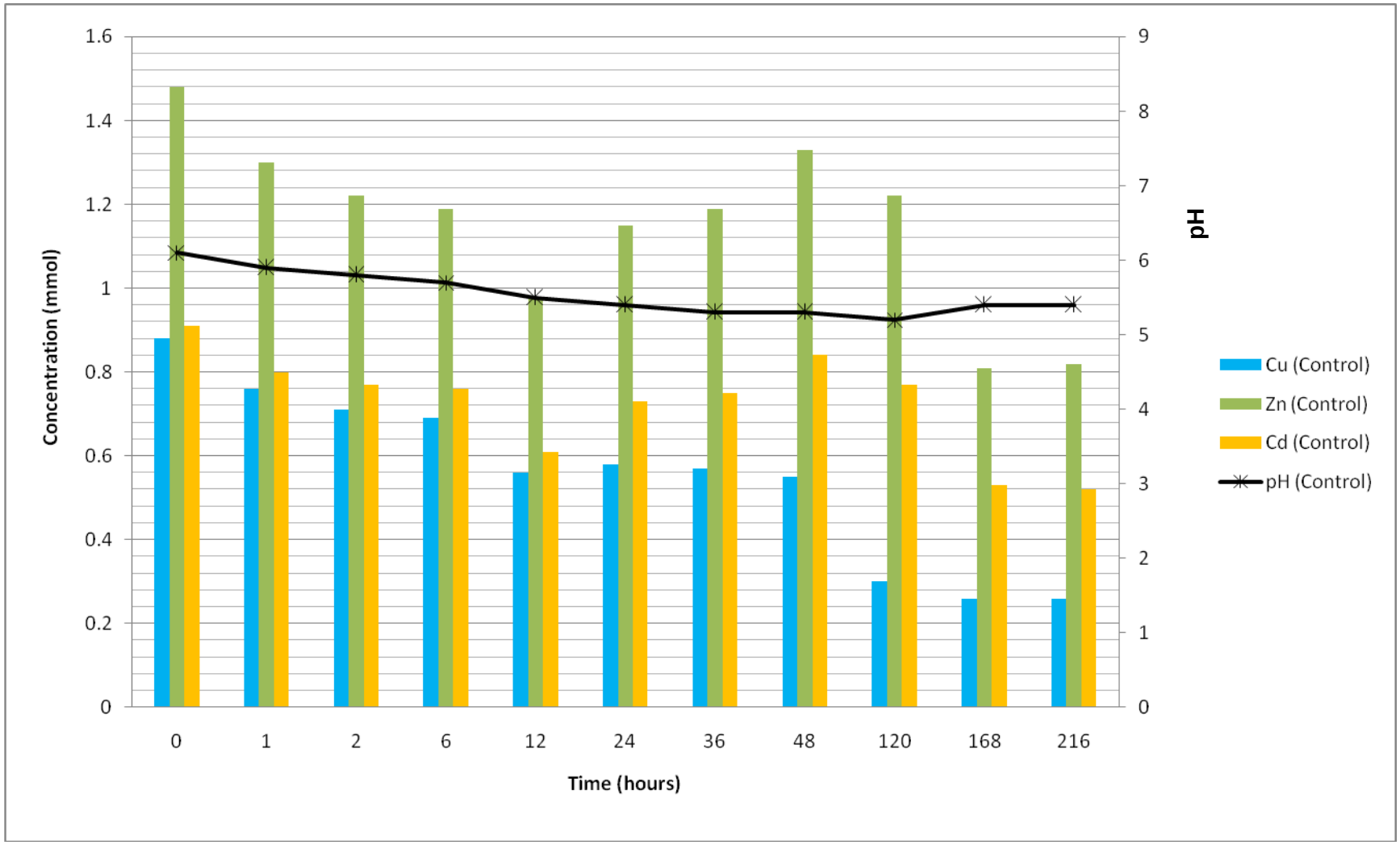

Figure C. 5: Residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactor A – Control (Experiment 2)

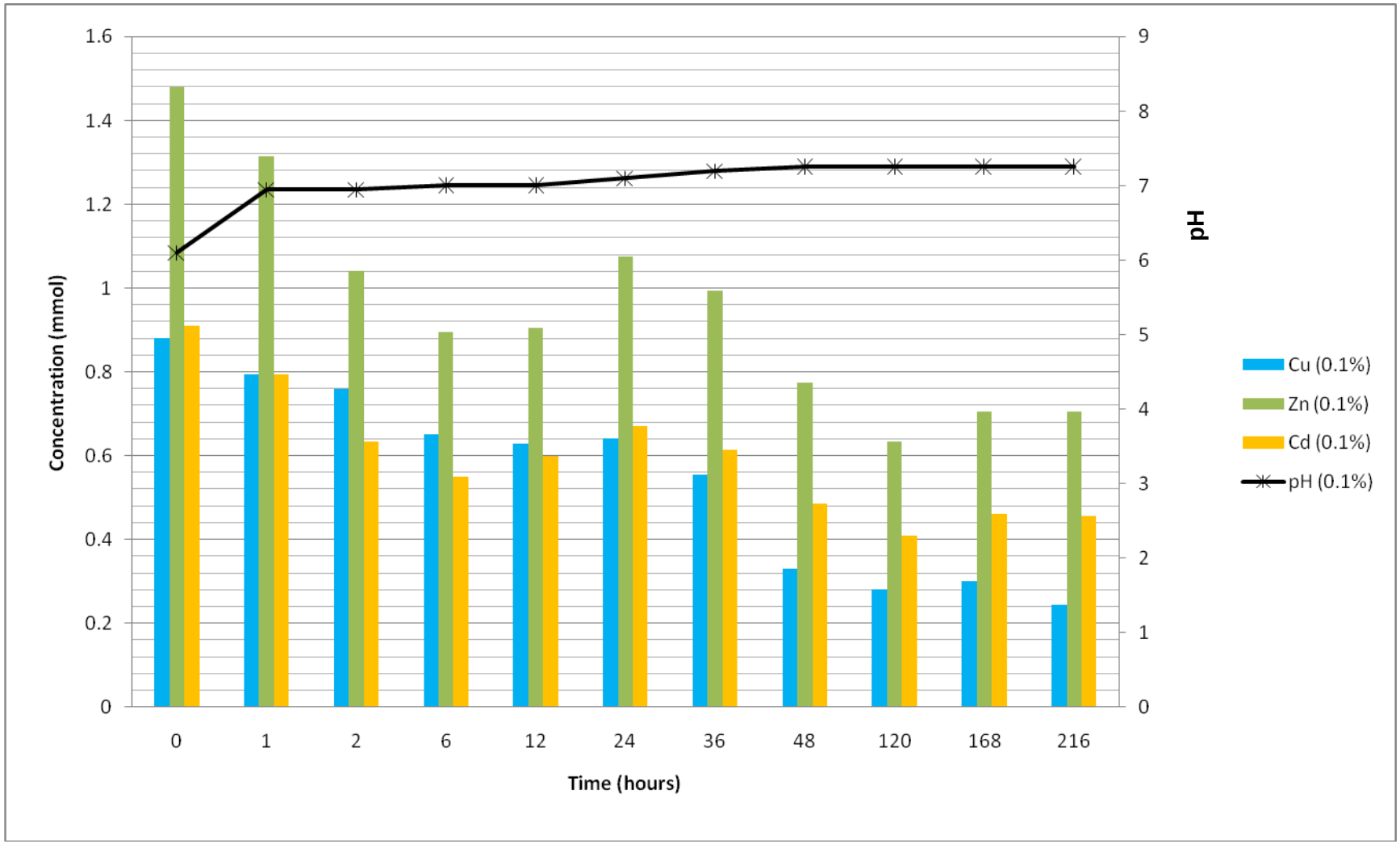

Figure C. 6: Average residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactors containing 0.1% (v/v) Voermolas (Experiment 2)

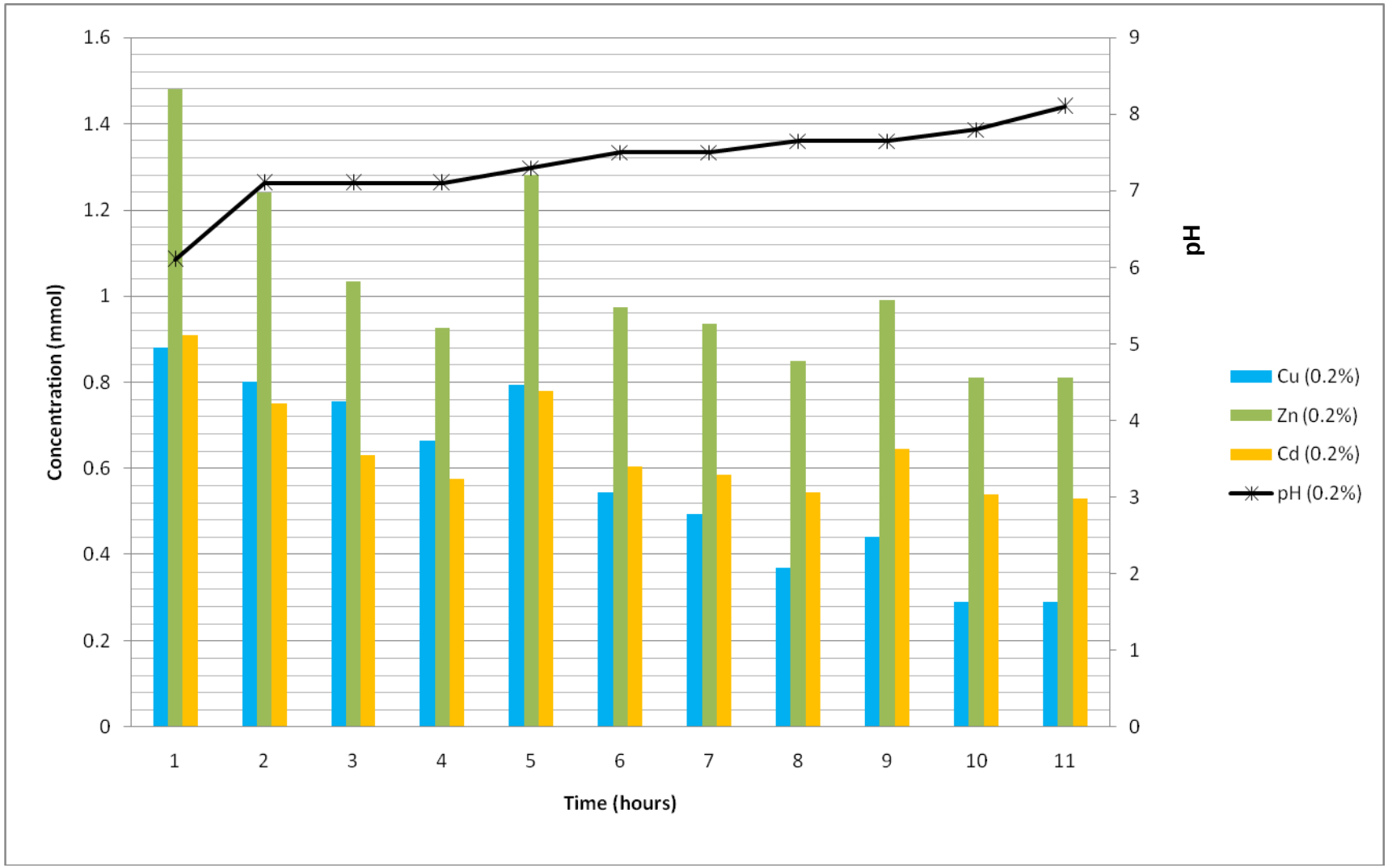

Figure C. 7: Average residual metal ion concentrations expressed in millimoles and corresponding pH values in Bioreactors containing 0.2% (v/v) Voermolas (Experiment 2)

Appendix D

Raw data collected during investigations reported in Chapter 4 and statistical analysis thereof.

