UNIVERSITY OF KWAZULU-NATAL

HUMIC ACID PRETREATMENT FOR ENHANCING MICROBIAL REMOVAL OF METALS FROM A SYNTHETIC 'WASTEWATER'

By

TSEGAZEAB DESTA

Submitted in fulfilment of the requirement for the Degree of Master of Science in Environmental Microbiology in the Discipline of Microbiology

School of Applied Environmental Sciences

Faculty of Science and Agriculture

University of KwaZulu-Natal

Pietermaritzburg campus

August 2004

ABSTRACT

The presence of heavy metal ions in waste streams is one of the most pervasive environmental issues of present times. A rotating biological contactor (RBC) was used to investigate the potential capacity of microbial biofilms in remediation of the metal ion species from a mixed metal contaminated effluent solution containing Cr^{+3}, Pb^{+2} and Cu^{+2}, each at a concentration of 200 mg l^{-1}. In the first part of this study the effectiveness of various support materials for the development of microbial biofilms capable of removing heavy metals from a synthetic effluent was investigated. EDX analysis showed that none of the support matrices investigated, viz. gravel, polyester batting and sand, adsorbed metal ions on their surfaces; hence, metal adsorption was due purely to microbial activities. The biofilms attached more firmly and uniformly to polyester batting than to gravel and sand. The characteristics of polyester batting which made it a superior support matrix were its surface roughness and porous hydrophilic nature, which provided a larger surface area for the adhesion of microorganisms and attraction of nutrients during the biofilm development process.

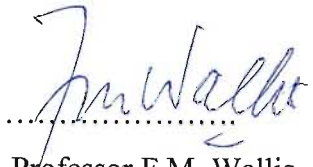
The selective accumulation of metal ion species by various microbial populations grown as biofilm using polyester batting as support matrix in separate compartments of a single-stage RBC bioreactor was examined. Lead ions were readily accumulated by almost all the microbial biofilms tested. Fungus-dominated biofilms selectively accumulated chromium ions whereas biofilms comprising mainly bacteria more readily accumulated copper ions from the mixed metal contaminated effluent solution. However, where interactions between the bacterial and fungal components were encouraged the mechanical stability of the biofilms was enhanced so that large amounts of all three metal ion species were removed by this biofilm.

The combined effect of a series of bench-scale columns containing liquid humic acid and a three stage RBC bioreactor on the removal of metal ion species from a mixed metal contaminated effluent was investigated. After seven days of treatment the combined system had removed approximately 99% of the Cr^{+3}, 98% of the Pb^{+2} and

90% of the Cu^{+2} ions from the mixed metal contaminated synthetic effluent. Complexation of the metal ions with humic acid was the predominant factor accounting for approximately 68-86% Cr^{+3}, 70-86% Pb^{+2} and 53-73% Cu^{+2} removal levels within the columns. A large proportion of the remaining Cr^{+3} and Pb^{+2}, but not of the Cu^{+2}, was removed in compartment 1 of the RBC. This suggested that the presence of the former two metals in solution might have reduced the removal of the Cu^{+2} ions from the system. The removal of substantially large amounts of the competing ions chromium and lead during the initial stages of the treatment process meant that copper was successfully taken up in the second and third RBC compartments. Hence, the economy of the treatment process was improved as larger quantities of the metal ions were removed in a shorter period of time than was possible when using the individual treatments (humic acid-metal complexation and biofilm adsorption) separately. More than 75%, 92% and 86% of the adsorbed Cr^{+3}, Pb^{+2} and Cu^{+2} ions, respectively, were recovered from the three RBC bioreactor compartments following repeated washing of the biofilms with 0.1 M HCl. This relatively easy desorption suggested that the metal ions were simply adsorbed onto the surfaces of the biofilm cells rather than being taken into the cytoplasm of the cells.

DECLARATION

I hereby declare that this research, unless otherwise stated, is the result of my own investigations completed in the Discipline of Microbiology, School of Applied Environmental Sciences, Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg.



Professor F.M. Wallis
(Supervisor)

T.G. Desta
August 2004

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and support of many people. I would like to thank the following people.

My supervisor, Professor Mike Wallis, for his advice, support and encouragement, I cannot thank him enough.

The Eritrean Human Resource Development (EHRD) for sponsoring and funding my studies.

Professor Mark Laing, for his help and encouragement.

The technicians in the Department Ingrid, Diane, Celeste and Kamenthren, for their help and advice.

The Electron Microscopy staff, Belinda, Priscilla and Vijay for their assistance with the Electron Microscopes, the EDX and with the Photographs.

Essac Habibi, Department of Soil Science for his assistance with the AAS.

The staff of the mechanical workshop for fixing my reactor.

Staff members and fellow postgraduate students in the Department for their support and help.

My family and friends for their prayers, support and encouragement. Special thanks goes to Fitsum for editing my thesis.

My faithful and beloved wife, Awet for her constant love, prayers, encouragement and patience during my studies.

To the Source of all knowledge and the Sustainer of all dreams GOD ALMIGHTY.
THANK YOU JESUS!

DEDICATION

To my father, Goje Desta, and my mother, Sara Solomon, for the support, prayers and encouragement during my studies.
May the visions of your heart replace the limitation of your eyes.

To my beloved wife, Awet and daughter, Wintana.

"For the Lord gives wisdom; from His mouth come knowledge and understanding" Proverbs 2:6.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Eritrea is a semi arid country, which receives very low annual rainfall and yet a large proportion of the population relies on agriculture for a living. Since the country has no wastewater treatment plant, municipal wastes join water bodies without proper processing. As a result, domestic and industrial waste products are becoming a major health problem in society. Industries such as textile, leather, paint and battery manufacturing, petroleum refining, metal-products and pulp and paper industries discharge large amount of wastewaters into the environment. These wastewaters are known to contain heavy metals such as chromium, lead and copper. Although trace amount of these metals are important for living organisms, at higher concentrations they are potentially toxic to a wide range of organisms. Treatment methods aimed at combating the problem must be introduced to reduce the hazardous effects of these wastes for man.

Conventional methods of metal removal from wastewaters, namely chemical precipitation, ion exchange, chemical oxidation-reduction, filtration and electrochemical treatment will be extremely expensive for the country. But an appropriate, low cost and efficient means of treatment of wastewaters prior to discharge into the environment must be developed. Over the past few decades rotating biological contactors (RBCs) have been shown to remove metal ions from industrial waste streams. This method relies on consortia of microorganisms that adsorb metals from wastewater and thus it may be used as an alternative to conventional systems of treatment. In the present study activated sludge obtained from Hammarsdale sewage works, which receives a high discharge of industrial wastes with a wide diversity of metal ions (Costley and Wallis, 2000), served as a source of inoculum for establishment of the biofilms subsequently used for metal removal. The initial concentration of 200 mg l^{-1} for each metal was based on previous findings relating to the metal tolerance limits of the inoculum.

1.2 Heavy Metals

Heavy metals comprise an ill-defined group of approximately 65 metallic elements, which have atomic weight between 63.5 g and 200.6 g and density greater than 5 g ml^{-1}, with diverse physical, chemical and biological properties. Generally they have the ability to exert toxic effects on microorganisms (Gadd, 1992). Living organisms require trace amount of some heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc. However, excess levels of these essential metals can be detrimental to organisms (Kennish, 1992). Toxicity of heavy metals may be listed in approximate decreasing order as follows: Hg, Cd, Cu, Zn, Pb, Ni, Cr, Al, Co. This sequence is very tentative and the position of each element in the series will vary with the species tested and the condition of the experiment (Abel, 1989).

The uptake of heavy metals and their use by living cells depend on the chemical and physical properties of each metal ion. An overabundance of any of these elements can cause build-up to an intracellular toxic level, which often results in death (Wood and Wang, 1983). The most important heavy metals in water pollution are chromium, copper, lead, cadmium, mercury, nickel and zinc (Abel, 1989). Of these chromium, lead and copper, which are frequently encountered into wastewaters from various industries are receiving great attention as environmental pollutants because of associated adverse health effects caused by these metals (Stephenson, 1987).

1.2.1 Chromium

Chromium is an element found in many minerals and is widely distributed in the earth's crust. It is considered to be essential for living organisms and a deficiency of chromium in animals can produce diabetes, arteriosclerosis, growth problems and eye cataracts (Gauglhofer and Bianchi, 1991). Over the past several decades increased quantities of chromium compounds have been used by man and introduced into the environment. The danger of environmental contamination depends on the oxidation state of chromium. Chromium occurs in two stable oxidation states, trivalent and hexavalent that have different chemical and physical properties (Moore and Ramamoorthy 1984). In its hexavalent form,

it is 100 to 1000 times more toxic than the more common trivalent state. The hexavalent chromium compounds are highly water-soluble whereas trivalent are less soluble and hence less toxic (Bader *et al.*, 1999). Hexavalent chromium is toxic to microorganisms even at low concentrations due to its ability to inhibit enzyme activity, however, some bacteria and fungi are able to tolerate the toxic concentrations of Cr (VI) in contaminated soils and sediments by reducing Cr (VI) to Cr (III) using a variety of electron donors (Bader *et al.*, 1999).

Chromium compounds are used as pigments and catalysts in the building industry, as a tanning agent in the leather industry and as dyes in the textile industry (Gauglhofer and Bianchi, 1991). The principal chromium emissions into surface waters are from metal finishing processes such as electroplating, pickling and bright dipping. The presence of abundant chromium anions in the water causes chronic adverse health effects such as dermatologic and respiratory tract disorders (Moore and Ramamoorthy 1984).

1.2.2 Copper

Copper is widely distributed in nature in the free state and complexed with sulphides, sulphates, arsenides, chlorides and carbonates. It is classified as intermediate between hard and soft acids in its chemical interaction with donor atoms (Moore and Ramamoorthy, 1984). Copper is very easily complexed and involved in many metabolic processes in living organisms some of which involve the redox potential of Cu (I) / Cu (II). Complex formation regulates copper homeostasis in soil and organisms and the biosynthesis of essential copper containing proteins and enzymes (Shceinberg, 1991). Copper is required in trace amount for the growth and function of organisms since it is a cofactor for numerous enzymes. Several copper containing proteins have been identified also in biological systems as electron carriers (Trevors and Cotter, 1990).

Copper is used in the electrical construction, plumbing and automotive industries (Moore and Ramamoorthy, 1984). Smelter activities, refining processes and industrial waste effluents contribute to copper contamination of the environment (Trevors and Cotter, 1990).

1.2.3 Lead

Lead is a member of the Group IV elements of the periodic classification and it has stable (+2) and (+4) oxidation states. Lead is classified as an intermediate acceptor between hard and soft acid in its interaction with oxygen and sulphur containing ligands (Moore and Ramamoorthy, 1984). Lead is one of the oldest metals known to man and it has been used in piping, building materials, solders, paint, ammunition and castings. In more recent times lead has been used mainly in storage batteries, metal products, chemicals and pigments (Moore and Ramamoorthy, 1984).

Lead and its compounds may enter the environment at any point during mining, smelting, processing, recycling or disposal and oil refining (Ewers and Schlipkoter, 1991). The major source of lead in humans is through the respiratory tract. This reflects the strong association of lead with urban airborne particles (Moore and Ramamoorthy, 1984). In humans, because of size and charge similarities, lead can substitute for calcium and be included in bone (Moore and Ramamoorthy, 1984). Lead in the body (90% in adults) is accumulated in bones (Bercovitz and Laufer, 1992) and this stored lead is not harmful to the body, but if high levels of calcium are ingested later the lead in the bone may be replaced by calcium and mobilized (Kennish, 1992). Once free in the system, lead may cause nephrotoxicity, neurotoxicity and hypertension. Children are especially susceptible to lead because developing skeletal systems require high calcium levels (Kennish, 1992).

1.3 Conventional Methods for Treatment of Heavy Metal Polluted Wastewaters

Removal of metals from industrial wastewater has conventionally been accomplished mainly by chemical precipitation, ion exchange, electrolytic processes and adsorption (Blanco *et al.*, 1999). Over the last two decades, many attempts have been made to remove heavy metals from waste streams by chemical processes using H_2SO_4, HCl, HNO_3, ethylene diamine tetraceric acid (EDTA) and other chemicals (Couillard and Shucaizhu, 1992). However, each of these processes either requires extensive capital investment and/or high running costs or merely transforms the polluting substance into another form, which may no longer be an acceptable approach (Wood, 1992). In addition, techniques such as

chemical precipitation produce toxic sludges in which metal recovery becomes difficult (Yan and Viraraghavan, 2001).

1.3.1 Chemical Precipitation

Chemical precipitation in wastewater treatment involves the addition of chemicals to alter the physical state of dissolved and suspended solids and to facilitate their removal by sedimentation (Tchobanoglous and Burton, 1997). The precipitation of soluble metal ions to form insoluble metal hydroxides is usually a function of pH adjustment through the addition of an alkali (Wood, 1992). Hexavalent chromate can be reduced to the trivalent state using this method (Janson *et al.*, 1982).

Chemical processes, in conjugation with various physical operations, have been developed for the complete secondary treatment of untreated wastewater, including the removal of nitrogen and phosphorus (Tchobanoglous and Burton, 1997). However, the generation of toxic sludges make the process inconvenient by creating a land disposal problem (Wood, 1992).

1.3.2 Ion Exchange

Ion exchange is a reversible chemical reaction, where an ion from the solution is exchanged for a similarly charged ion attached to a solid particle (Janson *et al.*, 1982). Originally it was developed to demineralise potable water and reduce hardness in domestic supplies. However, commercial ion exchange resins are available which can be used directly as metal removers. The process relies on the exchange of undesirable ions in the waste stream for sodium, hydrogen or other ions attached to the insoluble resin. (Wood, 1992).

Although every known metal has been recovered, separated, and purified by some ion exchange process at laboratory scale, it is economically impracticable to treat with ion exchange on a commercial scale (Janson *et al.*, 1982).

1.3.3 Electrolytic Treatment

Electrolytic processes use electrical energy to cause a chemical change within an electrolytic cell. The cells consist of an anode and cathode immersed in an electrolyte and externally connected together via an electrical circuit (Wood, 1992). Electrolytic processes have obvious advantages for the recovery of precious metals where the associated costs of the process may be offset by the value of the recovered metal deposited on the cathode (Wood, 1992).

1.3.4 Adsorption

Adsorption is the process of collecting soluble substances that are in solution on a suitable interface. The interface can be between the liquid and a gas, a solid, or another liquid (Tchobanoglous and Burton, 1997). In the past, the adsorption process has not been used in wastewater treatment, but demands for a better quality of treated wastewater effluent have led to an intensive examination and use of the process of adsorption on activated carbon (Tchobanoglous and Burton, 1997).

Activated carbon can be effective at removing noble metals from solution and burning off the impurities in the carbon pores can regenerate the exhausted carbon. The initial cost of the material and the subsequent expense of regeneration, which may result in the metal ions being released into the atmosphere, make this option unattractive (Wood, 1992). Low cost metal adsorbents which are easy to produce or readily available, have attracted much research interest. Novel adsorbents, for example peat moss, wool, hair, poultry feathers, coconut shell and even shredded tyre rubber, have all been found to adsorb metal ions from solution (Wood, 1992).

1.4 Biological Treatment of Heavy Metal Polluted Wastewaters

Biological processes have been used for treatment of metal contaminated wastewaters and over the years several processes have evolved. The processes are highly complex biologically and are mediated by inexpensive biological material such as algae, fungi and

bacteria (Andrews, 1971; Yan and Viraraghavan, 2001). Microorganisms have the ability to accumulate heavy metals, a process which is important industrially. Industrial effluents and wastewaters can be detoxified and valuable heavy metals recovered (Shumate and Strandberg, 1985). Such processes can also lead to a reduction in environmental pollution, since hazardous heavy metals are removed from waste streams before they are introduced to natural water systems (Gadd, 1988).

Microbiologically related technologies may thus provide an alternative process to the conventional physicochemical techniques of metal recovery and removal (Shumate and Strandberg, 1985). In these processes microorganisms play a predominant role in the accumulation, transport and deposition of heavy metals from their environment (Hutchins *et al.*, 1986).

1.4.1 Mechanisms of Microbial Heavy Metal Accumulation

Microbial cells can accumulate heavy metals by a variety of processes. Depending on the degree of metabolic dependence, both living and dead cells are capable of metal accumulation but there may be difference in the mechanisms involved in either case (Gadd, 1988). Many metals, such as Cu, Zn and Mn are essential for microbial growth at low concentration whilst others like Pb, Ag and Au are toxic towards living cells. Thus the choice of living and dead biomass is important for any envisaged process (Gadd, 1990). Microbial metal uptake capacities can often be divided into two main phases. The first phase, which can be also occur in dead cells, is metabolism-independent accumulation or adsorption to the cell wall and other external surfaces. The second, slower phase is metabolism-dependent transport across the cell membrane (Gadd, 1988; Wood, 1992). An understanding of the mechanisms by which microorganisms accumulate metals is important in the development of microbial processes for metal removal (Shumate and Strandberg, 1985).

Microorganisms have several mechanisms which may be involved in the process of removing soluble heavy metal ions from wastewaters. These range from physico-chemical interaction such as adsorption to the cell wall and other constituents, to mechanisms

dependent on metabolism-like transport, internal compartmentalisation and extracellular precipitation by excreted metabolites (Gadd, 1988). Hence, in a given microbial system, several mechanisms of metal uptake may operate simultaneously and/or sequentially (Gadd, 1990).

1.4.1.1 Metabolism-independent biosorption of heavy metals

Biosorption has emerged as a promising technique for metal removal, in which metal cations are readily adsorbed to negatively charged sites at the surface of microorganisms (Yan and Viraraghavan, 2001). This process is rapid, reversible and independent of temperature and energy of metabolism (Olson and Panigrahi, 1991). Adsorption may be divided into three types (Gadd, 1988):

- Electrical attraction or exchange adsorption, which involves the attraction of positively charged ions to negatively charged ligands in the cell material.
- Physical adsorption which involves van der Waal's forces where molecules can have transitional movement within the interface.
- Chemical adsorption, which involves chemical attraction between the adsorbate and the adsorbent.

Gram-positive bacteria possess selective affinities for several cations and they tend to be more efficient metal chelators than are Gram-negative bacteria. The cell wall of Gram-positive bacteria contains teichoic and teichuronic acids, which are the prime metal binding sites (Beveridge *et al.*, 1982). In Gram-negative bacteria the cell wall structure and chemical composition is different and in an experiment with *Escherichia coli* K-12 metal deposition was found to occur at the polar head regions of the membrane or on the peptidoglycan layer (Beveridge and Koval, 1981).

1.4.1.2 Metabolism-dependent intracellular accumulation of heavy metals

Metabolism-dependent intracellular uptake of metal ions may be a slower process than surface adsorption. It is inhibited by low temperature, the absence of an energy source and by metabolic inhibitors. The rate of intracellular uptake may also be influenced by the

physiological state of the cells, and the nature and composition of the external medium (Gadd, 1988). Although microbial transport systems are only vaguely understood, they are of a varying specificity and both essential and non-essential elements may be taken up from the external environment via such systems (Gadd, 1992). Many metals have been found to be taken up by this process, for example, Cd, Ag, Zn, Cu, Cr, Ni, U, Pb, Hg, Pd and Au (Shumate and Strandberg, 1985).