Table D. 1 : Bioreactor A: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor A	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	1.094	0.978	1.476	5.8	1.094	0.966	1.440	5.8	1.101	0.970	1.434	5.8
0.07	0.888	0.797	1.400	5.8	0.900	0.797	1.427	5.8	0.876	0.791	1.411	5.8
0.14	0.832	0.748	1.330	5.8	0.846	0.743	1.358	5.8	0.840	0.763	1.323	5.8
0.28	0.780	0.748	1.348	5.8	0.790	0.738	1.356	5.8	0.780	0.749	1.264	5.8
0.57	0.684	0.746	1.238	5.9	0.707	0.737	1.251	5.9	0.704	0.742	1.239	5.9
1	0.696	0.742	1.221	5.9	0.698	0.735	1.250	5.9	0.697	0.745	1.199	5.9
2	0.255	0.705	1.233	6.0	0.261	0.695	1.213	6.0	0.262	0.710	1.227	6.0
6	0.094	0.677	1.205	6.2	0.093	0.665	1.216	6.2	0.090	0.672	1.206	6.2
12	0.019	0.624	1.175	6.4	0.018	0.620	1.176	6.4	0.018	0.615	1.180	6.4
24	0.006	0.534	1.069	6.4	0.006	0.536	1.069	6.4	0.006	0.531	1.077	6.4
48	0.004	0.488	1.159	6.5	0.004	0.493	1.165	6.5	0.004	0.497	1.159	6.5
72	0.006	0.512	1.126	6.5	0.006	0.516	1.143	6.5	0.006	0.519	1.139	6.5
96	0.005	0.467	1.128	6.5	0.005	0.472	1.127	6.5	0.005	0.470	1.107	6.5
120	0.005	0.467	1.096	6.5	0.005	0.461	1.085	6.5	0.004	0.469	1.082	6.5
144	0.003	0.453	1.086	6.6	0.003	0.452	1.081	6.6	0.003	0.459	1.077	6.6
168	0.004	0.389	1.062	6.6	0.004	0.386	1.080	6.6	0.004	0.387	1.054	6.6
192	0.003	0.435	1.015	6.6	0.003	0.426	1.022	6.6	0.003	0.428	1.017	6.6
216	0.002	0.435	0.971	6.8	0.002	0.426	0.963	6.8	0.002	0.424	0.968	6.8
240	0.003	0.371	0.917	6.8	0.003	0.375	0.951	6.8	0.003	0.375	0.959	6.8
288	0.002	0.358	0.909	6.9	0.002	0.365	0.906	6.9	0.002	0.371	0.891	6.9
336	0.001	0.355	0.719	7.0	0.001	0.356	0.709	7.0	0.001	0.360	0.724	7.0

Table D. 2: Bioreactor A: Statistical analysis of the raw data for Bioreactor A. Metal ion concentrations are presented as millimolar values

Bioreactor A	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	1.096	0.971	1.450	5.8	0.004	0.006	0.023	0.0
0.07	0.888	0.795	1.413	5.8	0.012	0.003	0.014	0.0
0.14	0.839	0.751	1.337	5.8	0.007	0.010	0.019	0.0
0.28	0.783	0.745	1.323	5.8	0.006	0.006	0.051	0.0
0.57	0.698	0.742	1.243	5.9	0.013	0.005	0.007	0.0
1	0.697	0.741	1.223	5.9	0.001	0.005	0.026	0.0
2	0.259	0.703	1.224	6.0	0.004	0.008	0.010	0.0
6	0.092	0.671	1.209	6.2	0.002	0.006	0.006	0.0
12	0.018	0.620	1.177	6.4	0.001	0.005	0.003	0.0
24	0.006	0.534	1.072	6.4	0.000	0.003	0.005	0.0
48	0.004	0.493	1.161	6.5	0.000	0.005	0.003	0.0
72	0.006	0.516	1.136	6.5	0.000	0.004	0.009	0.0
96	0.005	0.470	1.121	6.5	0.000	0.003	0.012	0.0
120	0.005	0.466	1.088	6.5	0.001	0.004	0.007	0.0
144	0.003	0.455	1.081	6.6	0.000	0.004	0.005	0.0
168	0.004	0.387	1.065	6.6	0.000	0.002	0.013	0.0
192	0.003	0.430	1.018	6.6	0.000	0.005	0.004	0.0
216	0.002	0.428	0.967	6.8	0.000	0.006	0.004	0.0
240	0.003	0.374	0.942	6.8	0.000	0.002	0.022	0.0
288	0.002	0.365	0.902	6.9	0.000	0.007	0.010	0.0
336	0.001	0.357	0.717	7.0	0.000	0.003	0.008	0.0

Table D. 3 : Bioreactor B: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor B	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.440	0.828	1.325	5.8	0.448	0.839	1.319	5.8	0.439	0.827	1.307	5.8
0.07	0.380	0.663	1.295	6.0	0.375	0.666	1.307	6.6	0.374	0.666	1.314	6.6
0.14	0.362	0.632	1.131	6.6	0.363	0.634	1.133	6.9	0.370	0.622	1.128	6.9
0.28	0.361	0.588	1.093	6.9	0.363	0.590	1.091	6.0	0.360	0.586	1.057	6.0
0.57	0.362	0.572	1.011	5.8	0.364	0.566	1.013	5.8	0.340	0.568	1.018	5.8
1	0.352	0.536	0.983	5.9	0.356	0.538	0.983	5.9	0.354	0.530	0.981	5.9
2	0.328	0.476	0.957	6.4	0.318	0.476	0.975	6.4	0.326	0.473	0.966	6.4
6	0.295	0.448	0.886	6.4	0.299	0.444	0.889	6.4	0.296	0.449	0.856	6.4
12	0.272	0.391	0.861	6.6	0.274	0.387	0.843	6.6	0.272	0.392	0.848	6.6
24	0.268	0.414	0.806	6.5	0.263	0.421	0.811	6.5	0.264	0.419	0.811	6.5
48	0.265	0.225	0.599	5.8	0.265	0.227	0.602	5.8	0.265	0.223	0.598	5.8
72	0.228	0.207	0.514	5.8	0.229	0.203	0.514	5.8	0.229	0.204	0.505	5.8
96	0.227	0.178	0.413	5.9	0.228	0.180	0.410	5.9	0.226	0.184	0.421	5.9
120	0.226	0.169	0.417	6.2	0.224	0.162	0.408	6.2	0.225	0.164	0.413	6.2
144	0.219	0.168	0.410	6.8	0.218	0.162	0.404	6.8	0.216	0.158	0.404	6.8
168	0.205	0.158	0.368	6.8	0.205	0.156	0.368	6.8	0.208	0.156	0.354	6.8
192	0.200	0.159	0.360	7.0	0.203	0.155	0.357	7.0	0.200	0.155	0.359	7.0
216	0.201	0.142	0.320	6.5	0.201	0.143	0.321	6.5	0.200	0.138	0.321	6.5
240	0.200	0.125	0.290	6.5	0.201	0.125	0.289	6.5	0.199	0.122	0.291	6.5
288	0.180	0.115	0.285	6.6	0.180	0.111	0.283	6.6	0.179	0.110	0.283	6.6
336	0.126	0.103	0.243	6.5	0.126	0.103	0.242	6.5	0.126	0.103	0.242	6.5

Table D. 4: Bioreactor B: Statistical analysis of the raw data for Bioreactor B. Metal ion concentrations are presented as millimolar values

Bioreactor B	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.442	0.831	1.317	5.8	0.005	0.007	0.009	0.0
0.07	0.376	0.665	1.305	6.4	0.003	0.002	0.010	0.3
0.14	0.365	0.629	1.131	6.8	0.004	0.006	0.003	0.2
0.28	0.361	0.588	1.080	6.3	0.002	0.002	0.020	0.5
0.57	0.355	0.569	1.014	5.8	0.013	0.003	0.004	0.0
1	0.354	0.535	0.982	5.9	0.002	0.004	0.001	0.0
2	0.324	0.475	0.966	6.4	0.005	0.002	0.009	0.0
6	0.297	0.447	0.877	6.4	0.002	0.003	0.018	0.0
12	0.273	0.390	0.851	6.6	0.001	0.003	0.009	0.0
24	0.265	0.418	0.809	6.5	0.003	0.004	0.003	0.0
48	0.265	0.225	0.600	5.8	0.000	0.002	0.002	0.0
72	0.229	0.205	0.511	5.8	0.001	0.002	0.005	0.0
96	0.227	0.181	0.415	5.9	0.001	0.003	0.006	0.0
120	0.225	0.165	0.413	6.2	0.001	0.004	0.005	0.0
144	0.218	0.163	0.406	6.8	0.002	0.005	0.003	0.0
168	0.206	0.157	0.363	6.8	0.002	0.001	0.008	0.0
192	0.201	0.156	0.359	7.0	0.002	0.002	0.002	0.0
216	0.201	0.141	0.321	6.5	0.001	0.003	0.001	0.0
240	0.200	0.124	0.290	6.5	0.001	0.002	0.001	0.0
288	0.180	0.112	0.284	6.6	0.001	0.003	0.001	0.0
336	0.126	0.103	0.242	6.5	0.000	0.000	0.001	0.0

Table D. 5: Bioreactor C: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor C	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.306	0.828	1.295	6.8	0.308	0.830	1.307	6.8	0.307	0.827	1.314	6.8
0.07	0.281	0.583	1.061	6.9	0.284	0.583	1.047	6.9	0.280	0.582	1.058	6.9
0.14	0.287	0.535	1.039	7.2	0.288	0.531	1.046	7.2	0.289	0.534	1.044	7.2
0.28	0.223	0.520	0.962	7.1	0.223	0.509	0.968	7.1	0.224	0.508	0.964	7.1
0.57	0.201	0.484	0.956	7.3	0.201	0.487	0.946	6.9	0.200	0.494	0.949	6.9
1	0.198	0.420	0.821	6.9	0.197	0.416	0.810	7.3	0.197	0.416	0.824	7.3
2	0.182	0.336	0.664	7.3	0.182	0.343	0.663	7.3	0.180	0.338	0.660	7.3
6	0.164	0.205	0.623	7.6	0.164	0.209	0.611	7.6	0.165	0.212	0.612	7.6
12	0.166	0.201	0.475	7.5	0.158	0.198	0.488	7.5	0.163	0.197	0.489	7.5
24	0.163	0.147	0.373	7.5	0.159	0.147	0.373	7.5	0.158	0.146	0.370	7.5
48	0.171	0.106	0.277	7.7	0.172	0.107	0.263	7.7	0.173	0.107	0.266	7.7
72	0.169	0.098	0.256	7.9	0.169	0.100	0.255	7.9	0.169	0.099	0.253	7.9
96	0.163	0.095	0.238	8.0	0.165	0.094	0.238	8.0	0.164	0.094	0.238	8.0
120	0.161	0.089	0.223	8.3	0.159	0.089	0.214	8.3	0.159	0.091	0.224	8.3
144	0.161	0.049	0.173	8.5	0.160	0.049	0.170	8.5	0.161	0.049	0.172	8.5
168	0.149	0.046	0.149	8.5	0.149	0.046	0.156	8.5	0.148	0.045	0.156	8.5
192	0.144	0.041	0.151	8.1	0.145	0.041	0.153	8.1	0.143	0.041	0.148	8.1
216	0.132	0.041	0.138	8.3	0.132	0.039	0.135	8.3	0.131	0.039	0.137	8.3
240	0.130	0.043	0.117	8.4	0.130	0.042	0.118	8.1	0.130	0.042	0.115	8.1
288	0.124	0.032	0.108	8.1	0.123	0.031	0.111	8.4	0.123	0.032	0.111	8.4
336	0.112	0.040	0.117	8.5	0.112	0.040	0.117	8.5	0.113	0.039	0.117	8.5

Table D. 6: Bioreactor C: Statistical analysis of the raw data for Bioreactor C. Metal ion concentrations are presented as millimolar values

Bioreactor C	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.307	0.828	1.305	6.8	0.001	0.002	0.010	0.0
0.07	0.282	0.583	1.055	6.9	0.002	0.001	0.007	0.0
0.14	0.288	0.533	1.043	7.2	0.001	0.002	0.004	0.0
0.28	0.223	0.512	0.965	7.1	0.001	0.007	0.003	0.0
0.57	0.201	0.488	0.950	7.0	0.001	0.005	0.005	0.2
1	0.197	0.417	0.818	7.2	0.001	0.002	0.007	0.2
2	0.181	0.339	0.662	7.3	0.001	0.004	0.002	0.0
6	0.164	0.209	0.615	7.6	0.001	0.004	0.007	0.0
12	0.162	0.199	0.484	7.5	0.004	0.002	0.008	0.0
24	0.160	0.147	0.372	7.5	0.003	0.001	0.002	0.0
48	0.172	0.107	0.269	7.7	0.001	0.001	0.007	0.0
72	0.169	0.099	0.255	7.9	0.000	0.001	0.002	0.0
96	0.164	0.094	0.238	8.0	0.001	0.001	0.000	0.0
120	0.160	0.090	0.220	8.3	0.001	0.001	0.006	0.0
144	0.161	0.049	0.172	8.5	0.001	0.000	0.002	0.0
168	0.149	0.046	0.154	8.5	0.001	0.001	0.004	0.0
192	0.144	0.041	0.151	8.1	0.001	0.000	0.003	0.0
216	0.132	0.040	0.137	8.3	0.001	0.001	0.002	0.0
240	0.130	0.042	0.117	8.2	0.000	0.001	0.002	0.2
288	0.123	0.032	0.110	8.3	0.001	0.001	0.002	0.2
336	0.112	0.040	0.117	8.5	0.001	0.001	0.000	0.0

Table D. 7: Bioreactor D: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor D	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	1.473	1.100	1.442	6.0	1.468	1.095	1.415	6.0	1.483	1.071	1.436	6.0
0.07	1.331	0.978	1.401	5.8	1.322	0.966	1.382	5.8	1.336	0.970	1.393	5.8
0.14	1.325	0.906	1.358	5.8	1.326	0.898	1.361	5.8	1.318	0.905	1.343	5.8
0.28	1.315	0.931	1.250	5.8	1.306	0.922	1.254	5.8	1.309	0.918	1.259	5.8
0.57	1.281	0.880	1.221	5.6	1.290	0.878	1.250	5.6	1.269	0.864	1.199	5.6
1	1.094	0.800	1.185	5.5	1.094	0.801	1.186	5.5	1.101	0.801	1.177	5.5
2	0.990	0.796	1.140	5.1	1.005	0.793	1.151	5.1	0.991	0.798	1.134	5.1
6	0.730	0.757	1.008	4.9	0.691	0.751	0.993	4.9	0.682	0.751	1.009	4.9
12	0.618	0.722	0.996	4.7	0.660	0.752	0.983	4.7	0.688	0.759	0.983	4.7
24	0.641	0.676	0.984	4.7	0.640	0.678	0.979	4.7	0.637	0.679	0.980	4.7
48	0.600	0.668	0.978	4.7	0.607	0.673	0.974	4.7	0.607	0.676	0.982	4.7
72	0.581	0.656	0.960	4.7	0.588	0.654	0.965	4.7	0.584	0.656	0.952	4.7
96	0.493	0.648	0.950	4.6	0.503	0.646	0.925	4.6	0.513	0.669	0.934	4.6
120	0.478	0.619	0.878	4.5	0.484	0.620	0.882	4.5	0.465	0.624	0.870	4.5
144	0.418	0.612	0.778	4.5	0.412	0.614	0.760	4.5	0.409	0.615	0.757	4.5
168	0.383	0.550	0.702	4.4	0.387	0.546	0.692	4.4	0.381	0.552	0.702	4.4
192	0.332	0.512	0.689	4.4	0.339	0.510	0.691	4.4	0.336	0.512	0.690	4.4
216	0.337	0.453	0.656	4.6	0.324	0.453	0.662	4.6	0.322	0.451	0.649	4.6
240	0.304	0.427	0.634	4.6	0.315	0.433	0.629	4.6	0.316	0.434	0.639	4.6
288	0.302	0.414	0.624	4.6	0.300	0.410	0.632	4.6	0.299	0.408	0.633	4.6
336	0.288	0.405	0.615	4.4	0.286	0.410	0.607	4.4	0.288	0.406	0.604	4.4

Table D. 8: Bioreactor D: Statistical analysis of the raw data for Bioreactor D. Metal ion concentrations are presented as millimolar values

Bioreactor D	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	1.475	1.089	1.431	6.0	0.008	0.016	0.014	0.0
0.07	1.330	0.971	1.392	5.8	0.007	0.006	0.010	0.0
0.14	1.323	0.903	1.354	5.8	0.004	0.004	0.010	0.0
0.28	1.310	0.924	1.254	5.8	0.005	0.007	0.005	0.0
0.57	1.280	0.874	1.223	5.6	0.011	0.009	0.026	0.0
1	1.096	0.801	1.183	5.5	0.004	0.001	0.005	0.0
2	0.995	0.796	1.142	5.1	0.008	0.003	0.009	0.0
6	0.701	0.753	1.003	4.9	0.026	0.003	0.009	0.0
12	0.655	0.744	0.987	4.7	0.035	0.020	0.008	0.0
24	0.639	0.678	0.981	4.7	0.002	0.002	0.003	0.0
48	0.605	0.672	0.978	4.7	0.004	0.004	0.004	0.0
72	0.584	0.655	0.959	4.7	0.004	0.001	0.007	0.0
96	0.503	0.654	0.936	4.6	0.010	0.013	0.013	0.0
120	0.476	0.621	0.877	4.5	0.010	0.003	0.006	0.0
144	0.413	0.614	0.765	4.5	0.005	0.002	0.011	0.0
168	0.384	0.549	0.699	4.4	0.003	0.003	0.006	0.0
192	0.336	0.511	0.690	4.4	0.004	0.001	0.001	0.0
216	0.328	0.452	0.656	4.6	0.008	0.001	0.007	0.0
240	0.312	0.431	0.634	4.6	0.007	0.004	0.005	0.0
288	0.300	0.411	0.630	4.6	0.002	0.003	0.005	0.0
336	0.287	0.407	0.609	4.4	0.001	0.003	0.006	0.0

Table D. 9: Bioreactor E: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor E	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.302	0.837	1.298	6.8	0.282	0.839	1.312	6.8	0.263	0.830	1.314	6.8
0.07	0.243	0.551	1.128	6.9	0.243	0.544	1.105	6.9	0.244	0.552	1.126	6.9
0.14	0.217	0.497	1.005	6.9	0.219	0.508	1.013	6.9	0.220	0.513	0.996	6.9
0.28	0.201	0.494	0.955	7.1	0.201	0.496	0.955	7.1	0.200	0.493	0.964	7.1
0.57	0.196	0.478	0.882	7.2	0.194	0.489	0.846	7.2	0.193	0.485	0.843	7.2
1	0.190	0.372	0.725	7.3	0.196	0.366	0.752	7.3	0.199	0.367	0.757	7.3
2	0.118	0.364	0.741	7.3	0.118	0.362	0.721	7.3	0.116	0.356	0.726	7.3
6	0.079	0.252	0.723	7.5	0.079	0.249	0.711	7.5	0.080	0.250	0.720	7.5
12	0.138	0.217	0.560	7.5	0.135	0.215	0.558	7.5	0.138	0.213	0.554	7.5
24	0.171	0.160	0.544	7.6	0.181	0.159	0.540	7.6	0.183	0.159	0.548	7.6
48	0.175	0.127	0.369	7.7	0.175	0.129	0.381	7.7	0.176	0.129	0.378	7.7
72	0.164	0.084	0.272	7.9	0.167	0.085	0.267	7.9	0.166	0.084	0.266	7.9
96	0.163	0.050	0.204	8.0	0.162	0.061	0.203	8.0	0.160	0.060	0.198	8.0
120	0.157	0.043	0.171	8.1	0.158	0.043	0.171	8.1	0.155	0.043	0.173	7.9
144	0.159	0.053	0.183	8.1	0.155	0.053	0.180	8.1	0.154	0.053	0.181	8.1
168	0.136	0.045	0.155	8.3	0.138	0.046	0.153	8.3	0.139	0.045	0.153	8.3
192	0.155	0.035	0.129	8.3	0.154	0.034	0.128	8.3	0.154	0.032	0.128	8.3
216	0.139	0.036	0.116	8.4	0.137	0.035	0.114	8.5	0.138	0.036	0.113	8.4
240	0.129	0.033	0.113	8.5	0.140	0.034	0.114	8.5	0.140	0.033	0.112	8.5
288	0.133	0.029	0.106	8.5	0.134	0.030	0.105	8.5	0.133	0.030	0.104	8.5
336	0.114	0.024	0.087	8.6	0.113	0.024	0.086	8.5	0.114	0.024	0.086	8.5