Mechanisms of metal transport into the cell appear to be dependent on the establishment of an electrochemical proton gradient across the cell membrane, which has a pH gradient and transmembrane potential as its chemical and electrical components respectively, each of which can drive the transport of ionised solutes across the membrane (Gadd, 1990). The membrane potential was found to be responsible for electrophoretic mono-and di-valent cation transport in fungi but other gradients might be involved (Gadd, 1990). It should be stressed that this energy dependent uptake may not be a significant component of the total uptake of metals in some organisms, especially filamentous fungi and microorganisms possessing extracellular polysaccharide, slime or mucilage, which have high biosorptive capacity (Gadd, 1988). Furthermore, with toxic heavy metals permeabilization of the cell membranes can result in further exposure of intracellular metal binding sites and increased passive accumulation. Intracellular uptake may ultimately result in death of sensitive organisms, unless a means of detoxification is induced (Wood and Wang, 1983; Gadd, 1992).

1.4.1.3 Extracellular precipitation, binding and complex formation

Microorganisms produce large amounts of extracellular polymers that form capsules or loose aggregates around the cells. These polysaccharides have anionic properties and are capable of considerable binding of metal cations (Lester *et al.*, 1984). Slime layer have also been implicated in the binding of heavy metals species, however, it appears that this area has not received much attention. Although the production of such extracellular polysaccharides tends to vary greatly between microorganisms, the slime layer, together with extracellular polysaccharide matrices, appear to act as an efficient accumulation barrier and tolerance mechanism by preventing the entry of metal ions into the cell (Gadd,

1992). Some mention of crystallization of metals on microbial surfaces has already been made in relation to metabolism-independent accumulation, but it is often difficult to ascertain whether or not metabolism is involved in all cases (Lester *et al.*, 1984).

1.5 Microbial Resistance to Heavy Metals

Uptake of heavy metals and their use by living cells depend on the chemical and physical properties of each metal ion. An overabundance of these metals in the environment could lead to build-up a toxic level intracellularly, which often results in cell death (Wood and Wang, 1983). Some heavy metals have toxic effects on cells mainly as a result of their ability to denature proteins (Gadd and Griffiths, 1978). Others have the ability to form organometalic complexes, which are known to be particularly hazardous to aquatic organisms (Abel, 1989).

Microorganisms develop various detoxification mechanisms that enable them to resist toxic concentrations of heavy metals. These include cell wall involvement and metabolism of elements (Lester, 1987; Gadd, 1992). Some microorganisms have inherited the ability to resist high concentrations of toxic elements through their evolution under extreme environmental conditions (Wood and Wang, 1983). Other microorganisms have genetically determined mechanisms of resistance to polluted environments through the acquisition of extrachromosomal DNA molecules called plasmids (Misra, 1992). Each of these resistance mechanisms relies on inputs of cellular energy (Wood and Wang, 1983).

1.5.1 Mechanisms and Specific Examples of Microbial Metal Resistance

1.5.1.1 Cell wall

As mentioned in the previous section, many metal ions are deposited or precipitated within or on the surface of the cell wall and may not be toxic to the microorganism. *Micrococcus luteus* and *Azotobacter* species have been found to immobilize on the cell wall and membrane 99% of the lead from broth containing high concentration of lead salts, without a detectable effect on the viability of the cells (Lester, 1987). Less than 1% of the lead was

found in the cytoplasm (Lester, 1987). Several bacteria and some algae have been found to precipitate metal ions on their surfaces thereby preventing their cellular uptake (Olson and Panigrahi, 1991).

1.5.1.2 Hydrogen sulphide production

Microbial hydrogen sulphide production often has a significant effect on metal toxicity since most heavy metals form insoluble sulphides when reacted with H_2S. Consequently, an organism producing hydrogen sulphide will tend to be more tolerant to the presence of heavy metals in the environment (Olson and Panigrahi, 1991). For example, the sulphite reducer *Desulfovibrio desulfuricans* produces hydrogen sulphide, grows in high sulphide concentrations, and may not be affected by the addition of high concentration of heavy metals (Gadd and Griffiths, 1978). Likewise, copper and mercury tolerant strains of the yeast *Saccharomyces cerevisiae* produce more H_2S than do their non-tolerant parent strains, the metals being precipitated as insoluble sulphides (Gadd and Griffiths, 1978). Colonies of copper tolerant strains appear black or dark brown in the presence of copper and contain much copper sulphide (Ashida and Nakamura, 1959). Metal resistant strains of *Klebsiella aerogenes* were also found to precipitate Pb, Hg and Cd as insoluble sulphide granules on the outer surface of the cells (Aiken *et al.*, 1985).

In all cases the activity and toxicity of heavy metal is reduced due to extracellular precipitation and crystallization before the metal is able to accumulate intracellularly. Yeasts as well as algae have also been reported to remove a number of metal ions from the environment in the form of metal sulphide precipitates (Wood and Wang, 1983).

It has been noted that in some cases the sulphide-producing organisms may also offer protection to non-hydrogen sulphide producing sensitive microorganism in the surrounding environment (Gadd and Griffiths, 1978; Olson and Panigrahi, 1991). For example, when *D. desulfuricans* is grown in mixed culture with metal sensitive strains of *Pseudomonas aeruginosa*, the latter could tolerate a higher concentration of metal than it could in pure culture (Gadd and Griffiths, 1978).

1.5.1.3 Metal transformation

Metal transformation is an important biological process that can occur in many habitats and can be carried out by a wide range of bacteria and fungi. Metals cannot be broken down into other products but may undergo change in valence and/or conversion into organometalic compounds as a result of biological action (Gadd and Griffiths, 1978). Four major types of metal transformation in organisms have been defined. These are chelate formation by the binding of the metal to organic ligands, shift in metal valencies, substitution of one metal for another and biomethylation of metal by microorganisms (Lester, 1987).

Transformation involving change of valence has been studied with chromate and it was found that some microorganisms reduce Cr^{+6} to Cr^{+3}, which subsequently precipitates as Cr $(OH)_3$ extracellularly (Misra, 1992). Chromate-reduction occurs aerobically and anaerobically using a variety of electron donors in different bacterial strains (Bader *et al.*, 1999). The ability of bacteria to enzymatically reduce the toxic chromate anion to the less toxic Cr (III) form has potential application in the detoxification and immobilization of Cr (VI) in soil and water systems (Ohtake and Silver, 1994).

Certain microorganisms have been found to methylate metals into organometalic compounds. Metals that have been shown to undergo methylation include mercury and lead (Gadd and Griffiths, 1978). This metabolic transformation of metal ions by bacteria may represent a detoxification mechanism by which a bacterium can survive in an environment with high metal concentrations. Such transformations may result in accumulation of metals in the cytoplasm of the cell (Lester, 1987). Methylation can be catalysed by a wide variety of microorganisms including, both aerobic and anaerobic bacteria, yeasts and fungi. Although the products of methylation may be more toxic than the free metal, they are often volatile and can be released into the atmosphere (Gadd and Griffiths, 1978).

1.5.1.4 Genetically determined metal and antibiotic resistance

Many microbial metal resistance mechanisms are determined by genes located on plasmids, autonomously replicating circular extrachromosomal DNA molecules (Hughes and Poole,

1989), which can also control antibiotic resistance (Gadd and Griffiths, 1978). Plasmids and transposons are mobile genetic elements, which consist of genes that confer heavy metal resistance on cells containing them (Misra, 1992). Genes conferring resistance to metal ions and antibiotics can coexist in the same plasmid. This is an advantage from an evolutionary point of view in that genetic exchange between different strains, species, or genera is facilitated by relatively small extrachromosomal elements rather than by complex rearrangement within the chromosome (Misra, 1992).

Bacillus species isolated from sewage sludge were found to be resistant to ampicillin and it was concluded that heavy metal contamination of an ecosystem could provide a selective pressure for antibiotic resistant bacteria in that system (Gadd and Griffiths, 1978). However, it is now evident that drug and metal resistance are closely connected and often occur together in clinical isolates. Furthermore plasmids have been chiefly studied with regard to the transfer of antibiotic resistance (Olson and Panigrahi, 1991). For example, plasmid mediated copper resistance has been studied in *Klebsiella aerogenes* and it was found to be associated with enhanced resistance to chloramphenicol and gentamycin but increased sensitivity to streptomycin. On the other hand, in chromate resistant organisms, an associated resistance to nalidixic acid but not to the other three antibiotics tested was found (Olson and Panigrahi, 1991). The expression of many known metal ion resistance genes is inducible by the metal ion itself. When bacterial cells sense the metal ion, increased expression of genes conferring resistance to the metal ion takes place (Misra, 1992).

1.6 Environmental Effects on Heavy Metal Toxicity

Environmental factors such as pH, temperature, redox potential, the presence of organic constituents and other metallic ions may influence the chemical speciation and solubility of a compound, which in turn affect the toxicity of pollutants to microbiota (Brynhildsen *et al.*, 1988). Therefore, it is very important to study the relationship between physicochemical properties of the environment and the ability of microorganisms to tolerate the prevailing conditions.

1.6.1 Organic Constituents

One of the most important factors that influences the biological availability of any metal ion in a system is its binding to organic constitutes. If the metal ion is wholly or partially removed by binding, a decrease or complete disappearance of its toxic effect may result (Gadd and Griffiths, 1978). Organic materials such as humic and fulvic acids and proteins bind strongly to metal ions to form complexes. Humic substances have an appreciable exchange capacity due primarily to carboxyl and phenolic hydroxyl groups, and can form stable water-soluble and insoluble complexes with metals (Francis, 1990). Hence, the metals become biologically unavailable and the toxicity of the metal ion toward the microorganisms decreases (Gadd and Griffiths, 1978). In aquatic environments, metals such as copper and zinc can be bound and removed from the water by organic sediments, which effectively reduce the total metal ion concentration in solution, thus decreasing the toxic effect of certain metals to microbes (Milanovich *et al.*, 1975).

Compounds which can chelate metals, for example, citrate, cysteine and glutamate can also have a significant effect on microbial responses when included in growth media. In an experiment the toxic effect of copper on *Aerobacter aerogenes* was prevented by the addition of yeast extract and cysteine, and this was attributed to the ability of these compounds to bind copper (MacLeod *et al.*, 1967). In media without complexing agents, toxicity may be pronounced as indicated with *Chlorella pyrenoidosa* where a copper concentration as low as 5 $\mu g\ l^{-1}$ was toxic. Consequently, it was suggested that copper is not ordinarily present as the ionic form in natural waters but is usually complexed with organic materials such as polypeptides (Gadd and Griffiths, 1978). Most studies on the toxic effects of metals on microorganisms have involved high nutrient level media with glucose concentrations as high as 5 to 10 $g\ l^{-1}$. It is important when studying the effect of organic compounds on metal toxicity to ensure that the organic composition of the medium mimics the natural condition because most microbes live in energy and nutrient limited environments (Brynhildsen *et al.*, 1988).

1.6.2 Ion Interaction

In a natural environment, different negatively charged ligands are present at different concentrations, have different affinities for the same cation and compete for the heavy metals (Babich and Stotzky, 1980). Anions such as hydroxyl ions, phosphates, thiosulphates, chlorides, carbonates and bicarbonates form precipitates with metal ions and reduce the toxicity of heavy metals depending on their concentrations and the pH of the solution. The addition of such anions to a medium reduces metal toxicity (Gadd and Griffiths, 1978). In an experiment with *Klebsiella aerogenes* it was found that the presence of cadmium chromate antagonised the toxic effect of copper, hence its toxicity towards the cell was reduced (Gadd and Griffiths, 1978; Olson and Panigrahi, 1991). Thus, the presence of other metal ions in a medium affects the biological activity of heavy metal ions (Mohan, 1998).

Generally when metal ions act antagonistically to each other, either the toxicity of one of the metals may cause a conditioned deficiency of another physiologically important metal ion or competitive interaction at the level of metal ion uptake may become operative when other metals are present in sizable amounts in the medium (Mohan, 1998). Sometimes, however, the toxicity of certain metals is increased when other metal ions are present. In the case of the alga *Chlorella vulgaris*, an asymmetric respiratory response occurs when fluoride and copper ions are applied jointly; respiration is completely inhibited by the mixture, but individually the ions have little effect (Gadd and Griffiths, 1978). Likewise, in a study with *Klebsiella aerogenes* grown in a medium spiked with copper, the addition of zinc increases the lethal effect of the copper (Olson and Panigrahi, 1991).

1.6.3 pH

The pH can have a considerable effect on both the availability and toxicity of certain heavy metals in solution. At an acidic pH value heavy metals tend to exist as soluble, free ionic cations which increases their biological availability and consequently their toxicity is higher in an acidic environment (Hahne and Kroontje, 1973). On the other hand, at an alkaline pH the ionic cations precipitate as insoluble hydroxides or oxides. This decreases

their availability in the solution and as a result their toxicity is reduced (Hahne and Kroontje, 1973; Gadd and Griffiths, 1978). In an experiment on the fungus *Aureobasidium pullulans* it was found that an increase in the pH of the medium from 3.5 to 4.7 caused an increase in the toxicity of Cu^{+2}, which was related to an increase in the uptake of Cu^{+2} (Mohan, 1998).

It has been illustrated that in very acid soils, where toxicity is due to an abundance of iron, manganese, copper and zinc the toxic effect can be removed by adding lime, which raises the pH (Gadd and Griffiths, 1978). The optimum pH for adsorption of all metals, except gold, is between 4 and 5, where the metallic species are ionised and may bind to the biomass (Kuyucak and Volesky, 1988).

1.6.4 Oxidation-reduction Potential (Eh)

The oxidation-reduction potential of an environment can affect the availability and toxicity of heavy metals. Heavy metals that are deposited into an environment with a negative Eh may combine with any S^{2-} present to form insoluble sulphides, which are not available for uptake by microbes and, hence, may be rendered non-toxic (Mohan, 1998).

1.6.5 Temperature

The majority of aerobic wastewater treatments are operated at ambient temperatures 5-35 ^{0}C and the effect of temperature on process kinetics has been studied primarily in this range (LaPara *et al.*, 2000). However, elevated temperature can also affect the toxicity of heavy metals to microbes by altering the physiological state of the cells, rather than the chemical speciation or availability of metals (Collins and Stotzky, 1989).

1.7 Biofilms

Biofilms are discrete aggregation of microorganisms and their products, such as extracellular polymers, immobilized at an interface (Hamilton, 1987). At the solid-liquid interface and gas-liquid interfaces as well, nutrients tend to concentrate and create a

nutrient gradient condition. Motile microorganisms respond chemotactically towards the nutrient gradient established (Marshall and Bitton, 1980; Hsueh *et al.*, 1991), and as they arrived near the surfaces they become subject to short range attraction forces, such as hydrophobic, and van der Waals force, which are capable of holding the organisms at the surface of a solid-liquid interface (Marshall and Bitton, 1980). The cells then produce extracellular polymeric materials and become immobilized to inert material or adhere to each other to form biofilms (Bryers and Hamer, 1988).

Adhesion to surfaces is a common and well-known behaviour of microorganisms in natural oligotrophic habitats (Zobell, 1943). Bacterial adhesion and subsequent biofilm formation is the net result of the following physical, chemical and biological processes (Characklis, 1990):

1. Organic molecules accumulate at the substratum, resulting in a "conditioned" substratum,
2. Planktonic microbial cells are transported from the bulk water to the conditioned water to the conditioned substratum,
3. A fraction of the cells that reach the substratum reversibly adsorb to the substratum,
4. Some reversibly adsorbed cells desorb,
5. A fraction of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed,
6. The irreversibly adsorbed cells grow at the expense of substrate and nutrients in the bulk water, increasing biofilm cell numbers and forming other metabolic products,
7. Cells and other particulate matter attach to the biofilm, increasing biofilm accumulation,
8. Portion of the biofilms detach and are re-entrained in the bulk water.

The application of biofilm processes for biological wastewater treatment is becoming more popular because of the benefits offered by biofilms. These benefits are the active building up of biomass in the reactors and their upholding through attachment to the solid surface

(Rittmann, 1982). Immobilization provides a means for retaining microorganisms within a reactor at concentrations well above those that would exist due to suspended growth alone. Therefore, captured cell systems allow biological reactors to operate at a much higher loading rate than suspended culture reactors and hence increase the overall system productivity (Bryers and Hamer, 1988; Freitas dos Santos and Livingston, 1995). In addition to this, biofilm-associated cells are more resistant to many toxic substances such as heavy metals, antibiotics, chlorine and detergents (Watnick and Kolter, 2000) than to their planktonic counterparts.

1.7.1 Support Surface

The nature of the support surface plays a major role in biofilm development, by influencing the rate of cell accumulation as well as initial cell population distribution (Kolot, 1988). Depending on surface characteristics and physico-chemical interactions between the support surface and the microorganisms fixed films could be established quickly, uniformly and firmly to the support surface (Murray and Van Den Berg, 1981). As a result of the interaction microorganisms remain adsorbed to the surface of the supporting particle and aggregate to each other, forming a biofilm matrix throughout the interstices of the support particle (Bryers and Characklis, 1990). Some of the criteria of an ideal support material for a better microbial attachment are (Murray and Van Den Berg, 1981; Kolot, 1988):

- It should have high surface area to volume ratio
- It should provide an exposed surface over which biofilms can develop
- It should have rough and porous texture to permit bacterial adhesion
- It should be non-toxic to the cells and should not affect their metabolism
- It should be mechanically strong and resistant under operating conditions
- It should establish physico-chemical interactions such as electrostatic attraction
- It should be readily available.

Supports for microbial cells can be generally divided into two main categories, organic and inorganic (Kolot, 1988). Organic support materials provide a large variety of reactive groups such as carboxyl, amino and hydroxyl on their surface and they are preferable for

microbial attachment. On the other hand inorganic support materials, regardless of the fewer reactive groups on their surface, are widely used for microbial attachment (Kolot, 1988). A great variety of inorganic supports such as sand, gravel, brick, glass, ceramics and plastic materials have been used as supports for microbial attachment. Polystyrene sheets, needle-punched polyester and polyvinyl chloride (PVC) foils in various geometries have lately been in use with growing popularity (Qureshi *et al.*, 2001).

In biological wastewater treatment the polluting substance must be brought into contact with the microbial population for a sufficient period of time. As a result the pollutant is broken down and removed to a required extent (Winkler, 1983). Successful biological treatment depends on the development and maintenance of an appropriate, active and mixed microbial population in the system. This microbial population may be present as either a suspended growth as in activated sludge and anaerobic digestion processes, or a fixed film attached to support medium as in the trickling filter and rotating biological contactor (Barnes *et al.*, 1981).

1.7.2 Biofilm Thickness

Depending on the type and species of microorganisms and outside substrate concentration biofilms increase in depth and thickness, and the exchange of substances occurs only on one side of the biofilm. Therefore various gradients exist across the biofilm (Characklis, 1983). A nutrient gradient is formed by the respiring organisms at the upper layer and fermenting ones at the middle layer with the release of products such as ethanol. An oxygen gradient also develops, as a consequence of bacterial respiration in the upper layer (Hamilton, 1987). When the biofilm has reached a thickness of 10-225 μm condition at its base are anaerobic. The biofilm now approaches a state of maturity, with a high species diversity and stability (Hamilton, 1987). The microorganisms near the support medium surface then enter into an endogenous respiration phase and lose their ability of cling to the support surface. The shear forces formed as the support medium rotates through the wastewater cause the biofilms to slough off the support matrix. This is immediately followed by initiation of growth of a new slime layer (Bishop and Kinner, 1986).