Table D. 10: Bioreactor E: Statistical analysis of the raw data for Bioreactor E. Metal ion concentrations are presented as millimolar values

Bioreactor E	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.282	0.835	1.308	6.8	0.020	0.005	0.009	0.0
0.07	0.243	0.549	1.120	6.9	0.001	0.004	0.013	0.0
0.14	0.219	0.506	1.005	6.9	0.002	0.008	0.009	0.0
0.28	0.201	0.494	0.958	7.1	0.001	0.002	0.005	0.0
0.57	0.194	0.484	0.857	7.2	0.002	0.006	0.022	0.0
1	0.195	0.368	0.745	7.3	0.005	0.003	0.017	0.0
2	0.117	0.361	0.729	7.3	0.001	0.004	0.010	0.0
6	0.079	0.250	0.718	7.5	0.001	0.002	0.006	0.0
12	0.137	0.215	0.557	7.5	0.002	0.002	0.003	0.0
24	0.178	0.159	0.544	7.6	0.006	0.001	0.004	0.0
48	0.175	0.128	0.376	7.7	0.001	0.001	0.006	0.0
72	0.166	0.084	0.268	7.9	0.002	0.001	0.003	0.0
96	0.162	0.057	0.202	8.0	0.002	0.006	0.003	0.0
120	0.157	0.043	0.172	8.0	0.002	0.000	0.001	0.1
144	0.156	0.053	0.181	8.1	0.003	0.000	0.002	0.0
168	0.138	0.045	0.154	8.3	0.002	0.001	0.001	0.0
192	0.154	0.034	0.128	8.3	0.001	0.002	0.001	0.0
216	0.138	0.036	0.114	8.4	0.001	0.001	0.002	0.1
240	0.136	0.033	0.113	8.5	0.006	0.001	0.001	0.0
288	0.133	0.030	0.105	8.5	0.001	0.001	0.001	0.0
336	0.114	0.024	0.086	8.5	0.001	0.000	0.001	0.1

Table D. 11: Bioreactor F: Cu, Zn and Cd concentrations (mmol) and pH of each sample

Bioreactor F	Sample 1				Sample 2				Sample 3			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.271	0.828	1.295	6.8	0.273	0.839	1.307	6.8	0.272	0.827	1.314	6.8
0.07	0.251	0.535	1.186	6.9	0.248	0.529	1.200	6.9	0.248	0.531	1.178	6.9
0.14	0.221	0.529	1.117	6.9	0.222	0.526	1.106	6.9	0.221	0.527	1.118	6.9
0.28	0.202	0.485	1.087	7.1	0.202	0.487	1.092	7.1	0.204	0.484	1.103	7.1
0.57	0.210	0.416	0.926	7.2	0.215	0.425	0.928	7.2	0.216	0.428	0.936	7.2
1	0.219	0.371	0.845	7.3	0.218	0.372	0.837	7.3	0.214	0.374	0.829	7.3
2	0.201	0.314	0.821	7.3	0.201	0.316	0.834	7.3	0.200	0.315	0.840	7.3
6	0.190	0.233	0.628	7.5	0.186	0.239	0.629	7.5	0.188	0.230	0.621	7.5
12	0.189	0.150	0.597	7.5	0.190	0.148	0.580	7.5	0.188	0.148	0.581	7.5
24	0.189	0.132	0.385	7.6	0.187	0.133	0.384	7.6	0.187	0.133	0.389	7.6
48	0.187	0.087	0.355	7.7	0.186	0.096	0.347	7.7	0.186	0.095	0.341	7.7
72	0.181	0.070	0.193	7.9	0.179	0.070	0.193	7.9	0.181	0.071	0.195	7.9
96	0.173	0.049	0.151	8.0	0.170	0.048	0.151	8.0	0.173	0.048	0.151	8.0
120	0.170	0.045	0.121	8.1	0.168	0.045	0.120	8.1	0.168	0.044	0.120	8.1
144	0.168	0.030	0.087	8.1	0.165	0.030	0.086	8.1	0.168	0.030	0.086	8.1
168	0.167	0.028	0.086	8.3	0.169	0.028	0.086	8.3	0.166	0.028	0.087	8.3
192	0.162	0.027	0.084	8.3	0.161	0.027	0.084	8.3	0.160	0.029	0.085	8.3
216	0.156	0.024	0.074	8.4	0.155	0.024	0.075	8.4	0.155	0.023	0.072	8.4
240	0.154	0.022	0.072	8.5	0.155	0.021	0.072	8.5	0.156	0.021	0.072	8.5
288	0.132	0.018	0.071	8.5	0.133	0.017	0.068	8.5	0.137	0.016	0.056	8.5
336	0.102	0.017	0.060	8.5	0.101	0.016	0.056	8.5	0.101	0.016	0.056	7.9

Table D. 12: Bioreactor F: Statistical analysis of the raw data for Bioreactor F. Metal ion concentrations are presented as millimolar values

Bioreactor F	Average				Standard deviation			
Time (h)	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH	Cu^{2+}	Zn^{2+}	Cd^{2+}	pH
0	0.272	0.831	1.305	6.8	0.001	0.007	0.010	0.0
0.07	0.249	0.532	1.188	6.9	0.002	0.003	0.011	0.0
0.14	0.221	0.527	1.114	6.9	0.001	0.002	0.007	0.0
0.28	0.203	0.485	1.094	7.1	0.001	0.002	0.008	0.0
0.57	0.214	0.423	0.930	7.2	0.003	0.006	0.005	0.0
1	0.217	0.372	0.837	7.3	0.003	0.002	0.008	0.0
2	0.201	0.315	0.832	7.3	0.001	0.001	0.010	0.0
6	0.188	0.234	0.626	7.5	0.002	0.005	0.004	0.0
12	0.189	0.149	0.586	7.5	0.001	0.001	0.010	0.0
24	0.188	0.133	0.386	7.6	0.001	0.001	0.003	0.0
48	0.186	0.093	0.348	7.7	0.001	0.005	0.007	0.0
72	0.180	0.070	0.194	7.9	0.001	0.001	0.001	0.0
96	0.172	0.048	0.151	8.0	0.002	0.001	0.000	0.0
120	0.169	0.045	0.120	8.1	0.001	0.001	0.001	0.0
144	0.167	0.030	0.086	8.1	0.002	0.000	0.001	0.0
168	0.167	0.028	0.086	8.3	0.002	0.000	0.001	0.0
192	0.161	0.028	0.084	8.3	0.001	0.001	0.001	0.0
216	0.155	0.024	0.074	8.4	0.001	0.001	0.002	0.0
240	0.155	0.021	0.072	8.5	0.001	0.001	0.000	0.0
288	0.134	0.017	0.065	8.5	0.003	0.001	0.008	0.0
336	0.101	0.016	0.057	8.3	0.001	0.001	0.002	0.3

Appendix E

Table E 1: Average residual metal ion values as a percentage of initial concentration

Time (h)	Sample number	Reactor (Residual metal ion concentrations)						Standard deviation		
		Bioball BSP			Pine Bark BSP			Pine bark samples	Bioball samples	All Samples
		A Control	B Inoculum	C Biofilm	D Control	E Inoculum	F Biofilm			
0	1	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	0.000	0.000	0.000
0.07	2	88.0%	90.5%	78.7%	92.5%	78.8%	81.7%	0.072	0.062	0.061
0.14	3	83.2%	82.0%	76.4%	89.6%	71.3%	77.3%	0.093	0.037	0.064
0.28	4	81.0%	78.3%	69.6%	87.3%	68.2%	74.0%	0.098	0.060	0.073
0.57	5	76.2%	74.8%	67.1%	84.6%	63.3%	65.0%	0.118	0.049	0.081
1	6	75.6%	72.2%	58.7%	77.1%	53.9%	59.2%	0.121	0.090	0.100
2	7	62.2%	68.1%	48.4%	73.4%	49.8%	56.0%	0.123	0.101	0.100
6	8	56.1%	62.5%	40.5%	61.5%	43.2%	43.5%	0.105	0.113	0.100
12	9	51.6%	58.4%	34.6%	59.7%	37.5%	38.3%	0.126	0.123	0.113
24	10	45.8%	57.6%	27.8%	57.5%	36.3%	29.3%	0.147	0.150	0.134
48	11	47.1%	42.1%	22.4%	56.5%	28.0%	26.0%	0.170	0.130	0.136
72	12	47.1%	36.5%	21.4%	55.1%	21.4%	18.4%	0.204	0.129	0.154
96	13	45.4%	31.8%	20.3%	52.4%	17.3%	15.4%	0.208	0.125	0.155
120	14	44.3%	31.0%	19.3%	49.4%	15.3%	13.9%	0.201	0.125	0.153
144	15	43.7%	30.4%	15.6%	44.9%	16.1%	11.7%	0.180	0.141	0.148
168	16	41.4%	28.0%	14.3%	40.8%	13.9%	11.7%	0.162	0.136	0.138
192	17	41.2%	27.6%	13.8%	38.5%	13.0%	11.3%	0.152	0.137	0.134
216	18	39.7%	25.5%	12.7%	36.0%	11.9%	10.5%	0.143	0.135	0.130
240	19	37.5%	23.7%	11.8%	34.5%	11.6%	10.3%	0.136	0.128	0.122
288	20	36.1%	22.2%	10.9%	33.6%	11.1%	9.0%	0.136	0.126	0.121
336	21	30.6%	18.2%	11.0%	32.6%	9.3%	7.3%	0.141	0.099	0.111

Appendix F

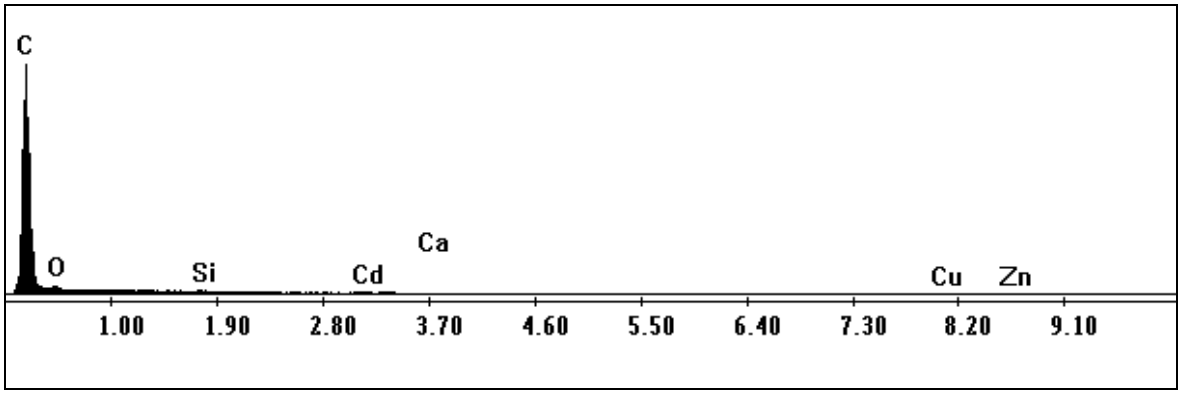

Figure F. 1: EDX Spectrum of the surface of a sample bioball to determine the inherent Cu, Zn and Cd content before exposure to the synthetic wastewater (T0).