Biofilms formed by mixed species are often thicker and more stable than mono-species biofilms. For example, in an experiment with an annular reactor, the mean thickness of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were 15 μm and 30 μm, respectively while a biofilm consisting of both species was 40 μm thick (Siebel and Characklis, 1991). This enhancement of biofilm thickness may be the result of one species enhancing the stability of another within a biofilm (James *et al.*, 1995). Similarly, a binary species of biofilm consisting of *Pseudomonas fluorescens* and *K. pneumoniae* detached from glass readily, but was more stable when a third species, *P. aeruginosa* was added. Thus biofilm stability can be considered a commensal interaction, where one species benefits from the ability of another to form a stable film (James *et al.*, 1995).

1.8 Processes Based on Attached Microbial Growth

An attached growth system relies on the ability of mixed microbial cultures to adhere to a fixed support material, independent of the retention time. The microorganisms attached to a solid substratum reach relatively high concentration and form biofilms (Murray and Van Den Berg, 1981; Bitton, 1994). In these processes the wastewater either flows over the film or the support surfaces move through the wastewater and as a result the biofilms remove the organic and inorganic pollutants (Bishop and Kinner, 1986). The physical attachment prevents the biomass washing out, thereby providing higher loading rate than in a suspended system. In addition to this, fixed film systems also exhibit increased resistance to the detrimental effects of toxic shock loading. Two important factors influencing microbial growth on the support material are the flow rate of wastewater and the size and geometric configuration of the particles (Bitton, 1994).

Fixed biofilm processes offer the following advantages (Rittmann *et al.*, 1988):

- They allow the development of microorganisms with a relatively low specific growth rate (e.g., methanogens)
- Lower capital costs
- They are less subject to variable or intermittent loading
- Ready availability of support materials such as sand, quartz and plastic

- Elimination of the need for clarification due to the attachment of biomass to the support medium
- They are suitable for small reactor sizes.

The two main types of fixed film reactors are, trickling filters and rotating biological contactors.

1.8.1 Trickling Filter

The trickling filter was introduced in 1890 and it is one of the earliest systems for biological wastewater treatment. It has four major components; these are a circular or rectangular tank, a wastewater distributor, an under-drain system and final clarifier (Bishop and Kinner, 1986). The filter medium used in trickling filters is stones (crushed limestone and granite), ceramic material, treated wood, hard coal, or plastic. Selection of filter media is based on factors such as specific surface area, void space, unit weight, media configuration and size, and cost. The smaller the size, the higher the surface area for microbial attachment and growth, but the lower the percentage void space (Bitton, 1994).

Influent wastewater is pumped up a vertical riser to a rotary distributor for spreading uniformly over the filter surface. The wastewater percolates, or trickles down over the biofilms growing on the filter media and is collected in an underdrain system (Hammer, 1996). The filter medium must provide both a high specific surface area for biofilm attachment and a high void space for oxygen transfer and liquid flow (McKinney, 1992). Many industrial effluents that contain toxic material, or low concentrations of organic materials of natural origin can be treated by trickling filters. The process seems better able to withstand a shock load of toxic materials than is the activated sludge system (Dart and Stretton, 1980).

1.8.1.1 Some advantages and disadvantages of trickling filter

Advantages: Trickling filters are attractive to small communities because of easy operation, low maintenance cost and reliability. Furthermore, they are very tolerant of changes in the

influent, and can be used for treatment of toxic industrial waste products and often withstand shock loads of toxic inputs. In addition to these, the sloughed biofilms can be easily removed by sedimentation (Bishop and Kinner, 1986; Bitton, 1994).

Disadvantages: High organic loading which results in excessive biofilm growth, may lead to clogging of the filter and also odour problems in trickling filters. Clogging restricts air circulation and thus results in low availability of oxygen (Bishop and Kinner, 1986; Bitton, 1994).

1.8.2 Rotating Biological Contactors (RBCs)

A rotating biological contactor (RBC) consists of a series of closely spaced circular plastic discs, which rotate partly submerged in the wastewater and microbial biofilms attached grow on the discs (Muyima *et al.*, 1997). Approximately 40% of the discs surfaces are submerged in wastewater and the submerged portion of the disc removes biological oxygen demand (BOD) as well as dissolved oxygen. The rotation of the discs provides aeration as well as the shear force that causes sloughing of the biofilm from the disc surface. Increased rotation improves oxygen transfer and enhances the contact between attached biomass and wastewater (Bitton, 1994). The sloughing action prevents bridging and clogging between adjacent support structures. The mixing action of the rotating discs keeps the sloughed biomass in suspension and prevents it from settling to the bottom of the RBC tank (Lin, 1987).

As the discs continuously rotate, the biofilms are alternatively exposed to the oxygen in the air and the wastewater, in which organic and inorganic material are sorbed by these microorganisms and biodegraded (Engelder and Matson, 1991). Accordingly, waste removal using RBC is based on gas-liquid contact at the interface, which is formed during the alternative exposure of biofilms on the rotating disc surface to the liquid and to the atmosphere (Hsueh *et al.*, 1991). The free space between the discs within the RBC must be large enough to prevent adjacent biological films from meeting and choking the contactor. This also depends on the speed of rotation that is usually between 1 and 3 rpm (Solt and Shirley, 1991). It is advisable for the RBC tank to be divided into at least 3-stages, and

sometimes 5-stages, to achieve high quality effluents. The total contact area required is dependent on the amount and proportion of BOD and ammonia to be removed. The RBC is usually covered to prevent the biological film being stripped off by wind and rain, and to prevent deposition of wind blow sand (Solt and Shirley, 1991).

RBC have some of the same advantages as trickling filters, for example low cost, low maintenance and resistance to shock loads, but not some of the disadvantages such as filter clogging (Bitton, 1994). In addition to these properties, RBCs differs from trickling filters by having long retention times and dynamic rather than stationary support matrices (Lin, 1987).

The advantages offered by RBCs are (Borchardt, 1971; Bishop and Kinner, 1986; Lin, 1987):

- Low operational and maintenance cost and simplicity of operation
- Production of a readily dewatered sludge that settles rapidly
- Bulking, foaming, or floating sludges are never a problem and no intense shear or turbulence can create foam even with a foamy substrate
- Succession of organisms is well established
- Longer contact times are possible
- No requirement of large reserve capacity, hence relatively low land requirement
- A large surface area for aeration
- Can effectively handle shock loading
- The ability to achieve a high degree of purification
- Effective sloughing off of the excess biomass.

RBC has some Disadvantages (Lin, 1987).

- Different organisms grow in laboratory and full-scale RBC units, and this may affect the efficiency of the treatment by making extrapolation difficulty
- RBCs may not be suitable for treating wastewaters containing high level of grease and oil

- Unbalanced biological growth will occur on the support matrices, if the rotation of the discs ceases.

1.9 Project Objectives

The main aims of this research project are:

- To develop an inexpensive but efficient treatment method which could be used by local industries to treat their wastewaters prior to disposal;
- To develop a laboratory-scale rotating biological contactor;
- To study the efficiency of a single and multi-stage rotating biological contactor in treatment of heavy metal polluted wastewater;
- To investigate the potential heavy metal adsorptive capacity of microbial populations adapted to withstand high concentration of various metals;
- To investigate the influence of various support matrices on biofilm development and subsequent metal accumulation;
- To investigate the efficacy of humic acid for the removal of metal ion species in solution;
- To investigate desorption of metal ion species from preloaded biomass using dilute HCl as a stripping agent.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Inoculum

Activated sludge containing microorganisms with marked heavy metal resistance, obtained from Hammarsdale sewage works, KwaZulu-Natal, South Africa, served as the source of inoculum responsible for removal of the metal ions under study.

2.2 Metal Salts

Metal salts used were:

Chromium: $Cr(NO_3)_3 .9H_2O$

Lead: $Pb(NO_3)_2$

Copper: $CuCl_2.2H_2O$ (Copper chloride was used simply because the nitrate salt was not available and provision was made to ensure its solubility in water).

2.3 Enrichment Medium

Voermolas is a thick dark brown liquid obtained from the refining of sugar cane and it is used in the production of alcohol and for feeding livestock. A detailed composition of voermolas is given in Table 2.1 (supplier, Tongaat Foods[1]) and the mean constituents are calcium, phosphorous and proteins. Furthermore, according to Phillips (2003) the C: N ratio was determined to be 31:1 using the Walkey Black oxidation procedure, hence; it could be an excellent source of carbon and energy for microorganisms. In addition to this, voermolas did not precipitate the metals used in this investigation in an acidic environment, (pH 4.00) which is a common problem in many complex media, including half strength nutrient broth **(Section 3.3.4)**. Therefore, 0.1% (v/v) voermolas was used as growth media in all the experiments to provide carbon and energy for the microorganisms.

[1] Tongaat Foods, (Pty) Ltd, P.O. Box 13, Maidstone, 4380. KZN, South Africa

Table 2.1 Chemical compositions of voermolas

Ingredient	Quantity (g kg^{-1})
Protein	56.50
Calcium	9.20
Phosphorous	1.10
Water	300.00

2.4 Synthetic Effluent

A synthetic effluent was prepared with separate aliquots from concentrated stock solution of each of the three metal ions to give a final concentration of 200 mg l^{-1}. Voermolas 0.1% (v/v) was fed into the reactors separately to supply carbon and energy to the developing biofilms. The pH of the synthetic effluent was adjusted to pH 4.00, in which the metal ions were kept in soluble form **(Section 3.3.2)**.

2.5 Complexing Agent

A water-soluble humic acid was used as a complexing agent to reduce the high concentration of metal ions in the effluent prior to its introduction into the RBC bioreactor compartments **(Chapter 5)**. The humic acid used in this study was a natural product derived from coal, which contained 26% wt/v humate as potassium salt. This product was supplied as a solution by Omnia Specialities[2] and the physical and chemical properties of the humic acid are indicated in Table 2.2. However, no detailed analysis of the product was made, as it was not important to the investigation.

[2] Omnia Specialities (Pty) Ltd, P.O.Box 69888, Brynston, South Africa

Table 2.2 Typical analysis of humic acid

Ingredients	Concentration (wt/v)
Total water-soluble humate	26.00 %
Total potassium	5.10 %
Total nitrogen	0.03 %
Total phosphorus	<0.10 %
Total calcium	0.02 %
Total magnesium	<0.10 %
Total sulphur	0.10 %
pH	11.1

2.6 Bioreactor Design

2.6.1 Rotating Biological Contactor Configuration

A rotating biological contactor **(RBC)**, consisting of three units connected in series, was operated as a single and/or multiple-stage reactor(s). The dimensions of the bioreactor compartments are given below in Table 2.3. Each bioreactor compartment comprises 6 plastic support discs to which gravel, polyester batting and sand (Chapter three), and polyester batting during subsequent experiments were attached to serve as potential a matrices for the development of the biofilms. Approximately 40% of the disc area was submerged in the medium and each bioreactor was supplied with inflow and outflow pipes to ensure that the volume of solution did not exceed this level.

Each bioreactor compartment had a volume of approximately 1300 ml. The rotation speed of the discs was maintained at 10 rpm because this speed was found to be favourable for the development of the biofilms while provided enough turbulence to keep the metals ions in suspension, and thus in contact with the immobilized biomass (Costley and Wallis, 1999).

Table 2.3 Dimensions of each compartment in a three-compartment rotating biological contactor

Bioreactor surface	Size/ area
Length of trough	20.0 cm
Width of trough	15.0 cm
Depth of trough	15.0 cm
Number of discs	6
Diameter of discs	12.5 cm
Thickness of discs	0.3 cm
Distance between discs	2.5 cm
Total area of discs	1473.2 cm^2
Submerged area of discs	589.2 cm^2

Figure 2.1 Side view of the three-compartment RBC bioreactor.

2.6.2 Flow Rate and Pump System

A Watson-Marlow pump was used for circulating the synthetic effluent through each bioreactor compartment separately at a flow rate of 0.9 ml min^{-1}. This flow rate was chosen since Costley and Wallis (2000) found that the system operated efficiently for the removal of metal ions, at a hydraulic retention time of 24 hours. Silicon tubing was used to connect the RBC units and the pump.

2.7 Metal Removal Analysis

2.7.1 Atomic Absorption Spectroscopy (AAS)

Atomic absorption spectroscopy (AAS) is currently perhaps the most widely used method and analytically convenient technique for trace metal analysis. It is particularly applicable where the samples are in solution or readily solubilized (Ebdon, 1982). The AAS consists of a hollow cathode lamp lined with the metal of interest and is used as a source of the discharged light beams (Ebdon, 1982). Liquid samples were injected directly into the instrument where they were converted to a vapour in the nebulizer, and then the ground state atoms (vaporized atoms) were excited by the burning flame. As the light beams from the hollow cathode lamp were directed through the flame into a monochromator, the detector measured the amount of the light absorbed by the atomised element in the flame. Each metal was analysed at its own characteristic absorption wavelength since the amount of energy at this wavelength absorbed by the flame is proportional to the concentration of the element in the sample.

Standard solutions (10, 50 and 100 mg l^{-1}) were carefully prepared from known stock standards of each metal ion and a calibration curve was obtained with precision of less than or equal to 1%. Beveridge *et al.* (1997) found that a precision of 1% is usually obtained if the standard solutions are prepared carefully. The instrument response was periodically checked after 50 samples to determine the accuracy of the standard curves. The following wavelengths were used for the metal ions under investigation: chromium 429.00 nm, copper 218.2 nm and lead 283.3 nm. To avoid any chemical interference encountered with an air-acetylene flame, a nitrous oxide-acetylene flame was used for the determination of the elements copper and chromium (Ebdon, 1982). In each case, a slot burner was used with a width of 100 mm for air-acetylene and 50 mm for nitrous oxide-acetylene, to increase the path length and to enable a specific portion of the flame to be viewed.

Prior to analysis samples were taken from each reactor and filtered through a nitrocellulose filter membranes (pore size 0.2 μm, 25 mm diameter) to remove any microorganism and colloidal particles present in the solution, and stored at 4 ^{0}C in sterile McCartney bottles.

The samples (2.5 ml) were diluted at least three fold with distilled water to be within the range of the standard calibration curve and the residual metal ion concentrations were quantified. The difference between the initial metal concentration and the residual metal concentration at each sampling time was taken to be the amount of metals accumulated on the surface of the biofilms over the intervals. Using these figures the percentage removal of the metals by the biofilms was determined.

Even though the AAS has a limitation of analysing only one metal element at a time, the fact that the system was equipped with an auto-sampler, and all the parameters were easily manageable using a computer, made AAS the preferred technique and all samples were thus analysed with this user-friendly instrument.

2.7.2 Statistical Analysis

Sample analyses were carried out in duplicate, unless otherwise stated. Mean values were computed and the general analysis of variance was performed using GeneStat 5 release 4.2 at a 5% probability level.

2.8 Environmental Scanning Electron Microscope (ESEM)

The environmental scanning electron microscope **(ESEM)** is one of the most important instruments used for viewing microbial cells. The ESEM is a versatile instrument in that: it can be operated at either low or high vacuum mode. As to Critical Point Drying **(CPD)** procedure, biofilm samples were fixed overnight in 3% glutaraldehyde in cacodylate buffer (pH 6.8), washed twice with 0.05 M cacodylate buffer (pH 7.02) for 30 min. The samples were then dehydrated in a cold ethanol series 10 minutes each in 30, 50, 70, 80, 90%, and 3 x 10 minutes in 100% ethanol. Samples were transferred into CPD baskets and dried in a Hitachi HCP-2 Critical Point Drier for 2 hours; sputter coated with a gold-palladium layer, and finally viewed using the ESEM at high vacuum mode and an accelerating voltage of 15 kV.

2.9 Energy Dispersive X-ray Microanalysis (EDX)

The electron beam/specimen interaction in the ESEM produces an X-ray signal. Using an X-ray spectrometer attached to the microscope, these signals can be collected to provide information on the elemental composition of the specimen. Spectrometry that relies on collecting energy of the X-ray signal emitted is called Energy Dispersive X-ray Microanalysis **(EDX)**. EDX equipped ESEM's are common in biological laboratories since EDX can detect a wide range of elements in a single sampling. The method used in EDX for quantification of the data from the samples was standardless.

For EDX analysis, wet biofilm specimens were air-dried and mounted on carbon stubs using carbon-based adhesives. The metal ions were then analysed in low vacuum mode by adjusting the working distance at 10 mm to obtain the most accurate results. Each element was then detected by its significant spectral peak and weight percentages were also quantified.

CHAPTER THREE

ATTACHMENT OF MICROOGANISMS TO VARIOUS SUPPORT MATRICES AND SUBSEQUENT METAL REMOVAL ABILITY OF THE BIOFILMS

3.1 Introduction

A large proportion of aquatic microbial populations are found attached to submerged solid surfaces (Fletcher, 1979). The attachment process requires a complex interaction between the microorganisms, the solid substratum and the liquid phase (McEldowney and Fletcher, 1986). Any variation in environmental conditions could affect the attachment of microbial population to a substratum (McEldowney and Fletcher, 1986). Thus, in assessing the ability of microorganisms to adhere to various attachment matrices and establish mature biofilms, the environmental growth conditions such as pH, temperature and medium composition should be kept constantly throughout the experiment.

Microorganisms, which adhere to various surfaces form biofilms. According to Kalmokoff *et al.* (2001) biofilm formation comprises two phases; the first step is reversible, where the microbes can be removed from the support surface by gentle washing. This is followed by the second stage in which the cells become irreversibly attached to the surface through the production of extracellular polysaccharides and form stable, mature biofilms. These sessile microorganisms have advantages in both growth and survival compared to their planktonic forms (Vatanyoopaisarn, 2000). Hence biofilms are found to be responsible for most of the microbial activities in natural and biotechnological situations, and have been found to be beneficial for wastewater treatment processes (Mueller *et al.*, 1992).

The capacity of the microbial cells to adsorb to the inert surfaces found in aquatic environments is well known. However, is has been noted that there are differences in both the extent and the rate of adsorption, depending on the surface selected (Blackman and Frank, 1996). That is, microbial attachments may depend on the surface characteristics such as roughness and porosity and/or physio-chemical interactions between the surface and the microorganisms (Murray and Van Der Berg, 1981). Accordingly, uneven surfaces (gravel

and sand) and porous material (polyester batting) were investigated to determine which support material offered the best surface for the development of microbial biofilms capable of removing heavy metals from a synthetic effluent.

3.2 Experimental Procedure

3.2.1 First Stage Enrichments

Liquid medium: 785 ml of 0.1% (v/v) voermolas was inoculated with 200 ml activated sludge obtained from Hammarsdale sewage works in 3 *l* Erlenmeyer flasks to which aliquots of the three metal salts: Cr $(NO_3)_3.9H_2O$, Pb $(NO_3)_2$ and Cu $Cl_2.2H_2O$ were added from the stock solution **(Appendix 1)** to give a final concentration of 50 mg l^{-1} for each metal ion. The flasks were aerated using fish tank air pumps. Air stones were attached to silicon tubing leading from the pumps to produce small bubbles with a large surface area for improved aeration. The flasks were kept at room temperature (18-28 ^{0}C).

This culture was used to start a series of enrichments conducted in the same manner, using 0.1% (v/v) voermolas spiked with increasing heavy metal ion concentrations of 100 and 150 mg l^{-1}. In this way a microbial population adapted to the metals under investigation was obtained.

3.2.2 Second Stage Enrichments

Second stage enrichments were also set up in 3 *l* Erlenmeyer flasks with 200 ml inoculum obtained from the first stage enrichments added to culture medium spiked with metal salts to obtain a final metal ion concentration of 200 mg l^{-1} for each metal. The volume was adjusted to 1000 ml with 0.1% (v/v) voermolas and the flasks were aerated using fish tank air pumps with attached air stones and kept at room temperature.

These microbial cultures adapted to the metal ions under investigation, were used as a source of inoculum for the bioreactor compartments. Weekly a fixed volume of the culture medium was removed from the flasks and replaced with an equal amount of fresh

0.1% (v/v) voermolas spiked with 200 mg l^{-1} of each of the three metals and to provide the microbial culture with adequate carbon and energy source.

The nature and composition of the inoculum was investigated using the biofilm samples removed from the air stones and subjected to ESEM analysis after fixation as to CPD procedure **(Section 2.8)** and the micrographs of the biofilms at various times were compared.

3.2.3 pH Profile Studies on Solubility of Metal Ions

3.2.3.1 Effect of pH on single-metal ions

A set of 13 Erlenmeyer flasks containing 0.1% (v/v) voermolas was prepared for each metal under investigation. Each flask within a set was spiked with a single aliquot of the specific metal ions to give a final concentration of 200 mg l^{-1} in a final volume of 50 ml. In each set the pH of the flasks was adjusted to 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 using either 1M HCl or 1M NaOH to determine the pH effect on solubility of the respective metal ions. The flasks were covered with Parafilm and after 24 h the contents of each were decanted separately into clean-centrifuged tubes and centrifuged at 3030 x g for ten minutes to remove colloidal particles that could clog up the AAS nebulizer. Metal analysis was performed on the supernatants using flame atomic absorption spectroscopy **(Section 2.7.1)**. The experiment was carried out in triplicate.

3.2.3.2 Effect of pH on mixed-metal ions

Two litres of stock solution of 0.1% (v/v) voermolas containing 200 mg l^{-1} of each of the three metal ions, Cr^{+3}, Pb^{+2} and Cu^{+2} were prepared in a 3 *l* Erlenmeyer flask. Thirty-nine 100 ml Erlenmeyer flasks were filled with 50 ml of the stock solution and the pH of triplicate flask was adjusted to pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with either 1M HCl or 1M NaOH. The Experiment was conducted under sterile conditions to avoid microbial contamination and all the flasks were covered with Parafilm to prevent evaporative lost. At each respective pH, 10 ml aliquots were removed from each of the three flasks and placed in clean tubes and centrifuged at 3030 x g for ten minutes.

The supernatants were transferred to clean McCartney bottles and metal analysis was performed by flame atomic absorption spectroscopy **(Section 2.7.1)**.

3.2.4 Metal Adsorptive Capacity of the Support Matrices

A flask experiment was carried out to determine if the three attachment matrices, viz. gravel, polyester batting and sand could adsorb the metals under investigation. Prior to placement into the metal solution, gravel and sand were pre-treated repeatedly with 2M HCl to remove any adsorbed charged ions that could interfere with the metals of interest. After thorough washing with deionised distilled water, the three support matrices were immersed separately in 50 ml Erlenmeyer flasks containing 200 mg l^{-1} of each of the three metals. The flasks were sealed with Parafilm to prevent evaporation and placed on a shaker. After 24 hours the support materials were analysed by EDX **(Section 2.9)** and liquid samples were taken for AAS analysis **(Section 2.7.1)** to determine the metal adsorption capacity of the support matrices.

3.2.5 Effect of Medium Composition on Dissolved Metal Ion Concentrations

An experiment was conducted to determine the effect of medium composition on metal ion concentration. Aliquots of each of the Cr^{+3}, Pb^{+2} and Cu^{+2} from the concentrated stock solutions **(Appendix 1)** were added to flasks containing either distilled water, half-strength nutrient broth, or 0.1% (v/v) voermolas to give a final metal ion concentration of 200 mg l^{-1} of each metal ion in a final volume of 100 ml. The pH of the medium was adjusted to pH 4.00 and the flasks were covered with Parafilm to prevent evaporative lost and after 24 hours; samples (2.5 ml) were taken from each flask. All the samples were diluted with distilled water to fall within the standard calibration range and analysed using AAS as in Section 2.7.1. The experiment was performed in triplicate and mean values were computed.

3.2.6 Biofilm Development in the RBC and Determination of the Metal Removal Capacity of the Biofilms

A laboratory-scale RBC was operated as described in Section 2.6.1 with gravel, polyester batting and sand as support matrices. Each support matrix was tested in a separate RBC bioreactor compartment and was attached to the discs using silicone glue. The effects of the support matrices on biofilm formation and subsequent metal uptake ability of the biofilms were determined.

Each bioreactor compartment was inoculated with 260 ml of the second stage enrichment culture and the working volume was made up to 1300 ml with synthetic effluent containing 200 mg l^{-1} of each of the three metal ions. For one week the bioreactors were operated as a batch culture to prevent the microbial cells from washing out of the system. Once the biofilms had started to develop the synthetic effluent was pumped into the reactors in a fed-batch mode. During the period of the biofilm development the minimum and maximum temperature of the laboratory was monitored daily and the pH and temperature of the effluent solution were measured weekly. Biofilms attached to the matrices under investigation were collected from the discs and analysed using ESEM.

After the biofilms were fully developed the bioreactor compartments were drained and washed with deionised distilled water for 24 hours to remove any metals adsorbed by the biofilms during their establishment. Fresh synthetic effluent was prepared as described in **section 2.4**, and pumped into each bioreactor compartment separate via silicon tubing. The whole system was set in recycling mode and the metal adsorption ability of the biofilms was determined. Liquid samples (2.5 ml) were taken before adding the effluent to the reactors to determine the initial metal concentration of the synthetic effluent and then after intervals of: 30 min, 1, 3, 6, 12 and 24 hours and thereafter at two day intervals for a period of two months. The samples were filtered using nitrocellulose filter membranes and stored at 4 ^{0}C in McCartney bottles until analysed by flame atomic absorption spectroscopy **(Section 2.7.1)**. Biofilm samples were taken after seven and 14 days exposure to the metal

ions and prepared for ESEM and EDX analysis as described in **sections 2.8** and **2.9** respectively.

3.3 Results and Discussion

3.3.1 Initial Enrichments for a Microbial Population Tolerant of Mixed Metal Ions

3.3.1.1 First stage enrichments

Many of the previous investigators have used half-strength nutrient broth for the establishment of biofilms and determination of the ability of the biofilms to remove metal ions from solution. However, half-strength nutrient broth was found to precipitate metals such as lead in an acidic medium where the pH is not favourable for the growth of most microbial cells **(Section 3.3.4)**.

Voermolas as a 0.1 % (v/v) aqueous solution was used in the preparation of a synthetic effluent, primarily to solve the problem of complexation of lead ions with organic compounds present in nutrient broth and, secondly, to provide the microbial cells with both a carbon and energy source. Accordingly the microorganisms, which had been previously exposed to metal ions, were able to grow in the presence of Cr^{+3}, Pb^{+2} and Cu^{+2}. The presence of such metal ions provides the necessary selection pressure for the isolation of metal resistant microbial cultures obtained initially from industrial wastewaters.

3.3.1.2 Second stage enrichments

Environmental scanning electron microscopy (ESEM) analyses of the biofilms taken from the surface of the air-stones revealed a variety of fungal mycelia, fungal spores and rod-and coccus-shaped bacterial cells **(Plate 3.1)**. The presence of a diverse microbial population in the enrichment medium could be an advantage for the accumulation of heavy metals from wastewaters, because a variety of microorganisms have been found capable of removing heavy metals from solution (Gadd, 1992). Furthermore, it was found that microbial associations are better able to withstand high metal ion concentrations than are pure or mono-cultures (Gadd, 1988).

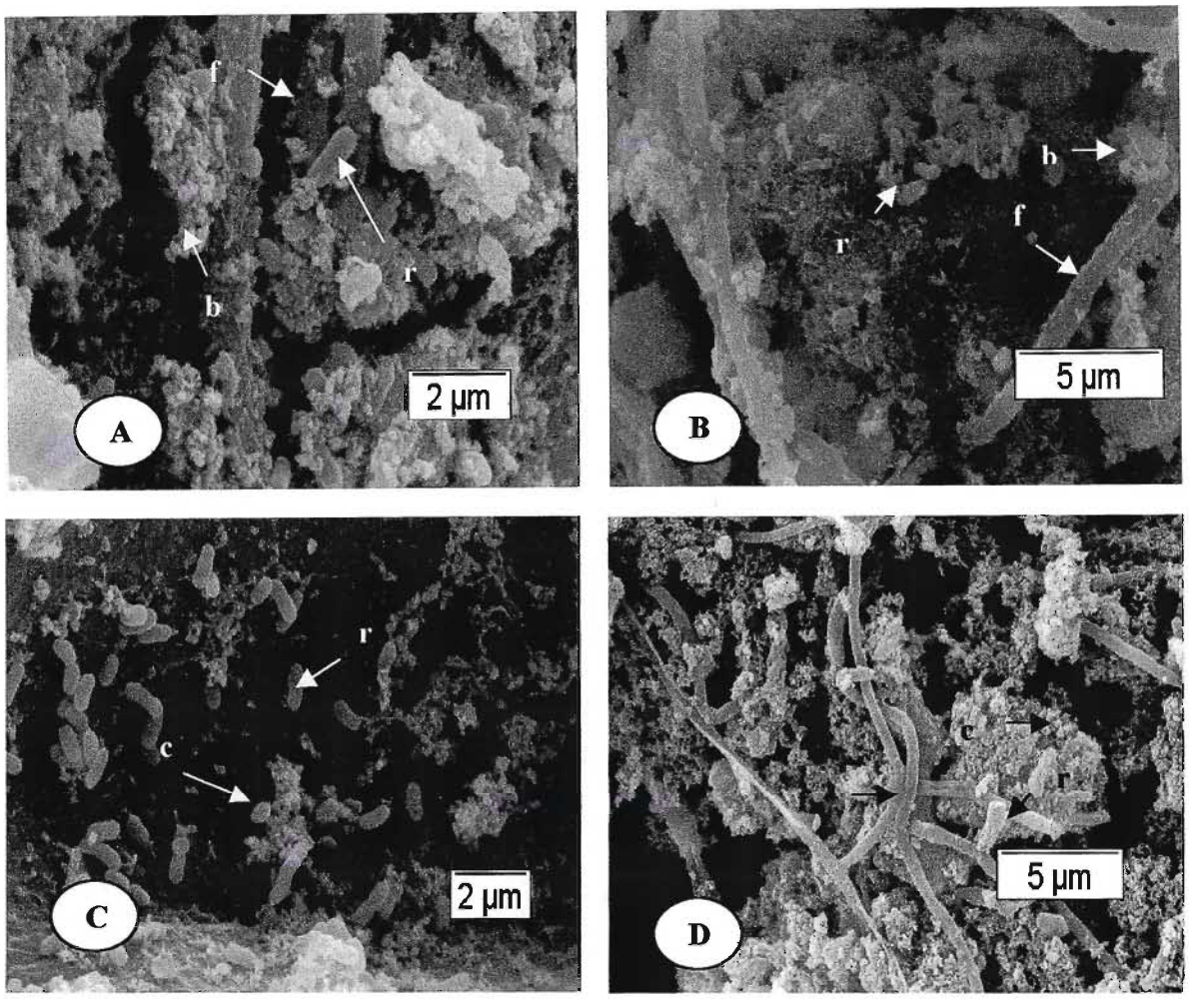


Plate 3.1 Scanning electron micrographs of the biofilm growing on the air stones in the second stage enrichment cultures in a mixed metal solution. A and B, showing filamentous organisms (f), clusters of bacterial cells (b) and some rod-shaped (r) bacteria; C, cocci (c) and rod-shaped (r) bacterial cells; and D, clusters of coccus-shaped bacteria (c), some rods (r) and filamentous organisms (f).

3.3.2 pH Profile Studies on Metal Ion Solubilities

3.3.2.1 Effect of pH on solubility of a single-metal ion species

In the experiment conducted to determine the effect of pH on the solubility of single metal ion species it was found that pH plays an important role in their precipitation. Table 3.1 shows the AAS results for the pH studies of chromium, lead and copper when present as sole metals in solution. The results indicated that as the pH increased from 2.0 to 8.0 the solubility decreased, hence, the metals precipitated as insoluble hydroxides. Gadd and Griffiths (1978) found that different metal ions have different pH ranges where they can be precipitated as insoluble hydroxides. Visual assessment and AAS analyses revealed that both chromium and copper precipitated between pH 5.5 and 6.0 whereas lead precipitated between pH 4.0 and 4.5. In single metal experiments a slight loss of concentration of both Cr^{+3} and Pb^{+2} occurred at lower pH values. However, as the experiment was conducted with mixed metals this had no significant effect on the results of any subsequent studies.

Table 3.1 Effect of pH on solubility of single metal ion species

pH	Mean metal ion concentration (mg l^{-1}) in solutions		
	Cr	Pb	Cu
2.0	169.92	144.44	200.22
2.5	158.82	140.07	200.18
3.0	158.84	132.32	200.64
3.5	155.83	128.13	197.83
4.0	155.16	124.07	197.15
4.5	155.01	105.69	196.45
5.0	153.84	83.85	197.01
5.5	143.73	68.47	186.26
6.0	13.83	57.08	134.59
6.5	4.32	41.91	48.83
7.0	1.65	10.52	7.44
7.5	0.00	1.63	2.90
8.0	0.00	0.00	2.20

3.3.2.2 Effect of pH on solubility of mixed-metal ion species

It is apparent from Figure 3.1 that metal ion solubility is a function of pH. As the hydrogen ion concentration decreased as the solution become more alkaline, so too did the solubility of the metal ions. Lead remained soluble at higher pH in the presence of other metals than it did when it was the sole element present. Hence, instead of precipitating between pH 4.0 to 4.5, the Pb^{+2} ions remained soluble in the presence of other metals at pH values between pH 5.5 to 6.0. The solubility of chromium on the other hand, did not show significant change in solubility; hence, it precipitated between pH 5.5 to 6.0 as insoluble hydroxide in the presence of other metal ions.

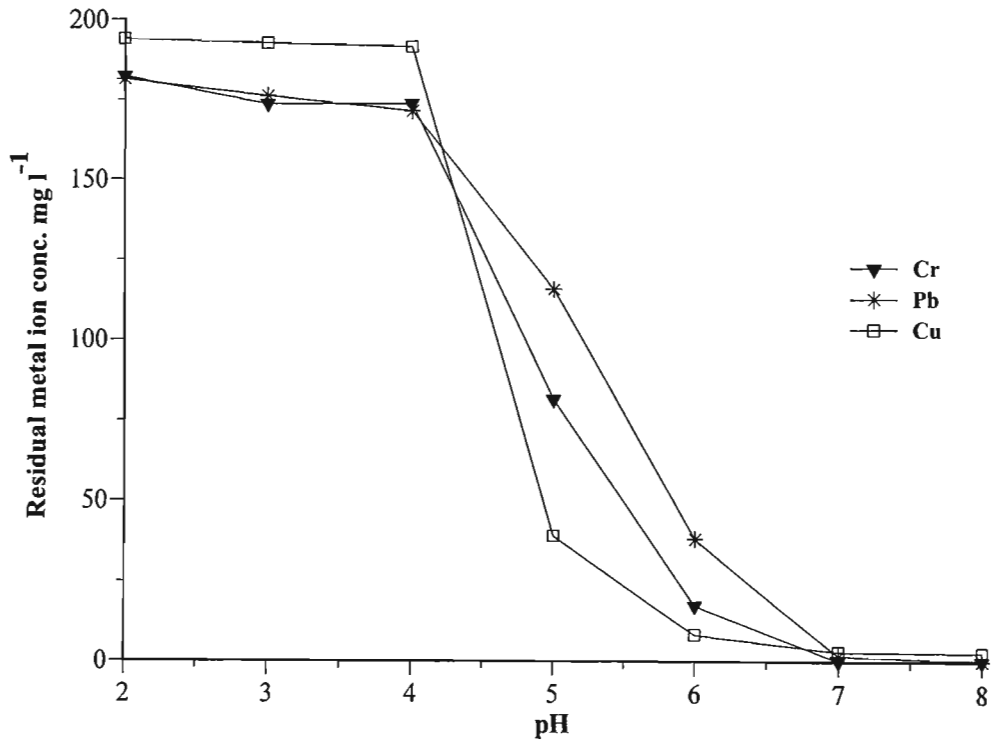


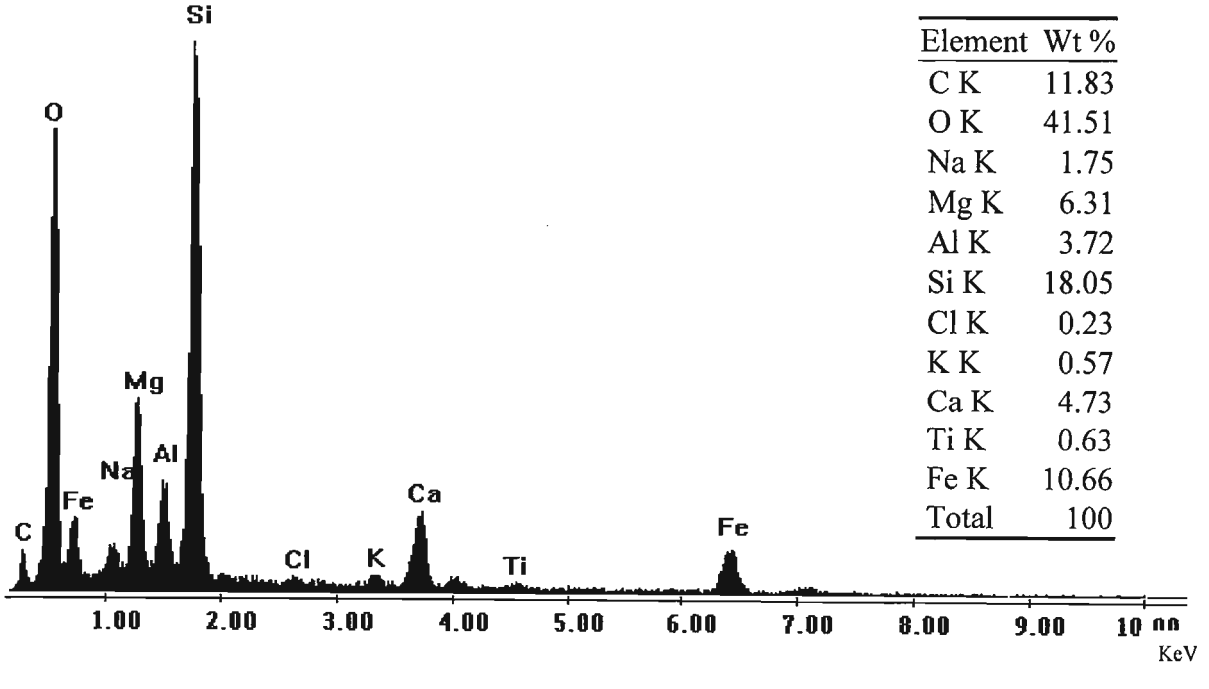
Figure 3.1 Solubility of mixed metal ions as a function of pH.