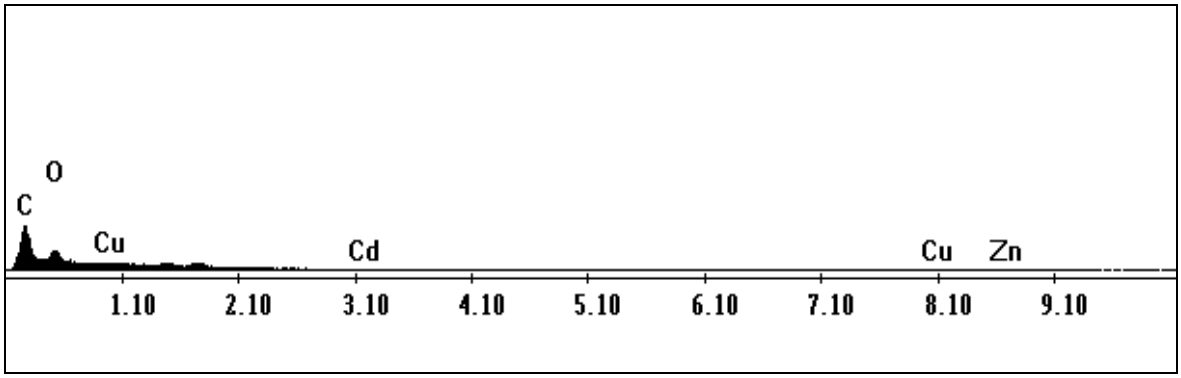

Figure F. 2: EDX Spectrum of the surface of a nugget of pine bark to determine the inherent Cu, Zn and Cd content before exposure to the synthetic wastewater (T0).

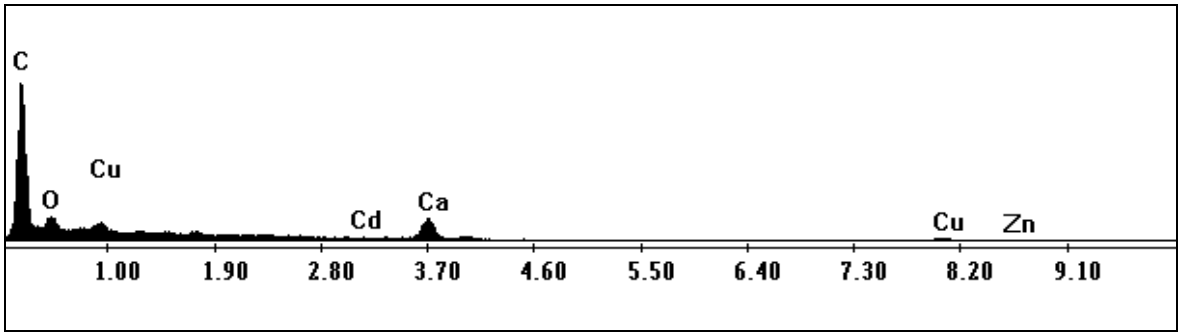

Figure F. 3: EDX Spectrum of the surface of a randomly selected bioball taken from control Bioreactor A after 48h.

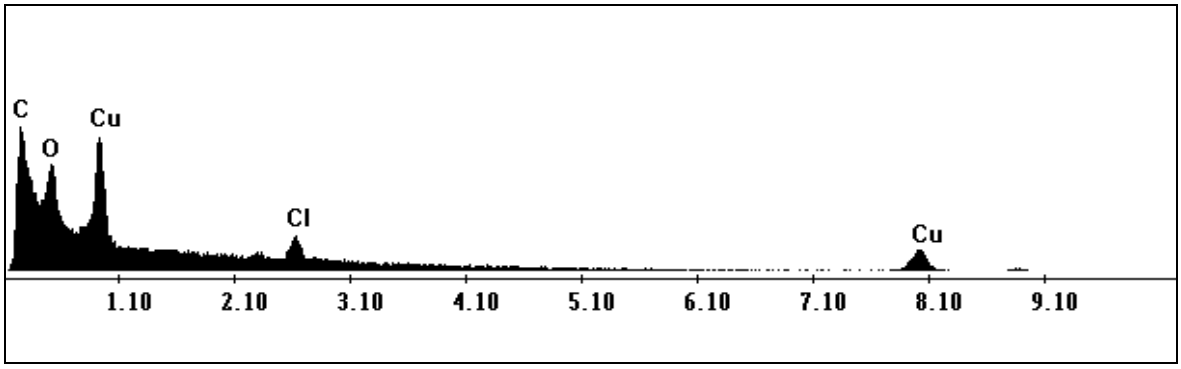

Figure F. 4: EDX Spectrum of the crystals formed on the surface of a bioball taken at random from Bioreactor A after 168h.

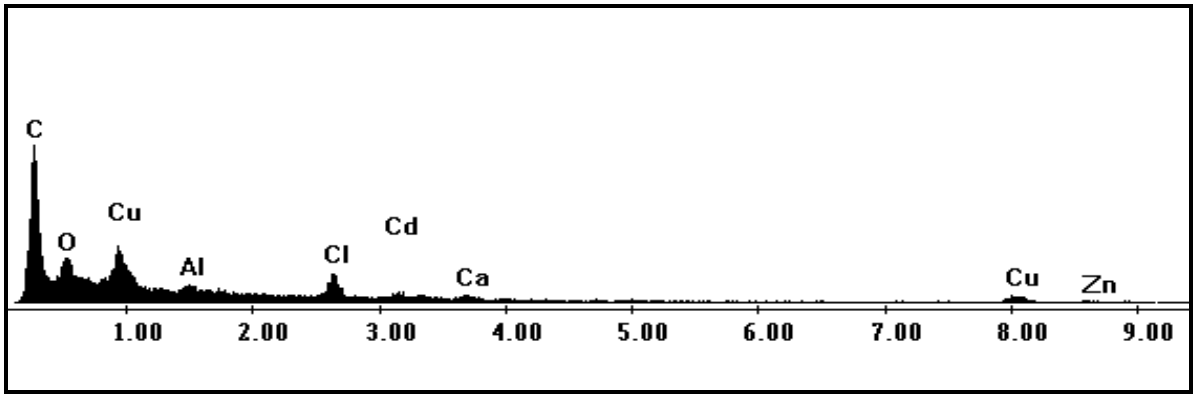

Figure F. 5: EDX Spectrum of microbial growth on a randomly selected bioball removed from Bioreactor A after 336h.

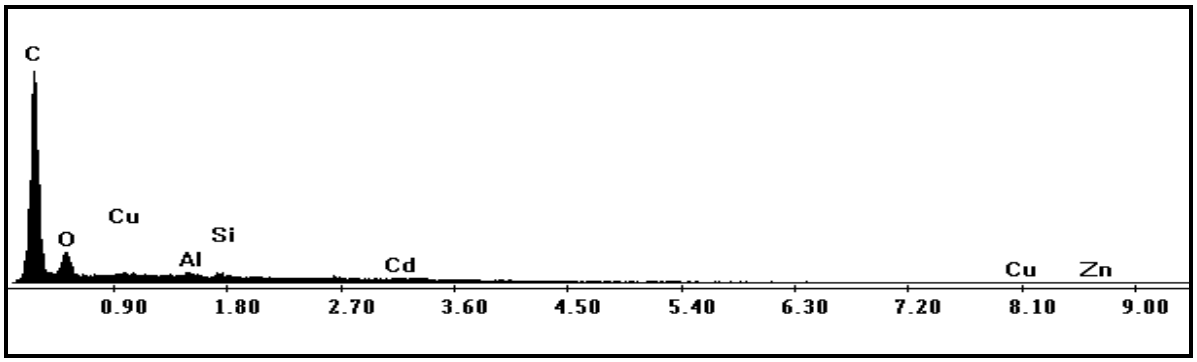

Figure F. 6: EDX Spectrum of the surface of a randomly selected pine bark nugget removed from control Bioreactor D after 48h.

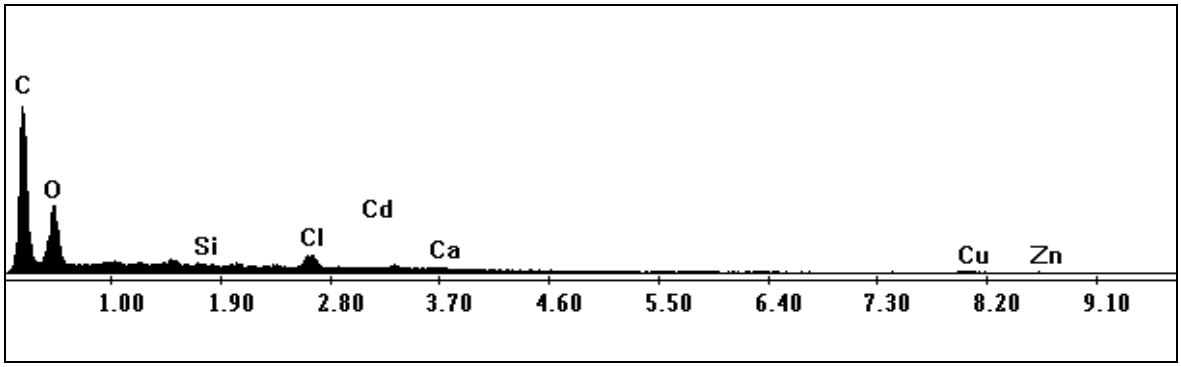

Figure F. 7: EDX Spectrum of the surface of a randomly selected pine bark nugget removed from control Bioreactor D after 168h.

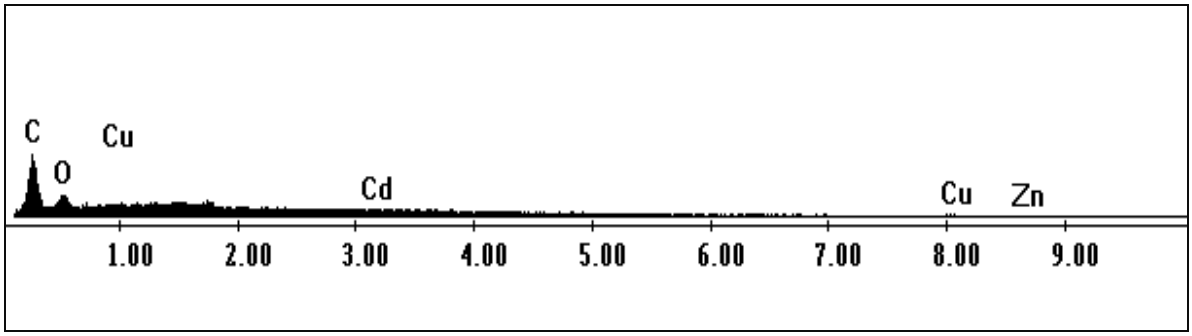

Figure F. 8: EDX Spectrum of the surface of a randomly selected pine bark nugget removed from Bioreactor D after 336h.

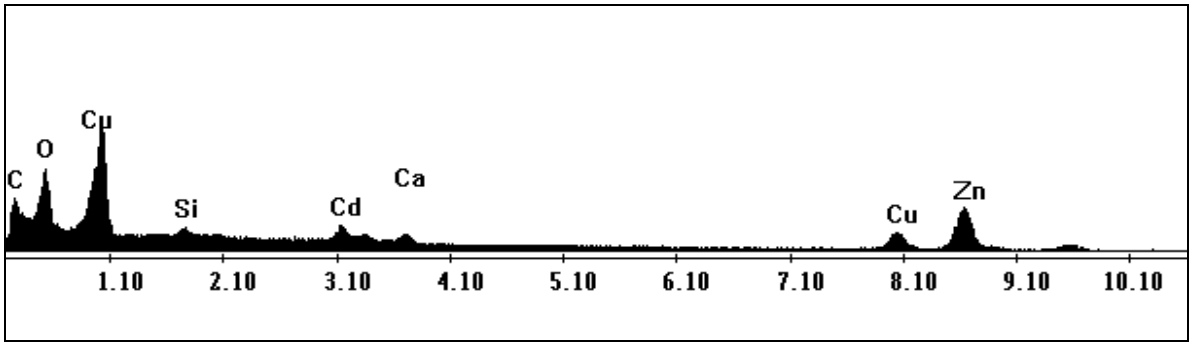

Figure F. 9: EDX Spectrum of the sediment collected from Bioreactor A (bioball control) after 336h

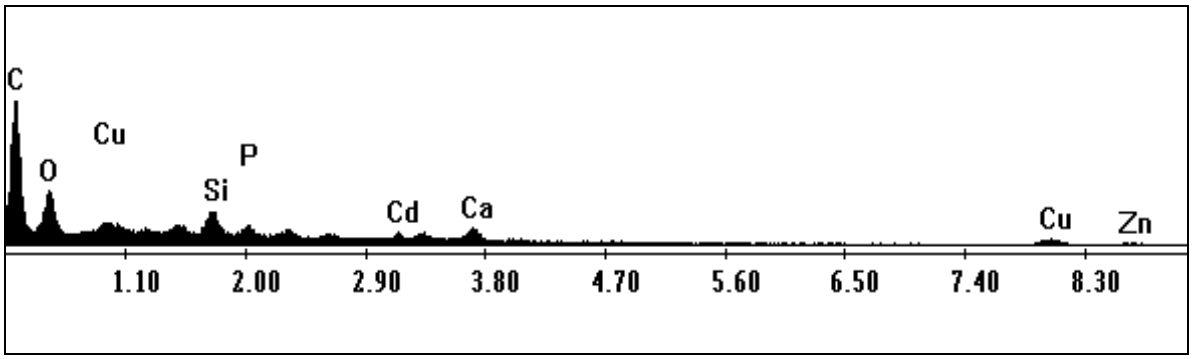

Figure F. 10: EDX Spectrum of the sediment collected from Bioreactor D (pine bark control) after 336h

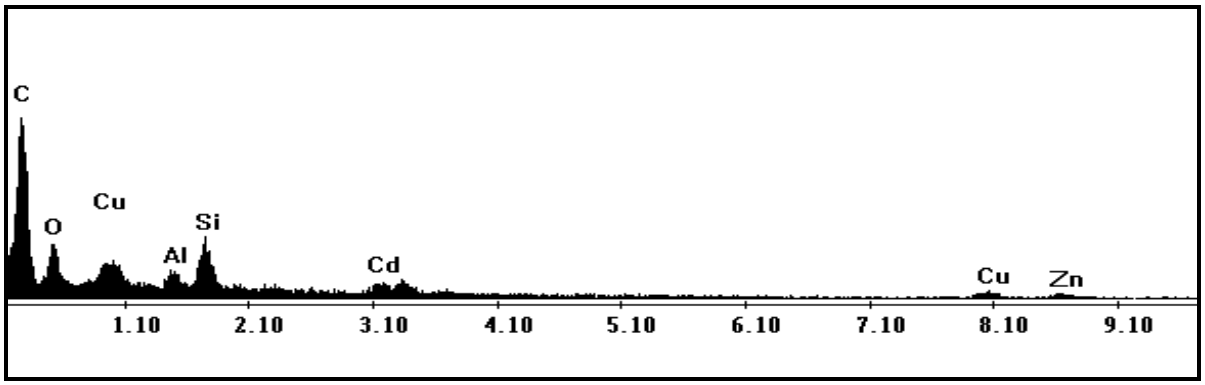

Figure F. 11: EDX Spectrum of a colonised area on the surface of a randomly selected bioball from Bioreactor B after 336h

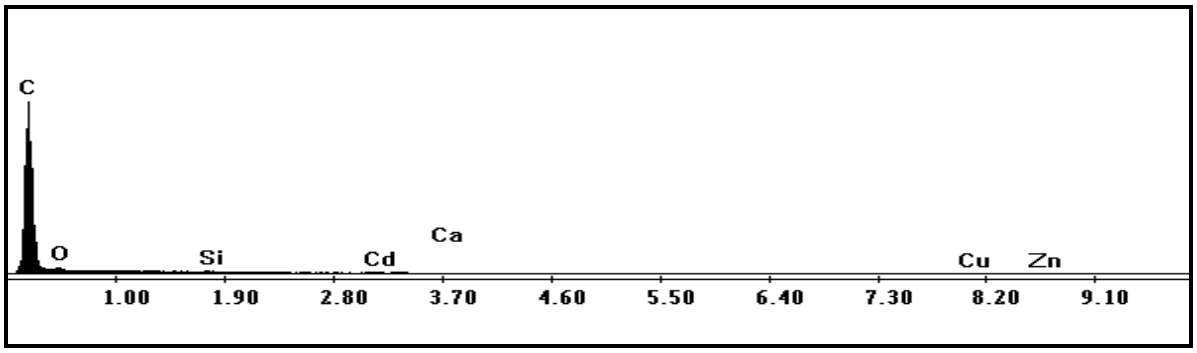

Figure F. 12: EDX Spectrum of a non-colonised area on the surface of a randomly selected bioball from Bioreactor B after 336h

Appendix G

Table G.1: Residual Cu^{2+} concentration ($mg.\ell^{-1}$) at two flow-through rates of $500\ell.h^{-1}$ (Experiment 1)

	Flow-through rate	
Time (h)	$500\ell.h^{-1}$	$1000\ell.h^{-1}$
0	107.8	107.8
2	89.4	86.2
4	66.2	61.9
6	28.9	28.7
12	9.0	10.4
24	3.8	4.6
48	2.4	4.0
72	1.8	3.5
120	1.7	3.3
168	1.7	3.3
Percentage Decrease	98.4%	96.9%
Biosorption Capacity	0.62 $mg.g^{-1}$	0.61 $mg.g^{-1}$

Table G.2: Residual Zn^{2+} concentration ($mg.\ell^{-1}$) at two flow-through rates of $500\ell.h^{-1}$ (Experiment 2)

	Flow-through rate	
Time (h)	$500\ell.h^{-1}$	$500\ell.h^{-1}$
0	105.6	105.6
2	85.2	85.5
4	60.6	61.1
6	25.6	26.1
12	8.4	9.3
24	6.7	5.9
48	4.3	5.0
72	3.8	4.4
120	3.4	4.2
168	3.4	4.2
Percentage Decrease	96.8%	96.0%
Biosorption Capacity	0.60 $mg.g^{-1}$	0.60 $mg.g^{-1}$

Table G.3: Residual Cd^{2+} concentration ($mg.\ell^{-1}$) at two flow-through rates of $500\ell.h^{-1}$ (Experiment 3)

	Flow-through rate	
Time (h)	$500\ell.h^{-1}$	$500\ell.h^{-1}$
0	105.7	105.7
2	83.4	83.9
4	57.9	58.5
6	24.1	24.4
12	10.1	10.4
24	7.1	7.4
48	6.3	6.4
72	5.8	5.9
120	5.6	5.8
168	5.6	5.8
Percentage Decrease	94.7%	94.5%
Biosorption Capacity	0.59 $mg.g^{-1}$	0.59 $mg.g^{-1}$

Biosorption Capacity ($mg.g^{-1}$) = Volume(ℓ) [$Conc_{initial}$-$Conc_{final}$] ($mg.\ell^{-1}$) / Sorbent mass (g)