Copper on the other hand, which remained soluble over a wide range of pH in the absence of other metals, showed radical change in concentration and started to precipitate between pH 4.0-4.5 when mixed with other metal ions, hence, the solubility decreased in the presence of other metal ions. The formation of insoluble heavy metal precipitates is one of many factors limiting the bioavailability of heavy metals in many aquatic ecosystems (Gadd and Griffiths, 1978). Thus it is apparent that the bioavailability and hence the

toxicity of copper can be reduced in the presence of other metals because the presence of both lead and chromium ions seem to cause precipitation of copper ions. In the same way, Olson and Panigrahi (1991) in an experiment with *Klebsiella aerogenes* were found that the presence of cadmium chromate reduced the toxic effect of copper. In general, metals exist as free ionic cations at an acid pH but at an alkaline pH the ionic cations precipitate as insoluble hydroxides or oxides (Gadd and Griffiths, 1978). Throughout the experiment the pH of the synthetic effluent was adjusted as close as possible to pH 4.0 to avoid the precipitation of metal ions.

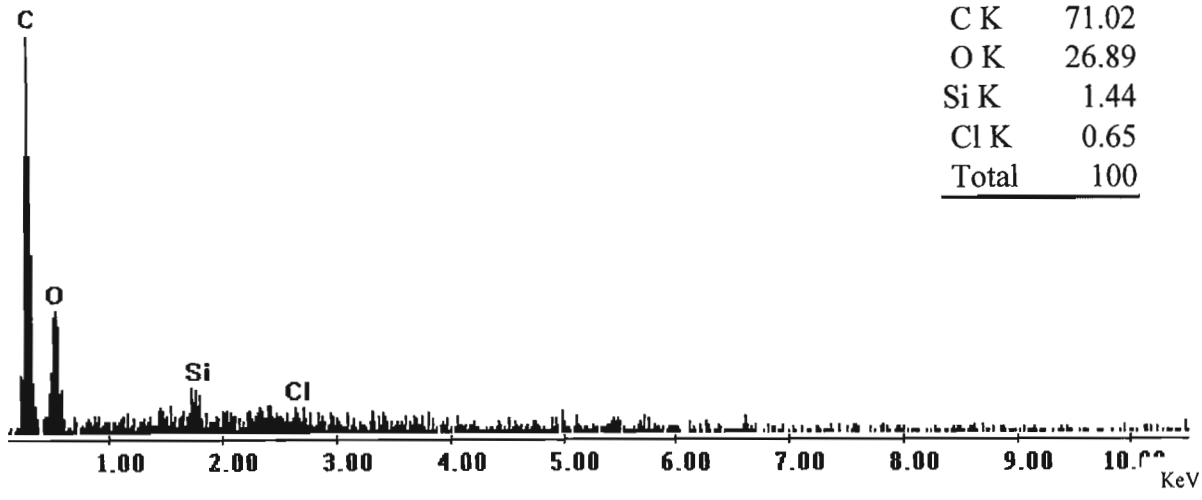
3.3.3 Metal Adsorptive Capacity of Support Matrices

With respect to the propensity of the different attachment matrices to adsorb metal ions from solution, EDX analysis (Figure 3.2) showed that none of the support matrices used in this investigation adsorbed metal ions on their surfaces. This was also verified by AAS analysis of liquid samples (data not shown) which revealed no significant change in the metal ion concentrations with time had occurred. Thus the three attachment matrices were used for biofilm development in the RBC and for subsequent metal removal studies.



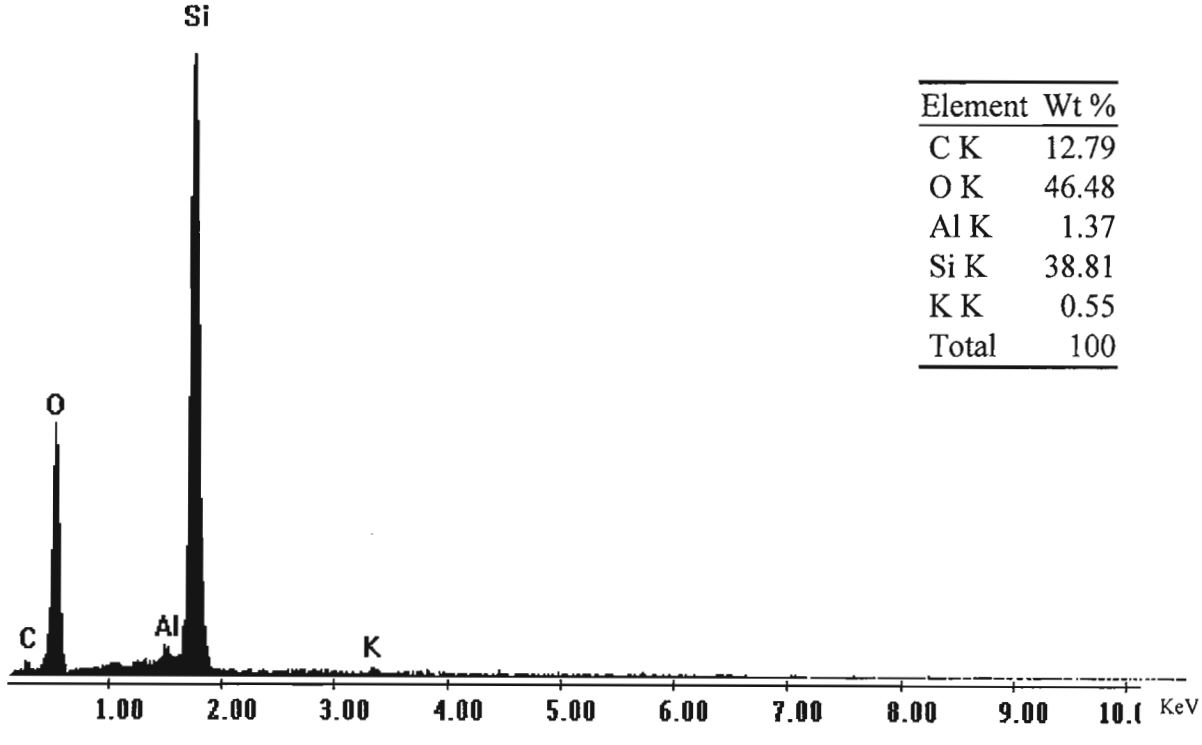
Element	Wt %
C K	11.83
O K	41.51
Na K	1.75
Mg K	6.31
Al K	3.72
Si K	18.05
Cl K	0.23
K K	0.57
Ca K	4.73
Ti K	0.63
Fe K	10.66
Total	100

Figure 3.2 EDX quantification of the elements occurring in the gravel used as a support matrix.



Element	Wt %
C K	71.02
O K	26.89
Si K	1.44
Cl K	0.65
Total	100

Figure 3.3 EDX quantification of the elements occurring in the polyester batting used as a support matrix.



Element	Wt %
C K	12.79
O K	46.48
Al K	1.37
Si K	38.81
K K	0.55
Total	100

Figure 3.4 EDX quantification of the elements occurring in the sand used as a support matrix.

3.3.4 The Effect of Medium Composition on Availability of Metal Ions

In a study with an *Alcaligenes faecalis* culture, Prahalad and Seenayya (1988) found that the strength of the nutrient broth used influenced the availability of metal ions to the microorganisms and suggested that the effect was due to the metal complexing with the compounds in the growth medium. Hence, when formulating the synthetic effluent used in this investigation the effect of distilled water, 0.1% (v/v) voermolas, and half-strength nutrient broth on the behaviours of metal ions in solution was investigated. The pH of the medium was adjusted to pH 4.0 in each case to standardise the environmental conditions under which all media were tested and then the residual metal ion concentrations were determined by AAS.

Table 3.2 Residual metal ion concentrations in the different media after mixing for 24 hours

Metal spiked medium	Metal ion concentration (mg l^{-1})		
	Cr	Pb	Cu
Distilled water	175.98	177.89	189.85
0.1 % (v/v) voermolas	177.24	177.56	191.84
Half-strength nutrient broth	166.89	29.91	201.02

As indicated in Table 3.2 there was no significant difference between the residual concentrations of the three metal ions in distilled water and 0.1% (v/v) voermolas. However, half-strength nutrient broth was caused precipitation of the Pb^{+2} ions as insoluble oxides thereby causing a significant reduction in its availability in the medium. Because of this complexation with lead ions, half-strength nutrient broth was not used in the preparation of the synthetic effluent. To permit the evaluation of biofilms for removal of heavy metals from industrial waste streams, a chemically defined medium that complexes minimally with metals is required (Hsieh *et al.*, 1985). Brynhildsen *et al.* (1988) found that the nutritional state of an organism could influence its sensitivity to metals; particularly those metals taken up by an energy-dependent transport system may accumulate more rapidly in cells growing in energy source rich media. Accordingly, 0.1% (v/v) voermolas which minimally complexed with the metals while concomitantly providing the necessary

carbon and energy source for microbial cells, was used in formulating the synthetic effluent throughout this investigation.

3.3.5 Biofilm Development on the RBC Discs and Determination of the Metal Removal Ability of the Biofilms

3.3.5.1 The effect of attachment matrices on the development of biofilm

Of the three support matrices used polyester batting was most quickly colonized by the microorganisms during the biofilm formation process. Kolot (1988) suggested that porous support matrices are most suitable for microbial immobilization because they usually provide more surface area for microbial loading and the pores also protect the cells from turbulence in reactors operating at high flow rates. Thus, polyester batting, because of its porous nature, provided a larger surface area for microbial adhesion and subsequent biofilm formation. In addition, the biofilm was also firmly adhered to the irregular surface and was thus not easily sloughed off the RBC discs while rotating in the liquid medium. This findings correlated with the results obtained by McEldowney and Fletcher (1986), who found that hydrophilic surfaces produce firmer bacterial adhesion than hydrophobic surfaces, as the hydrophilic surfaces are strongly polar and have high-energy consequently, tend to absorb micro and macromolecules more strongly than low energy hydrophobic surfaces.

Surface roughness of the support matrix is also another property which increases the surface area for microbial attachment and offers the microorganisms protective from fluid dynamic shear forces (Verran *et al.*, 1991). After investigating the adsorption of *Pseudomonas aeruginosa* on various surfaces Mueller *et al.* (1992) ascertained that the rate of adsorption increased with increasing surface free energy and surface roughness of a support matrix. In the present study it was found that the initial stage of surface adhesion and subsequent microbial colonization were slightly better on gravel than on the sand particles. EDX spectral analysis **(Figure3.2)** revealed that the gravel comprised of various metallic and non metallic elements like silicon, iron, calcium, aluminium, magnesium and titanium on its surface and these predominant positively charged elements might attract the

negatively charged microbial cells resulting in more rapid adhesion and surface colonization. On the other hand the initial adhesion and colonization processes on the sand particles were very limited, possibly due to the presence of relatively large amount of silicon **(Figure 3.4)** on its surface. Silicon, one of the non-metallic elements is a major constituent of glass, and Hsieh *et al.* (1985) and Kolot (1988) found that the charges on glass surfaces are unknown and/or negatively charged and this limits the choice of sites for microbial attachment. From this it can be inferred that the initial surface adhesion and colonization processes on the sand support matrix was limited due to the abundance of silicon ions on the surface of the sand particles.

After two weeks the biofilms started to develop on the surface of the discs. However, the surface coverage was irregular with some areas showing no colonization. Since the biofilms were not fully formed most of the cells were damaged during CPD sample preparation and thus the ESEM electromicrographs were unclear **(Plate 3.2, A)**. After four weeks biofilms had colonized all three of the attachment matrices tested. Biofilm specimens were taken from the discs with minimal disruption and prepared for investigation in the ESEM to determine the morphology and nature of the component microorganisms. High magnification the biofilm samples **(Plate 3.2 B and C)** showed the presence of some rod- and coccus-shaped bacterial cells. However, since the pH of the synthetic effluent was maintained at pH 4.0 the fungal cells appeared to subsequently dominate the bacterial cells. After seven weeks thick contiguous biofilms had developed on all three of the attachment matrices tested. ESEM analysis **(Plate 3.2 D, E and F)** of the specimens removed at week 7 revealed that the biofilms were comprised of mainly filamentous organisms, including a preponderance of fungal mycelia, with some spore bearing structures. The thickness of the biofilms was roughly the same in all three reactors, ranging between 2-3 mm on both sides of the discs. In all three reactors the nature and type of microorganisms present were very similar; hence only some representative electron micrographs of the biofilms are presented here.

Plate 3.2 Scanning electron micrographs showing the development of the biofilms with time. A, week 2 initial colonization, biofilms still rudimentary; B and C, week 4; high magnification reveals the presence of rods (r) and cocci (c) on the surface of support matrices; D, week 6; some clusters of bacteria (b) but fungal mycelia (fm) predominant; E and F week 7; mature biofilms, comprising mainly fungal hyphae with some spores attached (fs), and actinomycetes (ac).

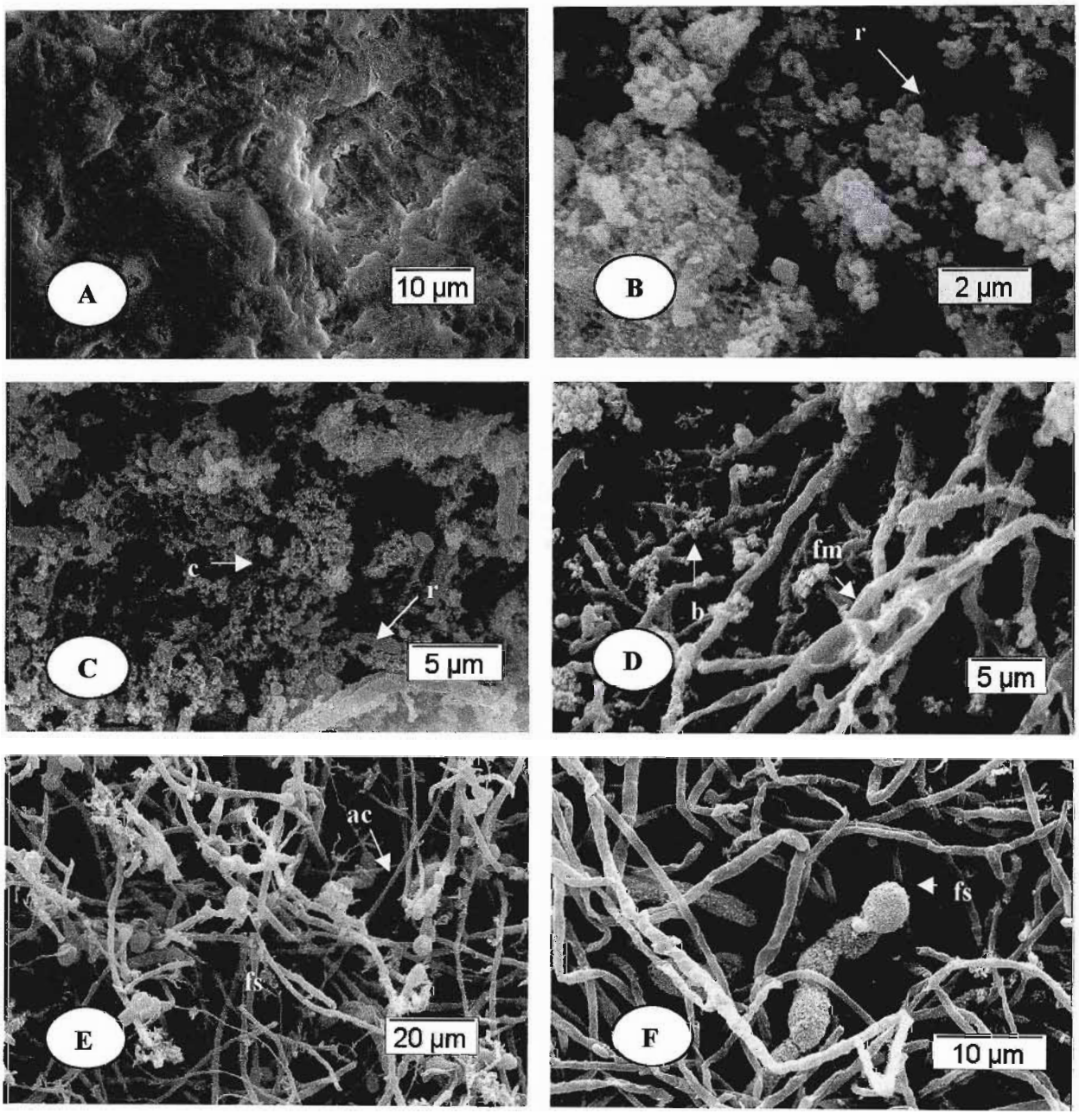
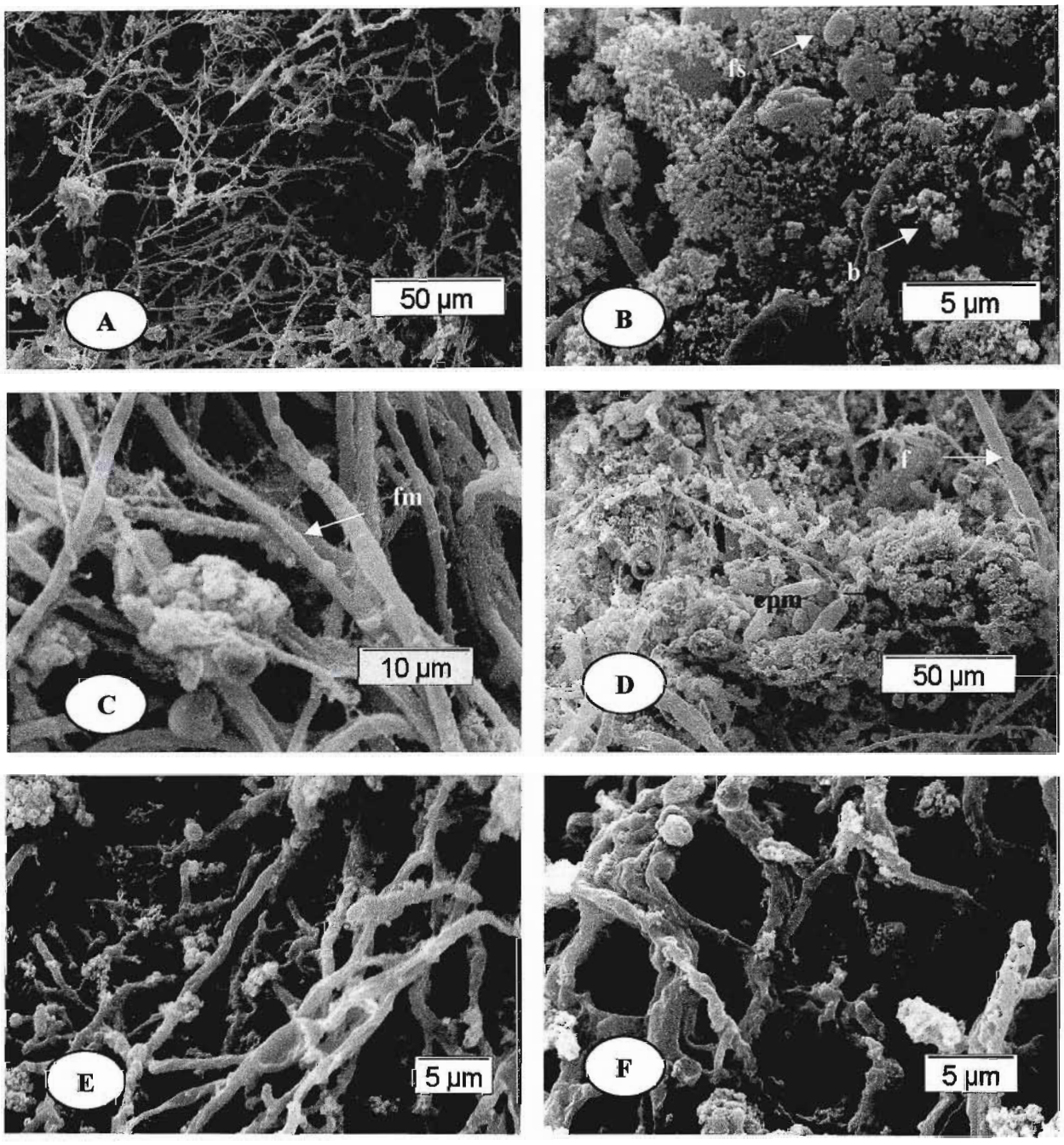

A
10 µm
B
r
2 µm
C
c
r
5 µm
D
fm
b
5 µm
E
ac
fs
20 µm
F
fs
10 µm

Plate 3.3 Scanning electron micrographs of biofilm samples removed at various times. A, low magnification of a biofilm removed after two weeks; B and C, high magnification of samples removed after four weeks, illustrating the presence of numerous filamentous organisms, clusters of bacterial cells (b) and fungal spores (fs); D, low magnification of samples removed at the end of the experiment (after 8 weeks) showing the presence of filamentous (f) organisms with some extracellular polysaccharide matrix (epm); E and F, show detail of the filamentous organisms at high magnification taken at the end of the experiment (week 8).