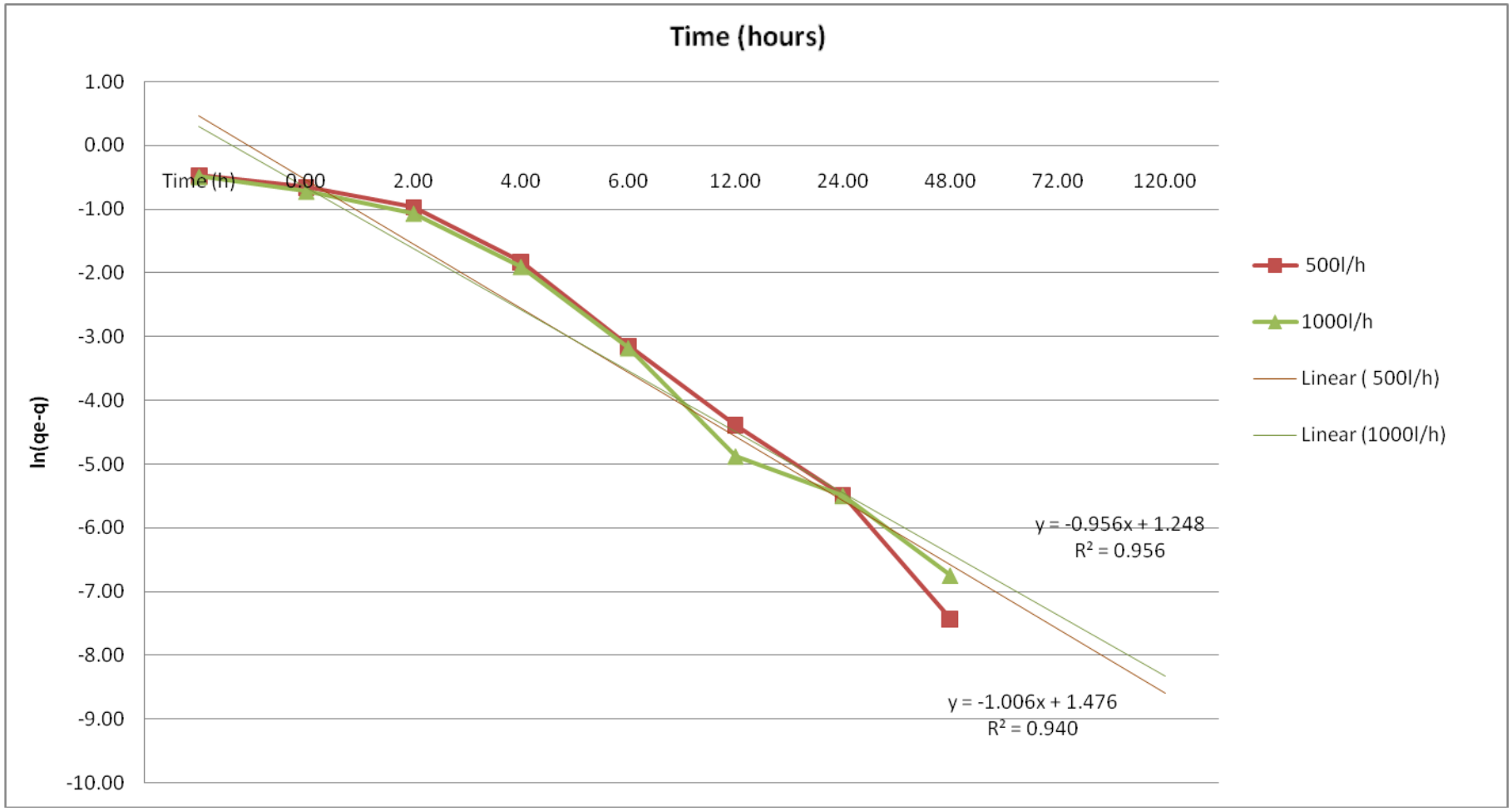

Figure G. 1: Pseudo-first-order plot of the biosorption kinetics of Cu^{2+}

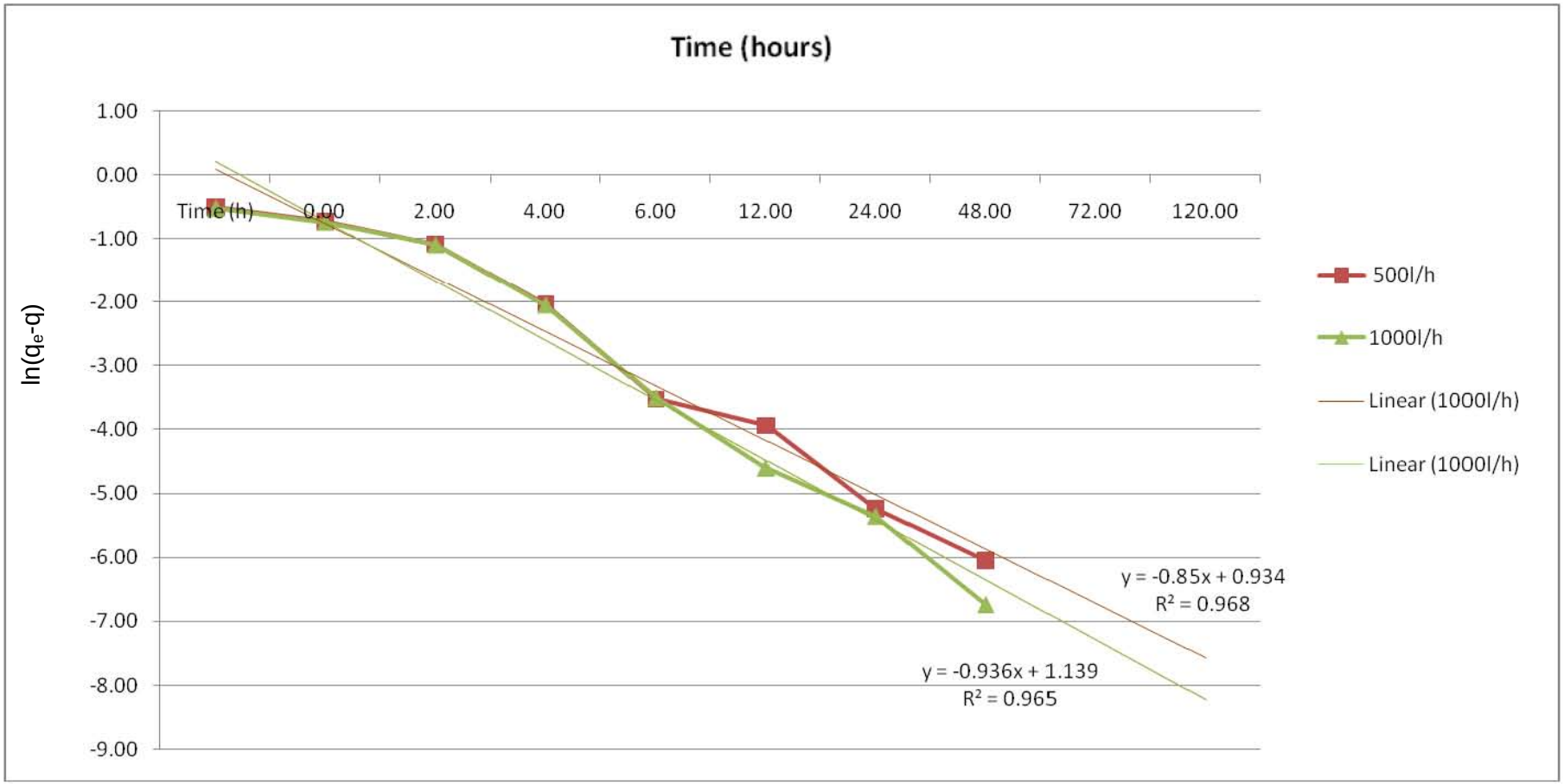

Figure G. 2: Pseudo-first-order plot of the biosorption kinetics of Zn^{2+}

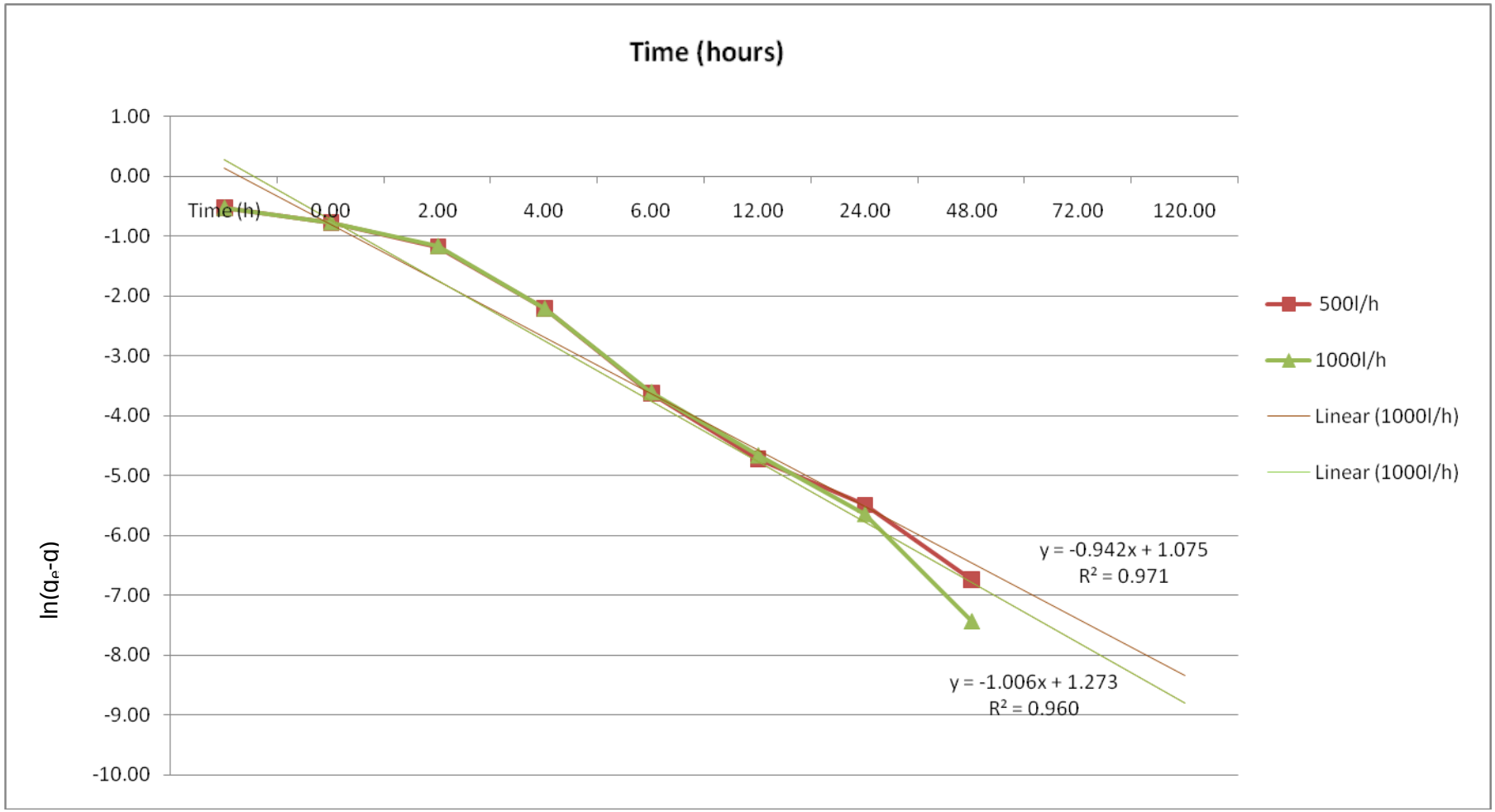

Figure G. 3: Pseudo-first-order plot of the biosorption kinetics of Cd^{2+}

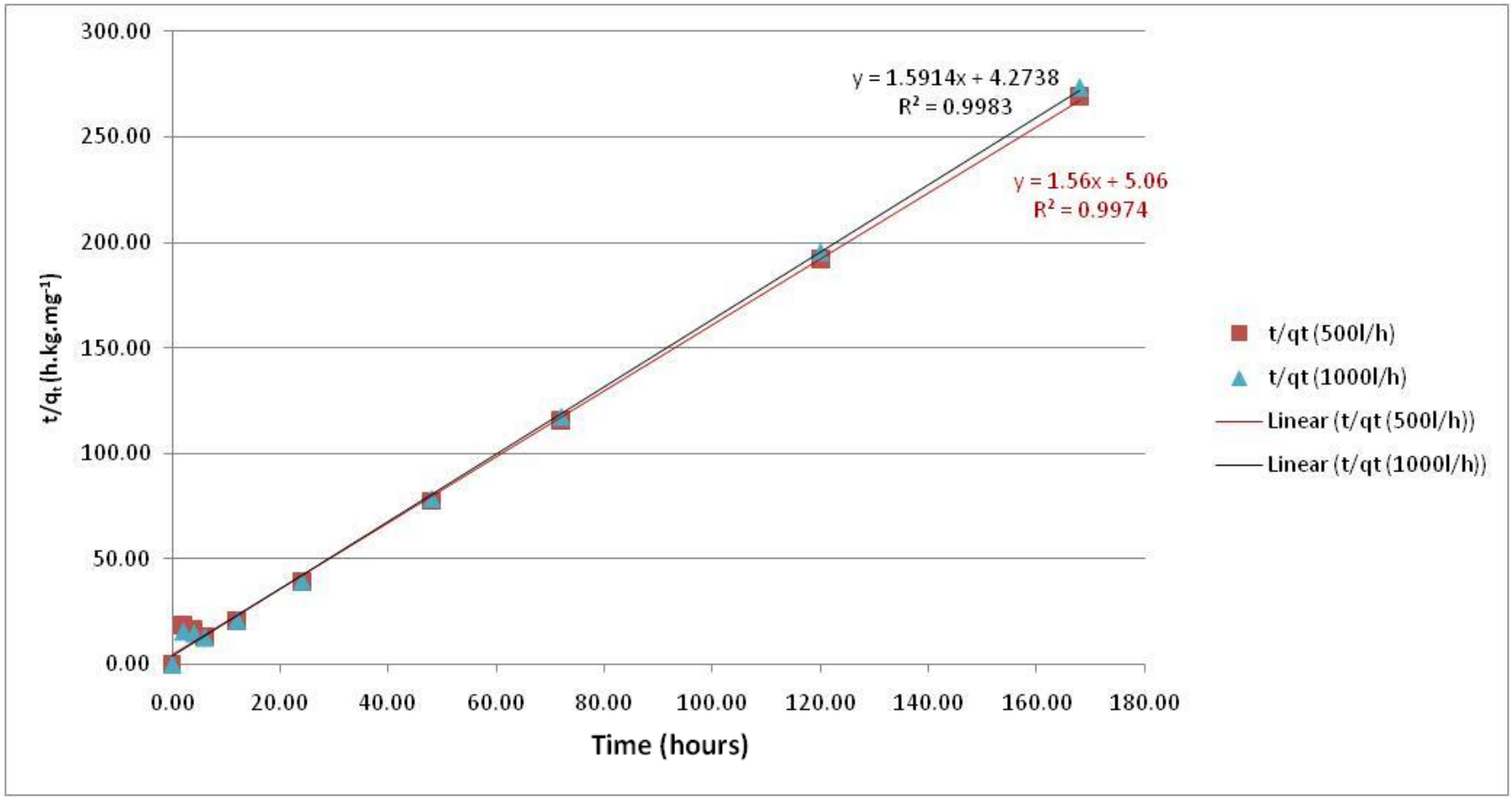

Figure G. 4: Pseudo-second-order plot of the biosorption kinetics of Cu^{2+}

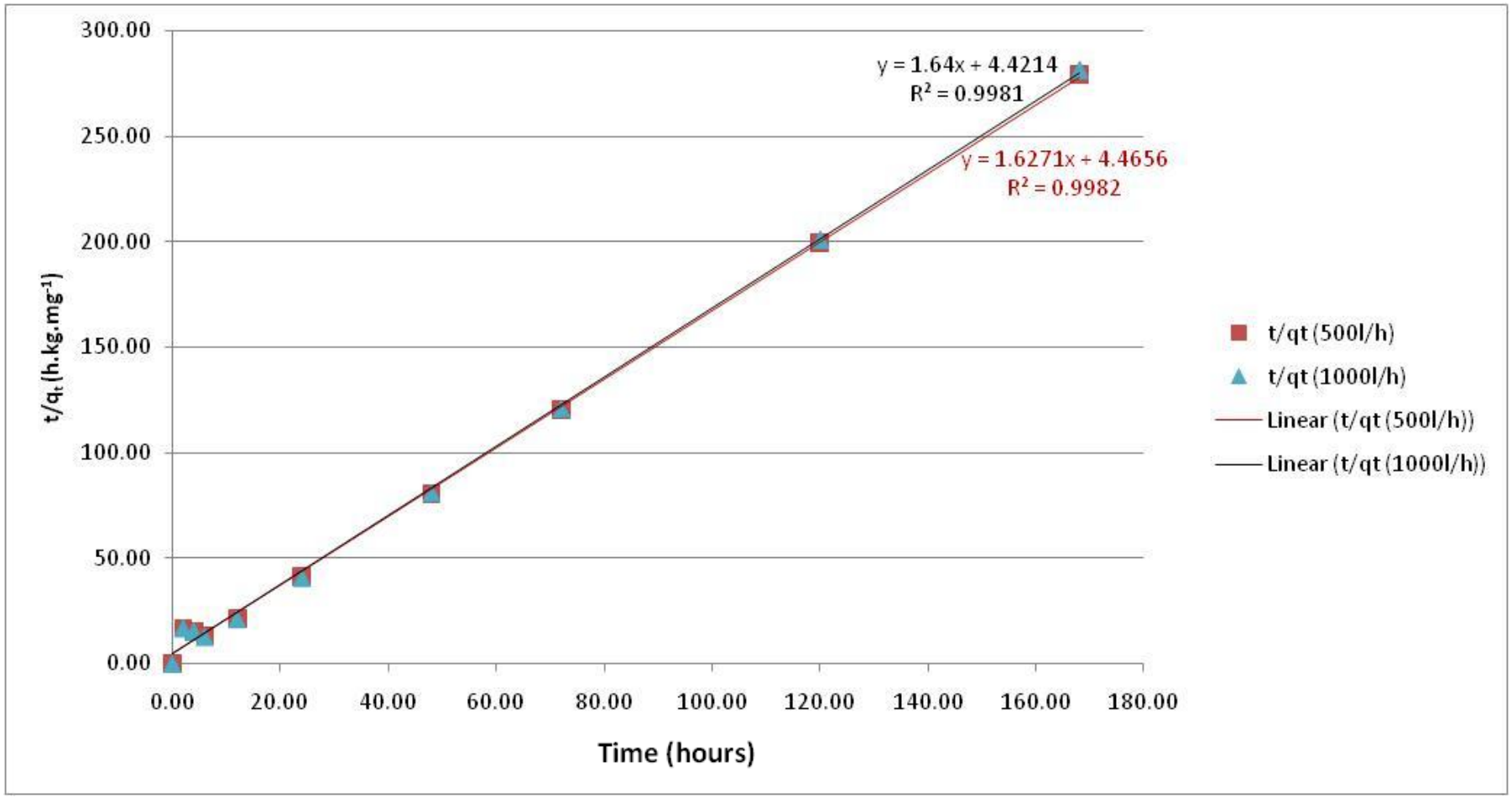

Figure G. 5: Pseudo-second-order plot of the biosorption kinetics of Zn^{2+}

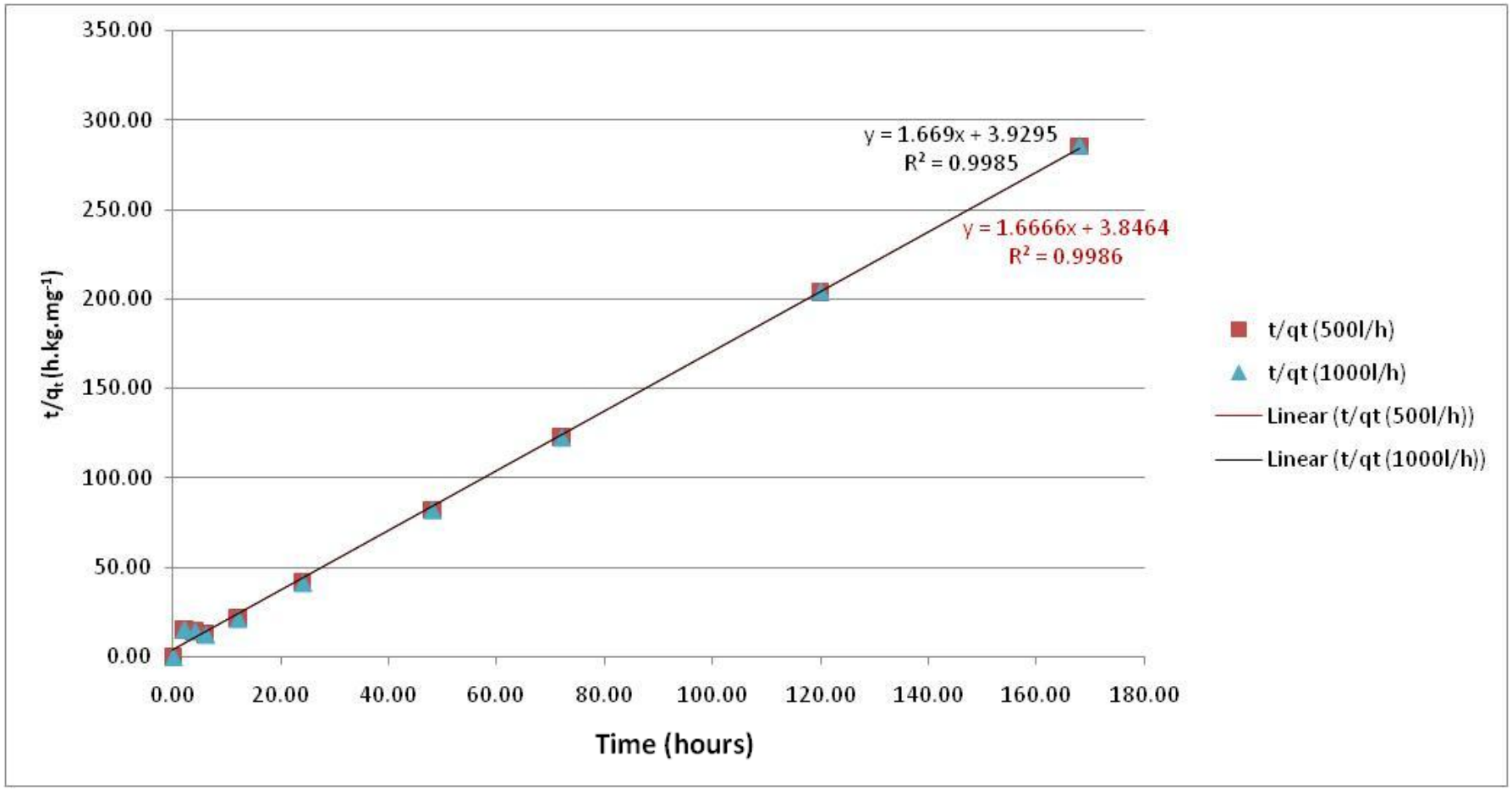

Figure G. 6: Pseudo-second-order plot of the biosorption kinetics of Cd^{2+}