A
50 µm
B
fs
b
5 µm
C
fm
10 µm
D
f
epm
50 µm
E
5 µm
F
5 µm

3.3.5.2 Biological metal uptake by the system

After full establishment of the biofilms on the RBC discs, metal containing synthetic effluent was pumped to each bioreactor compartment separately at a flow rate of 0.9 ml min^{-1} **(Section 2.6.2)** and the metal removal ability of the biofilm in each compartment was determined. Atomic absorption spectroscopy readings of the samples indicate that the maximum metal removal rate occurred during the first 30 minutes of contact between the metal ions and the biofilms. During the subsequent 48 hours the biofilms continued to remove significant amounts of the metals from solution; thereafter, however, an increase in the residual metal concentration was exhibited in all three bioreactor compartments.

According to Gadd (1988) microbial metal accumulation is a two-phase process. During the first phase metabolism-independent accumulation or adsorption onto the cell wall and other external surfaces occurs. This is followed by the second, slower phase where metabolism-dependent transport across the cell membrane occurs. Hence, from Day 1 to Day 3 of the experiment the residual metal ion concentrations of all three metals declined sharply, which possibly could be due to rapid accumulation of the metal ion species onto the cell walls of the microbial population. However, after Day 3 the metal ion concentrations started increasing, which could be due to either saturation of the metal binding sites on the surface of the biofilms or evaporative loss of liquid from the system, thereby causing an increase in metal ion concentration of the synthetic effluent.

Since the system was operated in fed-batch mode and the synthetic effluent was circulated between the reservoirs and the RBC bioreactor compartments; the metal ion concentration of the influent solution was periodically measured and compared against the effluent solution. AAS results after one week (data not shown) revealed that the metal ion concentration of the influent solution exceeded the initial concentration in all the bioreactor compartments. The main cause of the increase in concentration was possibly the evaporative loss of liquid from the system, which caused the effluent to become more and more concentrated. To counteract this loss of liquid, diluted voermolas (0.1% v/v) was pumped into the reactors at very low flow rate (0.2 ml min^{-1}) to replenish the loss of water

from the system while concomitantly serving as a source of carbon and energy for the biofilms. Accordingly, the biofilms continued to remove heavy metals from the system over the entire sixty-day duration of the experiment **(Appendix 2)**.

Chromium Removal

In all three bioreactor compartments chromium was more efficiently removed than the other two metals by the biofilms. Initially the concentration of Cr^{+3} ions decline rapidly, after 4 days the residual concentration returned to its original value **(Appendix 2)**. However when voermolas was pumped into the bioreactor compartments, chromium was removed efficiently from the system over the sixty days period of the experiment.

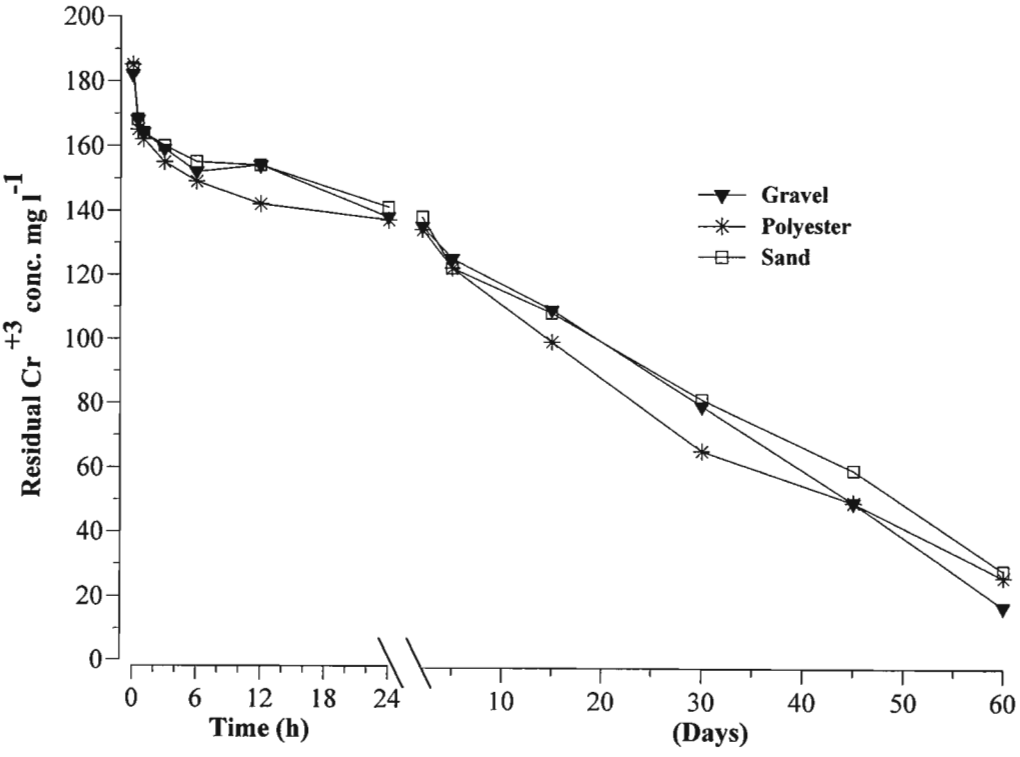


Figure 3.5 Residual Cr^{+3} concentrations in solution after various periods of metal uptake by biofilms developed on different surfaces.

On comparing the three-bioreactor compartments, the biofilms formed on polyester batting were seen to remove large amount of Cr^{+3} over a prolonged period of time. However, the rate of metal removal later decreased perhaps due to desorption of the metal ions from the biofilm. Similarly, the biofilms formed on the gravel and sand attachment matrices were removed appreciable amounts of chromium ions from the system. In fact towards the end of the experiment the biofilms formed on gravel were found to have adsorbed the highest amount of chromium ions. The overall level of chromium removal by the biofilms formed on gravel, polyester and sand was: 90%, 85% and 83%, respectively.

Lead Removal

As with chromium, lead showed a marked decrease in concentration until saturation of biofilms occurred after approximately 48 hours contact time, where an increase in residual lead concentration was observed. After addition of voermolas lead removal from the system increased. Figure 3.6 illustrates the removal of lead ions by biofilms growing on the three attachment matrices. The three reactor compartments displayed more or less similar lead removal rates with the gravel biofilms showing slightly higher removal than the polyester and sand attached biofilms. The percentage removal of lead ions by the system was computed and found to be 85 % by the biofilm growing on gravel and 78 % by those colonising both the polyester and sand surfaces.

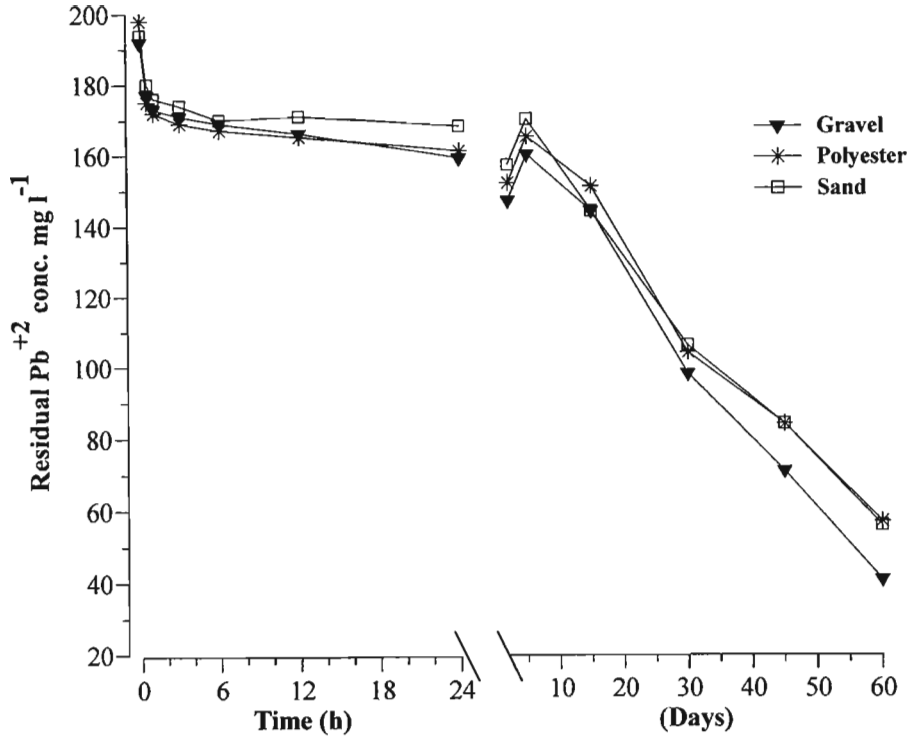


Figure 3.6 Residual Pb^{+2} concentrations in solution after various periods of metal uptake by biofilms developed on different surfaces.

Copper Removal

During the first two days, copper showed the same trend as chromium and lead, namely a rapid decline in residual concentration, followed by an increase in concentration. For the remainder of the experiment, however, copper uptake was quite small compared to that of the other two metals. This might be due to either the lower affinity of copper ions for the microbial surface or the presence of other metal ions might competitively suppress the uptake of copper from the effluent solution. Wong *et al.* (1993) found that the presence of high amount Pb^{+2} ions in a solution strongly inhibited the recovery capacity of microbial cells for Cu^{+2}, possibly due to its high electro-negativity [Pb^{+2}, 2.33 and Cu^{+2}, 1.90], so that Pb^{+2} shows a higher affinity towards the negatively charged microbial surface than does Cu^{+2}.

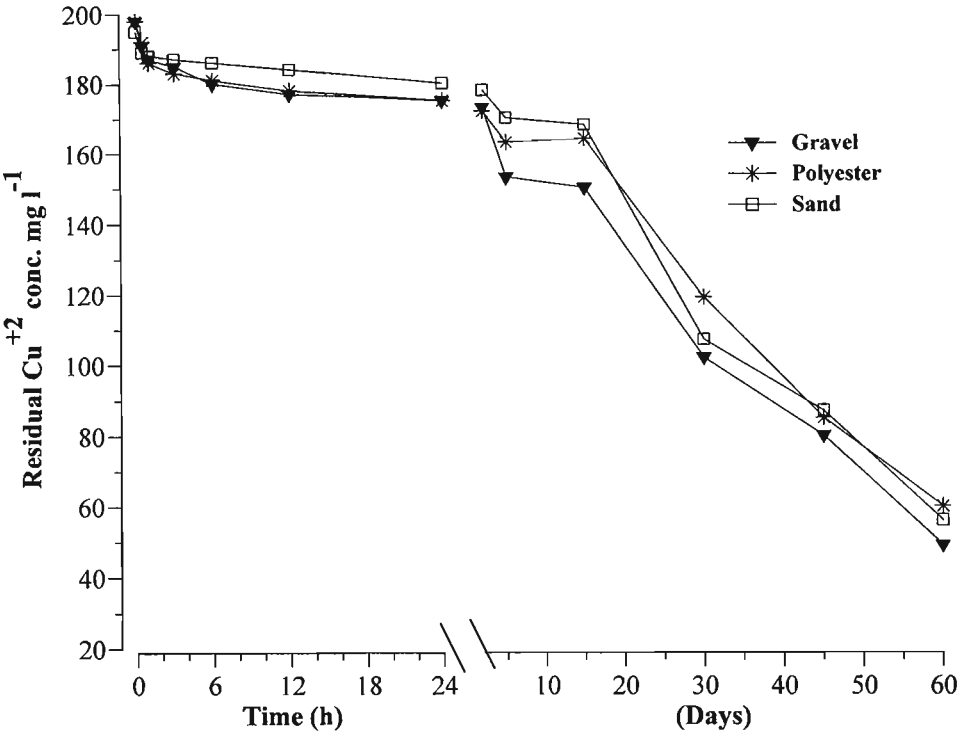


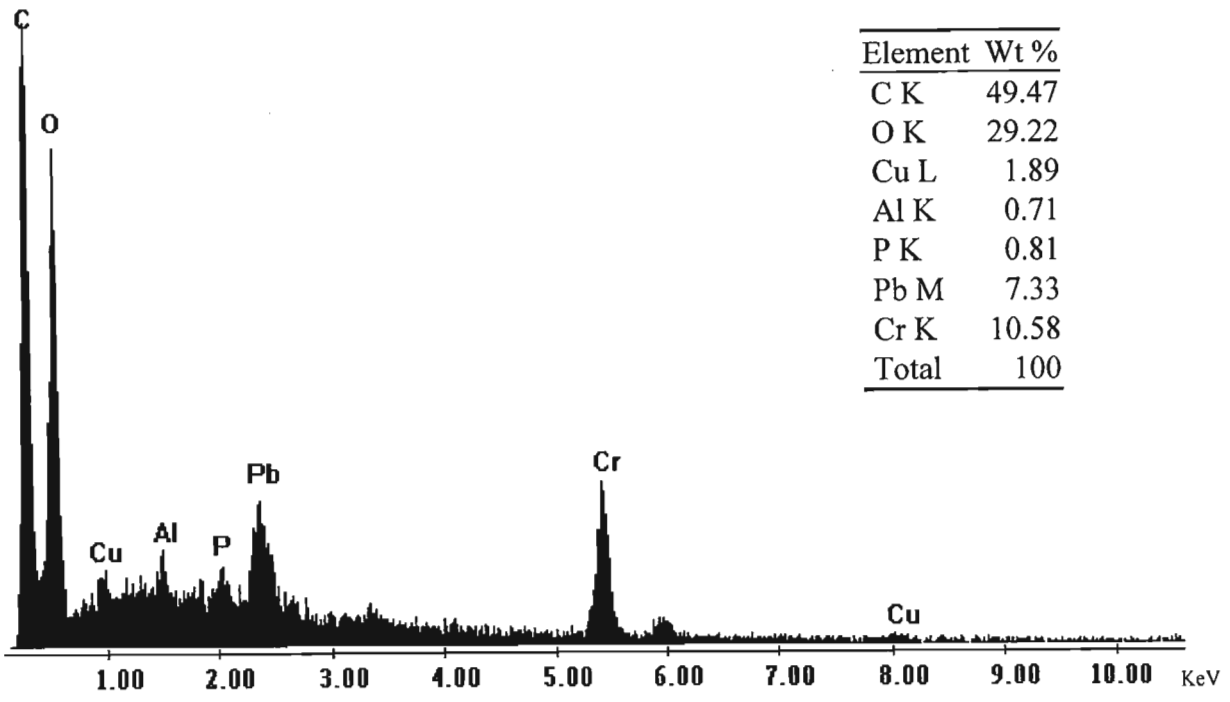
Figure 3.7 Residual Cu^{+2} concentrations in solution at various periods of metal uptake by biofilms developed on different surfaces.

Generally the AAS results indicated that, the biofilms grown on gravel removed about 77%, while those established on sand and polyester removed about 73% and 71% of the copper ions from the system, respectively. However, the EDX spectral analysis of the biofilms removed from all the support matrices showed that only trace amounts of copper ions were associated with the biofilms **(Figure 3.8-3.10)**. This possibly indicated that some of the copper ions were precipitated as insoluble hydroxides and thus became unavailable to the microorganisms.

As the exposure time increased the metal removal ability of the biofilms slowed down slightly over the period Day 20 till the termination of the experiment on Day 60. Furthermore, EDX spectral comparison of the metal ions accumulated by the biofilms at various period of time also illustrates this phenomenon clearly **(Figure 3.8-3.10)**. For example, the EDX analysis of the biofilms removed after two weeks showed the presence of fairly high concentration of Cr^{+3} and Pb^{+2} whereas only trace amount Cu^{+2} was present.

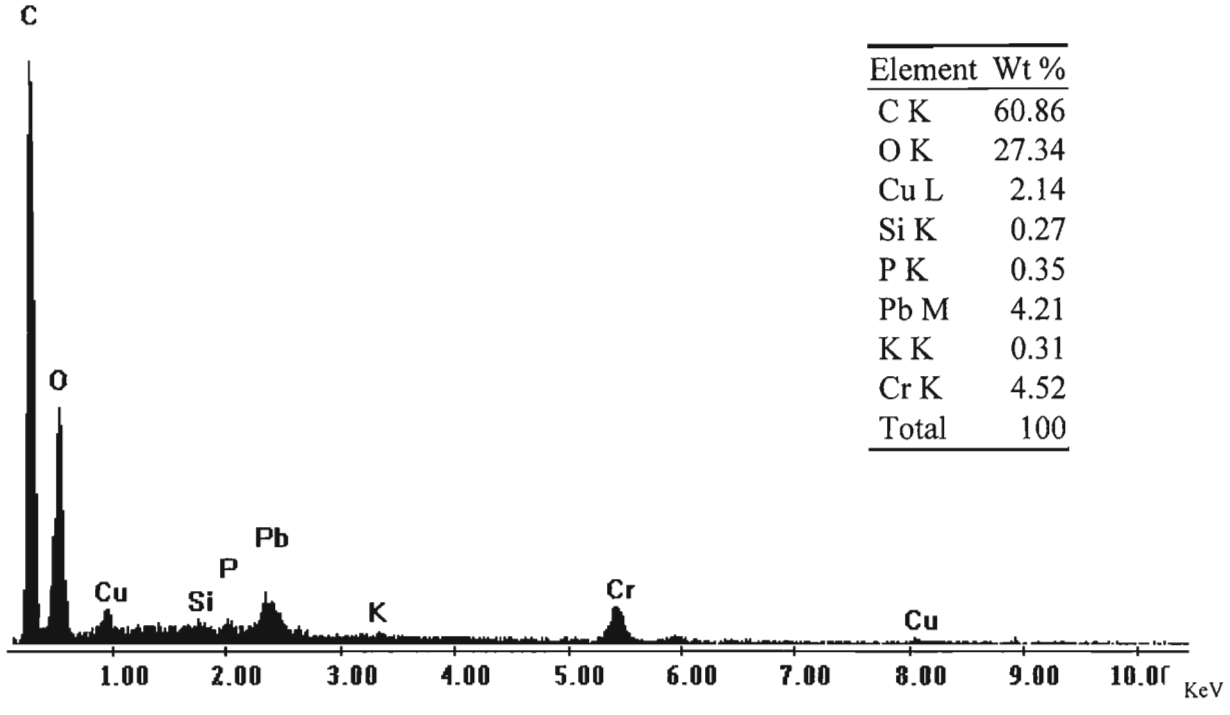
However, at the end of the experiment (Day 60) the spectral peaks of the three metals were smaller but the peak for carbon had increased tremendously, which could be due to the organic build-up on the surface of biofilms. Some elements like aluminium, silicon, potassium and phosphorus were found in trace amounts associated with the biofilms, these elements could be deposited either from the attachment matrices or the organic compound voermolas, which contains some non-metallic elements. Overall metal removal by the biofilms in the three bioreactor compartments followed the same trend, that is Cr> Pb>Cu. The reactor with gravel as support matrix was found to be relatively better than those with polyester and sand were used as the supports at removing the three metal ions under investigation.

A



Element	Wt %
C K	49.47
O K	29.22
Cu L	1.89
Al K	0.71
P K	0.81
Pb M	7.33
Cr K	10.58
Total	100

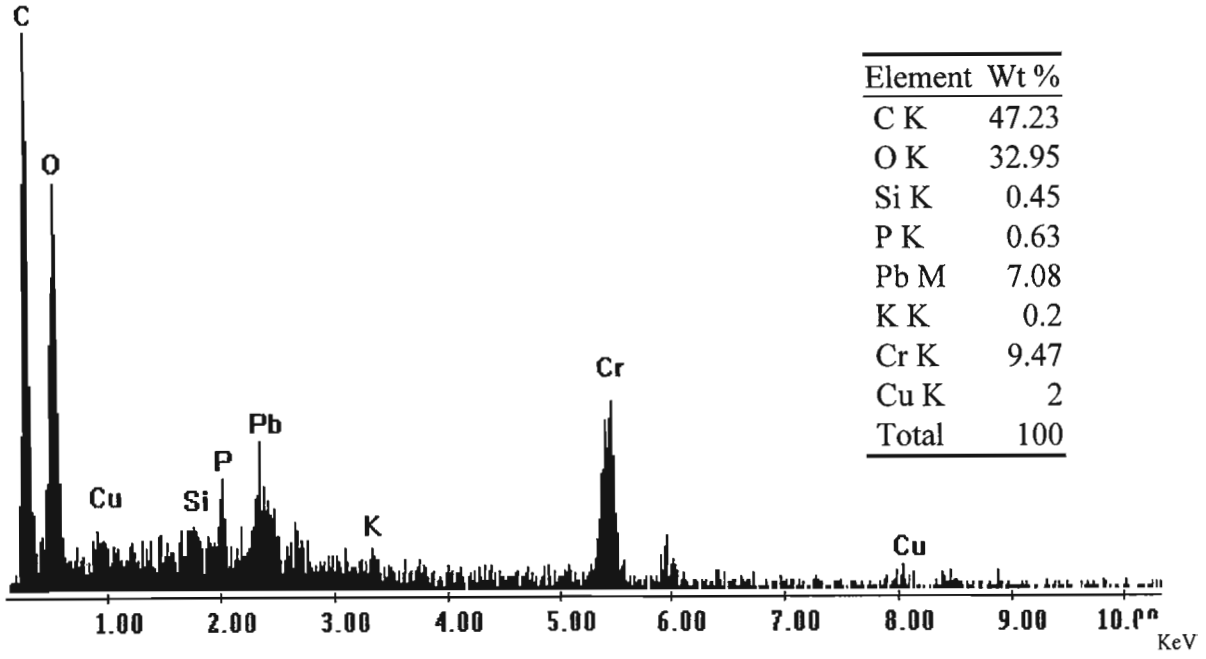
B



Element	Wt %
C K	60.86
O K	27.34
Cu L	2.14
Si K	0.27
P K	0.35
Pb M	4.21
K K	0.31
Cr K	4.52
Total	100

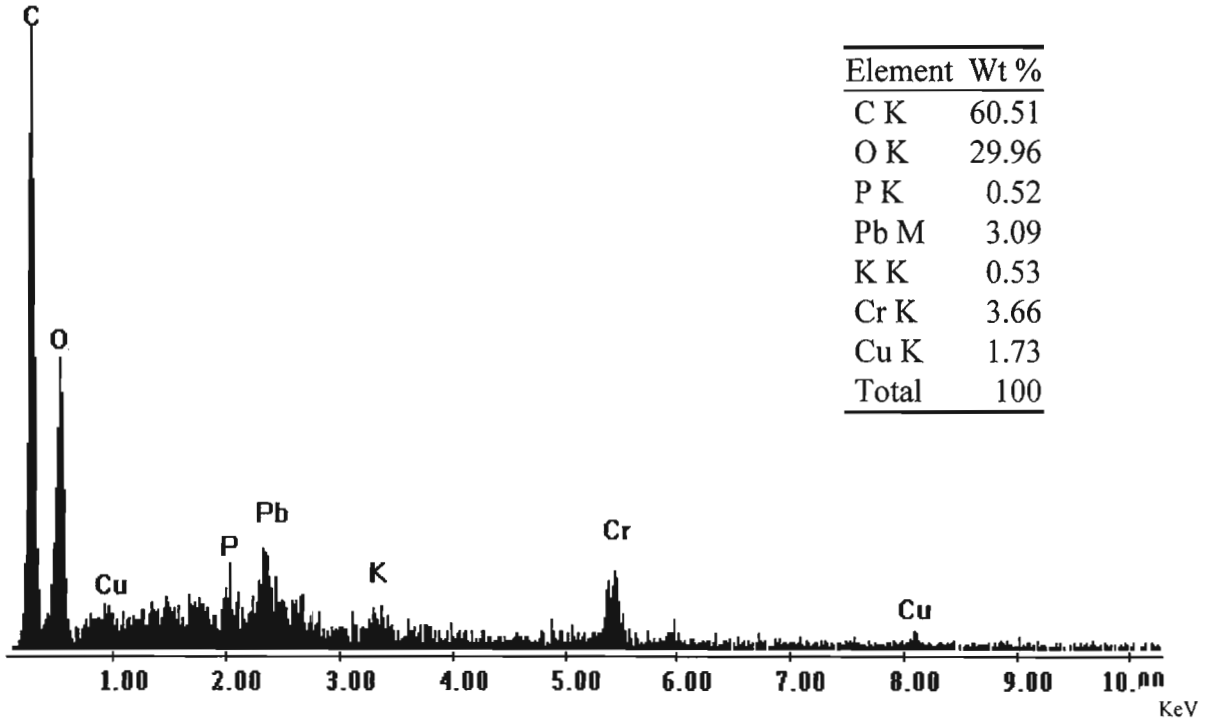
Figure 3.8 EDX quantification of metals associated with biofilm established on gravel matrix; A, after two weeks and B, at the end of the experiment (week 8).

A



Element	Wt %
C K	47.23
O K	32.95
Si K	0.45
P K	0.63
Pb M	7.08
K K	0.2
Cr K	9.47
Cu K	2
Total	100

B

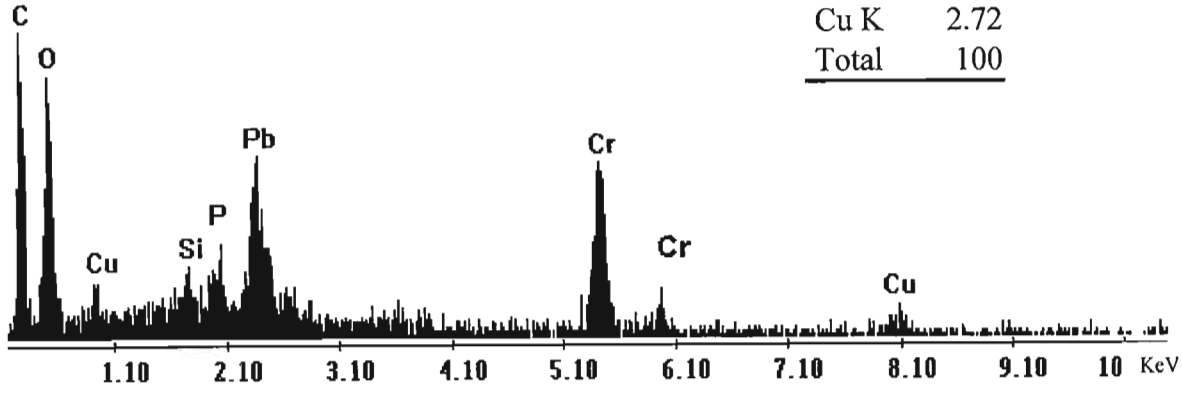


Element	Wt %
C K	60.51
O K	29.96
P K	0.52
Pb M	3.09
K K	0.53
Cr K	3.66
Cu K	1.73
Total	100

Figure 3.9 EDX quantification of metals associated with biofilm established on polyester matrix; A, after two weeks and B, at the end of the experiment (week 8).

A

Element	Wt %
C K	47.2
O K	28.35
Si K	0.91
P K	1.29
Pb M	9.96
Cr K	9.58
Cu K	2.72
Total	100



B

Element	Wt %
C K	60.7
O K	27.11
Cu L	1.45
Si K	0.77
P K	0.85
Pb M	3.86
K K	0.7
Cr K	4.56
Total	100

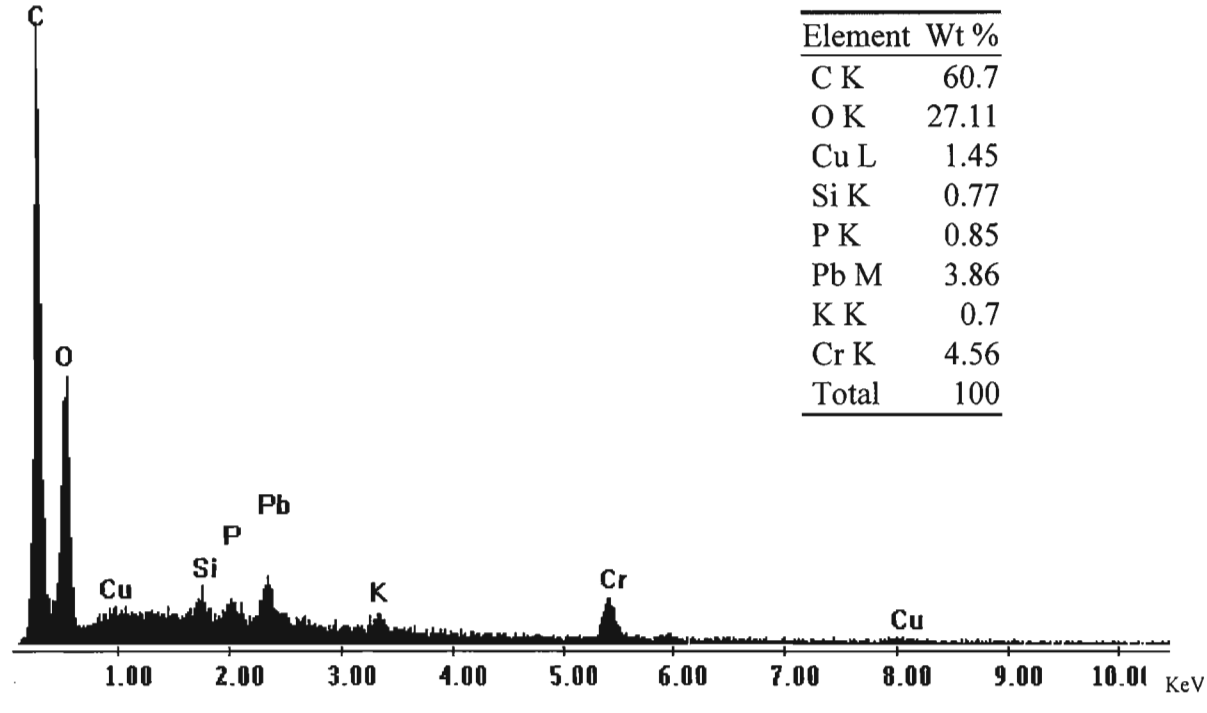


Figure 3.10 EDX quantification of metals associated with biofilm established on sand matrix; A, after two weeks and B, at the end of the experiment (week 8).

A. Gravel attached biofilms

B. Polyester batting attached biofilms

C. Sand attached biofilms

Plate 3.4 Surface views of biofilms attached to different support matrices, at the end of the experiment (Day 60).

Table 3.3 Overall mean residual metal ion concentrations in the three-bioreactor compartments for all three-support matrices used

Support Matrix used for Biofilm growth	Cr^{+3} conc. (mg l^{-1})	Pb^{+2} conc. (mg l^{-1})	Cu^{+2} conc. (mg l^{-1})
Gravel	74.6	92.6	102.3
Polyester batting	69.4	102.3	110.1
Sand	78.3	102.5	109.9
LSD (5% level)	14.41	13.44	14.18
P-values	NS	NS	NS

NS = Non Significant

Analysis of variance of the residual metal ion concentration in the three-bioreactor compartments was computed as indicated in Table 3.3 and it was found that there were not significant differences between the amounts of metals removed by the biofilms on the different attachment matrices at each sampling time. However, the biofilms grown on the polyester batting showed most efficient removal of chromium from the system, followed by gravel and then sand. On the other hand, both lead and copper ions were best removed by the biofilms attached to gravel, and the biofilms attached to both polyester batting and sand showed more or less similar effect levels of removal for lead and copper. Hence, it should be emphasised that once the biofilms were fully established, metal ion adsorption was purely an effect of the microbial activities and not of the attachment matrices. The EDX spectra also showed that the metals were associated almost exclusively with the biofilms.

With all three-support matrices time of contact of the biofilms with the metal contaminated effluent played a significant role in removal of metals; thus the larger the exposure the greater the amount of metals ions removed from the system **(Appendix 2)**. However, the energy required to operate the system for the time necessary to remove approximately 80-85% of the three metals studied, suggested that microbial systems are not economically feasible with initially high metal ion concentrations. Therefore, for effective remediation of heavily contaminated effluent, microbial processes should be preceded by pre-treatments involving application of humic substances to precipitate much of the metal ions by

complexation with the humic acid. This would reduce the toxic effects of the metals prior to introduction into the microbial systems and would improve the metal removal process by shortening the contact time needed for effective removal, thus saving much of the energy spent in running the process. Similar suggestion have been reported by Costley (1999), who concluded that biological accumulation processes need not be complete systems on their own, and may not be applicable to all situations but may be used as 'Polishers' to the existing conventional physicochemical remediation processes. This phenomenon was investigated further and will be discussed in Chapter 5.

3.3.6 Conclusions

Biofilm development was found to be better on the surface of the porous, hydrophilic polyester batting than on the gravel and sand. Although the surface irregularities of the latter two matrices serve as anchoring points for microbial attachment, because of their heaviness both gravel and sand added extra-weight to the RBC discs, which in turn caused stress damage to the RBC motor during operation. Thus, polyester batting provides an ideal attachment matrix for industrial scale RBC bioreactors because of its porous and rough surface which increases surface area for microbial attachment; its hydrophilic nature which allows quick colonisation; and its light weight. In addition it is also cheap and readily available.

CHAPTER FOUR

SELECTIVE METAL ACCUMULATION BY IMMOBILIZED BACTERIAL, FUNGAL AND MIXED MICROBIAL POPULATIONS ATTACHED TO A POLYESTER BATTING SUPPORT MATRIX IN A RBC BIOREACTOR

4.1 Introduction

The impacts and long-term ecological effects of pollution on the biosphere have stimulated research to evaluate the interactions between pollutants, the environment and the microbiota (Babich and Stotzky, 1977a). Microorganisms are highly efficient bioaccumulators of soluble and particulate forms of metal ions, especially when external concentrations are low. Various microorganisms, including actinomycetes, bacteria, algae, fungi and yeasts are known to accumulate heavy metals from their external environments (Gadd, 1988). However, pure culture studies have shown that various bacterial, actinomycete and fungal species differ in their sensitivity and preferences for particular metal ion species (Babich and Stotzky, 1977b).

Several investigations have showed that immobilized bacterial cells have high surface area to volume ratios and, hence, could have a large capacity for metal ion adsorption from effluents (Mullen *et al.*, 1989). Studies also showed that Gram-positive bacterial cells accumulate higher amount of heavy metals than do isolates of Gram-negative cells (Da Costa and Duta, 2001). Fungi on the other hand, are also well suited to this purpose since their cell walls and membranes strongly complex metal ions (Gadd, 1988). They thus can play an important role in the removal of metal ions from contaminated environments.

In the present study bacterial, fungal, and mixed bacterial and fungal populations were immobilized on a polyester batting support matrix in separate RBC compartments and the preference of the different biofilm population for particular metal ion accumulation was determined. The objective was to select the best microbial association to form a biofilm for removal of heavy metals from metal contaminated effluents in a continuous process.

4.2 Experimental Procedures

4.2.1 Establishment of Various Biofilms in the RBC Bioreactor Using Polyester Batting as Support Matrix

To investigate the selective accumulation of heavy metals by various microorganisms and to find which microorganism have the highest metal binding capacity, various enrichments were prepared (as described in Section 3.2.2) in Erlenmeyer flasks at different pH ranges to be used as inoculum in the bioreactor compartments. Each RBC bioreactor compartment was inoculated with 260 ml microbial culture from the second stage enrichment and the final volume was made up to 1300 ml with synthetic effluent containing 200 mg l^{-1} of Cr^{+3}, Pb^{+2} and Cu^{+2} ions. The culture medium was maintained at different pH values in the separate RBC compartments to establish the fungi, bacteria and mixed microbial biofilms. Polyester batting was attached to the RBC discs to serve as support matrix for the establishment of the biofilms. During the initial stage of biofilm development the bioreactor compartments were operated in batch culture mode to prevent microbial washout from the system. Once the microbes had started to colonize the surface of the polyester batting, synthetic effluent was pumped to each bioreactor compartment in a fed-batch mode from a different reservoir. The pH and temperature within each compartment were measured weekly and the minimum and maximum laboratory temperatures were recorded. The biofilms attached to the support matrix in each compartment were collected separately with minimal destruction for ESEM analysis.

4.2.2 Accumulation of Heavy Metals by the Biofilms

After mature biofilms had developed within the RBC, each compartment was drained and washed for 24 hours with 1300 ml acidified water (0.01M HCl) to desorb any metal ions bound during the biofilm establishment phase. The bioreactor compartments were drained and the biofilms-rinsed thoroughly with deionised distilled water for another 24 hours to neutralise the system. Following this, fresh synthetic effluent prepared as described in Section 2.4, was pumped into each bioreactor compartment from separate reservoir and the whole system set-up in recycling mode. Samples (2.5 ml) were taken before adding the

effluent to the bioreactor compartments to determine the initial metal concentration in the synthetic effluent, and then after intervals of: one, three, six, 12 and 24 hours and thereafter at two day intervals for a period of 12 days. The samples were filtered through nitrocellulose filter membranes (pore size 0.22 μm, 25 mm diameter) and stored at 4 ^{0}C in McCartney bottles until analysed by flame atomic absorption spectroscopy **(Section 2.7.1)** to determine the metal adsorption capacity of the biofilms. Biofilm samples were taken at various times and finally at the end of the experiment (week 8) for the ESEM and EDX analysis after sample preparation as described in sections 2.8 and 2.9, respectively.

4.3 Results and Discussion

4.3.1 Development of Biofilms on RBC Discs Lined with Polyester Batting as Support Matrix

Biofilms are formed by the initial adsorption of microorganisms to the support matrix followed by the growth of the microorganisms, the production of extracellular polymer substances and the entrapment of other microbial cells from the liquid medium (Characklis *et al.*, 1990). According to Bishop and Kinner (1986) in most wastewater treatment systems biofilms are visible on the support matrices within a few days after start-up; however, microscopic films develop within hours of the process. A similar pattern was observed during the establishment of the fungal, bacterial and mixed bacterial-fungal biofilms in the present investigation. Soon after inoculation of the bioreactor compartments, the microbial cells adhered to the surface of the polyester support matrix and eventually, after several days, they had formed small colonies which then grew to form larger colonies which ultimately covered the entire disc surface.

Microorganisms have different maximum, minimum and optimum pH levels for growth. Some grow in very acidic environments (pH 1-2) while others thrive in very alkaline environments (pH 10-11). However, most bacterial species prefer growing in the pH range 5.5 to 8.0. By contrast fungi grow well in a pH range from 4 to 5.5 (Babich and Stotzky, 1977a). During biofilm development the pH of the culture medium in each bioreactor

compartment was maintained at a values which favoured establishment of the desired type of microbial biofilm organisms. Hence, the culture medium in the first compartment was kept acidic (pH 4.0 to 4.5) to promote the growth of fungal biofilms while the second bioreactor compartment was maintained at pH 6.0 to 7.0 to grow bacterial biofilms. The third bioreactor compartment was maintained at pH 5.0 to 6.0 to allow for growth of biofilms comprising both bacterial and fungal components.

Environmental scanning electron microscopy (ESEM) investigation of biofilms removed from the first bioreactor compartment after 4 weeks indicated the presence of masses of fungal mycelium and some fungal spores **(Plate 4.1 A and B)**. After six weeks the fungal mycelium had developed more fully and become entangled to form a thick biofilm **(Plate 4.1 C and D)**. Low magnification of the fungal biofilms removed at the end of the experiment revealed the presence of fungal mycelia and some spores, which had developed on the surface of the polyester support matrix **(Plate 4.1 E)**. High magnification showed the presence of some accumulated extracellular granular material **(Plate 4.1 F)**.

High magnification of the biofilms removed from the second bioreactor compartment after four weeks revealed the presence of various bacterial morphotypes including filamentous organisms, rods and cocci together with some fungal mycelium and possibly some yeast cells **(Plate 4.2 A and B)**. After six weeks the bacterial cell population had increased and numerous rod shaped bacteria and filamentous organism were evident on the surface of the support matrices together with extracellular fibrillose material **(Plate 4.2 C)**. Biofilm samples removed at the end of the experiment revealed the possible presence of metal deposits on the surface of the bacterial biofilms **(Plate 4.2 D)**. High magnifications of the samples illustrate the presence of numerous bacterial cells including rods and cocci associated with some filamentous organisms and large amount of extracellular fibrillose material **(Plate 4.2 E and F)**.

The micrographs of the biofilm removed from the third bioreactor compartment at week 4 show the presence of fungal hyphae, some of which have a spore attached **(Plate 4.3 A)**. High magnification revealed the presence of numerous bacterial cells mixed with the fungal

hyphae **(Plate 4.3 B)**. After six weeks, however, various microbial cells, including some rod-shaped bacteria, fungal mycelia and possibly some actinomycete mycelium were observed **(Plate 4.3 C and D)**. Micrographs of the biofilm samples removed at the end of the experiment (week 8) revealed the presence of large clusters of bacterial cells mixed with much smaller numbers of fungal and/or possibly yeast cells **(Plate 4.3 E and F)**.

Plate 4.1 Scanning electron micrographs illustrating the development of the fungal biofilm on the surface of the RBC discs (using polyester as support matrix) at different times. A and B, high magnification of the biofilms removed at week 4, showing the presence of fungal mycelium (fm) and some spores (fs); C and D, fully developed fungal biofilm removed at week 6; showing a thicker entangled fungal mycelium with some fungal spores present; E, low magnification of the biofilms removed at the end of the experiment illustrating further development of the fungal mycelium biomass (fm); F, higher magnification of the biofilms showing various types of fungal hyphae and some spore masses and possibly extracellular materials.

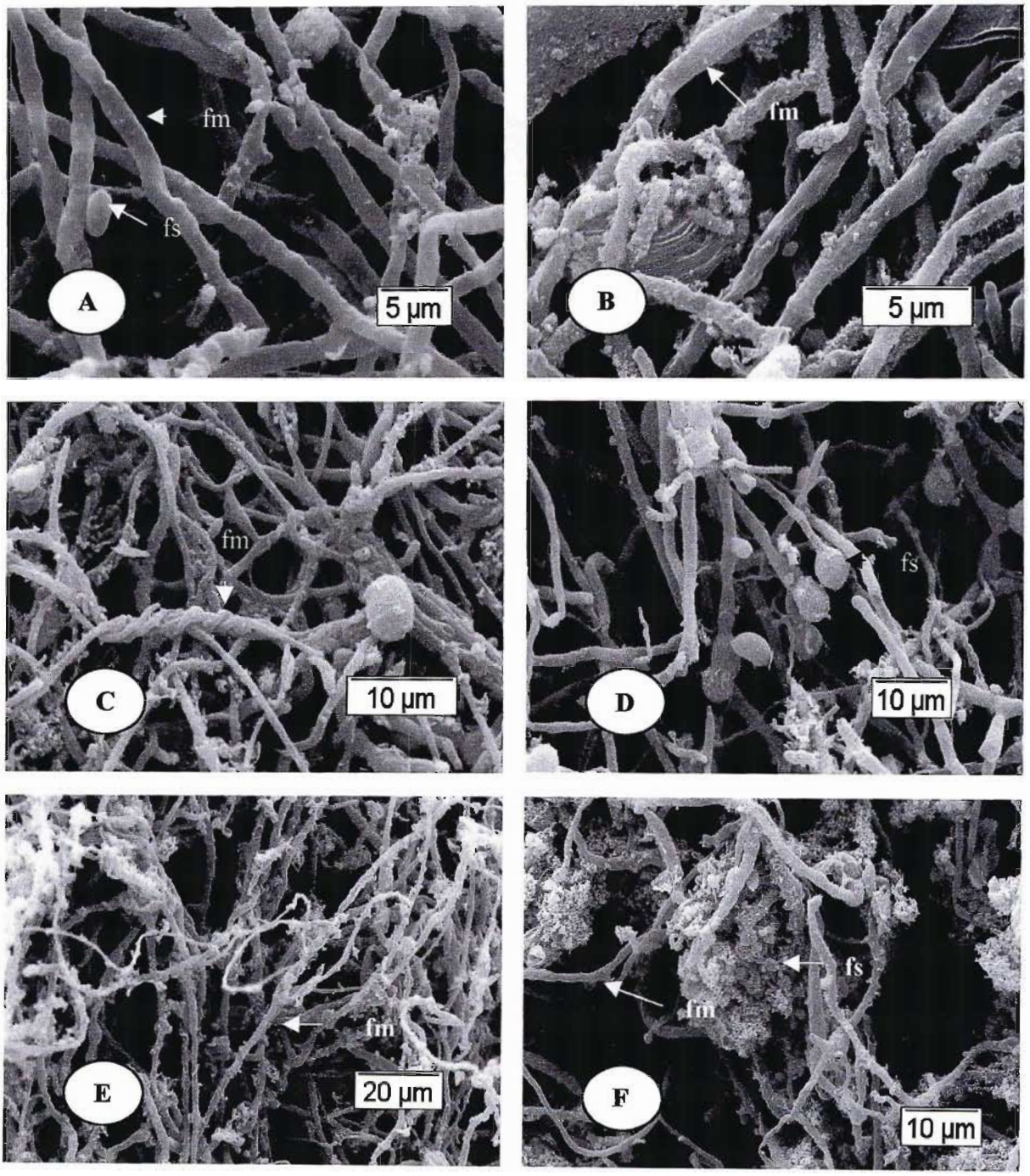

fm
fs
A
5 µm
fm
B
5 µm
fm
C
10 µm
fs
D
10 µm
fm
E
20 µm
fs
fm
F
10 µm

Plate 4.2 Scanning electron micrographs illustrating the development of the bacterial biofilm on the surface of the RBC discs after different periods of time. A and B, high magnification of the biofilms removed at week 4; showing the presence of filamentous organisms (f), numerous rods (r) and cocci (c) and some fungal spores (s) and possibly yeast cells; C, fully developed bacterial biofilm removed at week 6; showing the presence of numerous rods (r) and some filamentous organisms together with strands of extracellular material; D, high magnification of the biofilms removed at the end of the experiment (week 8) illustrating clusters of bacterial cells (b), some rod-shaped bacteria and spores (s) and, possibly, metal deposits on the surface of the biofilm; E and F, high magnification of the biofilms showing various bacterial morphotypes including cocci and rods.

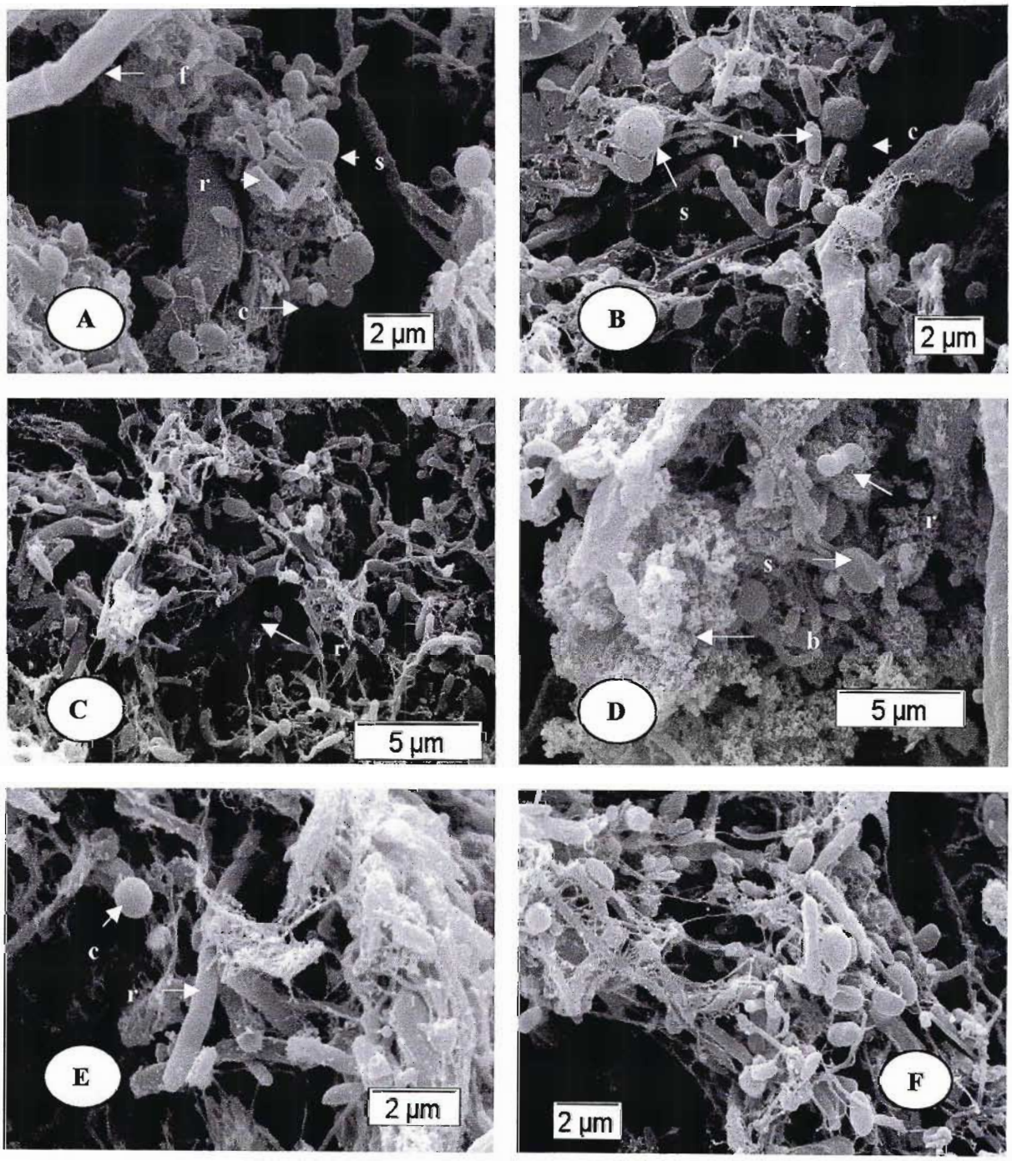
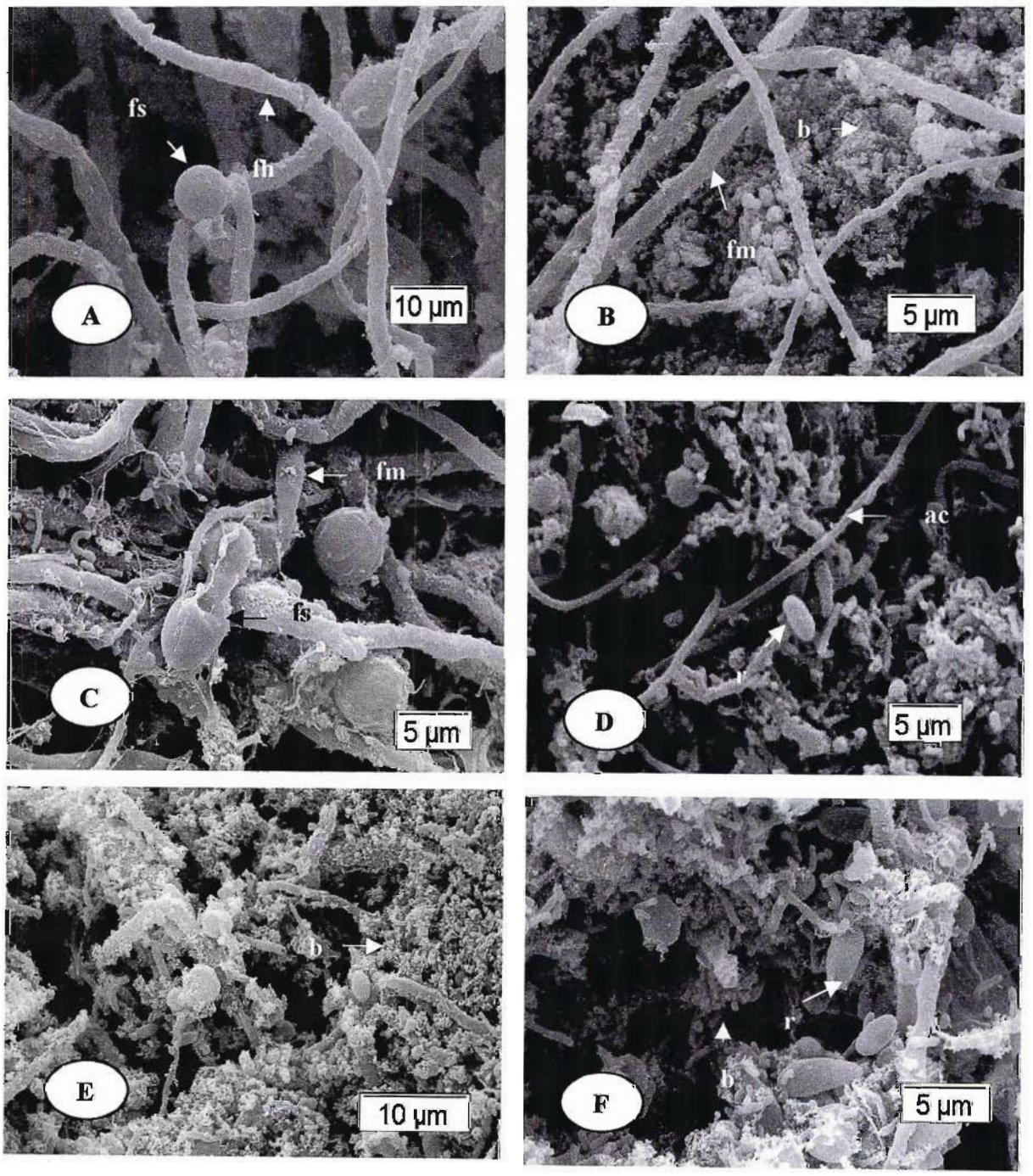

f
r
s
c
A
2 µm
r
c
s
B
2 µm
r
C
5 µm
r
s
b
D
5 µm
c
r
E
2 µm
2 µm
F

Plate 4.3 Scanning electron micrographs illustrating the development of the mixed bacterial and fungal biofilm on the surface of the RBC discs at different times. A, biofilm removed at week 4 showing the presence of fungal hyphae (fh) and some fungal spores (fs); B, high magnification of the biofilm removed at week 4 showing the presence of a consortium of microbial morphotypes including fungal mycelia (fm) and cluster of bacterial cells (b); C, illustrates the presence of fungal cells (fm) and spores (fs) with adhered bacterial cells and D, illustrates the presence of numerous rod-shaped bacteria, actinomycete-like organisms (ac) in a mature biofilm removed at week 6; E and F, biofilms removed at the end of the experiment (week 8) illustrating large clusters of bacterial cells (b) and smaller number of, possibly, yeast cells on the surface of the biofilms.


fs
fh
A
10 µm
b
fm
B
5 µm
fm
fs
C
5 µm
ac
r
D
5 µm
b
E
10 µm
r
b
F
5 µm

4.3.2 Selective Accumulation of Heavy Metals by the Different Microbial Biofilms Developed in the RBC Bioreactor Compartments

4.3.2.1 Fungi

Fungi are industrially important for the removal of heavy metals from contaminated environments, since they often exhibit marked tolerance towards metals and other adverse conditions, for example, low pH. They have large numbers of metal binding sites on their cell walls and may also show high levels of intracellular accumulation (Gadd, 1988). A wide variety of ligands including carboxyl, amino, phosphate and hydroxyl groups are present on the cell walls of fungi, which may be involved in the removal of heavy metals from waste streams; however, the relative importance of each of these chemical groups is difficult to determine (Strandberg *et al.*, 1981).

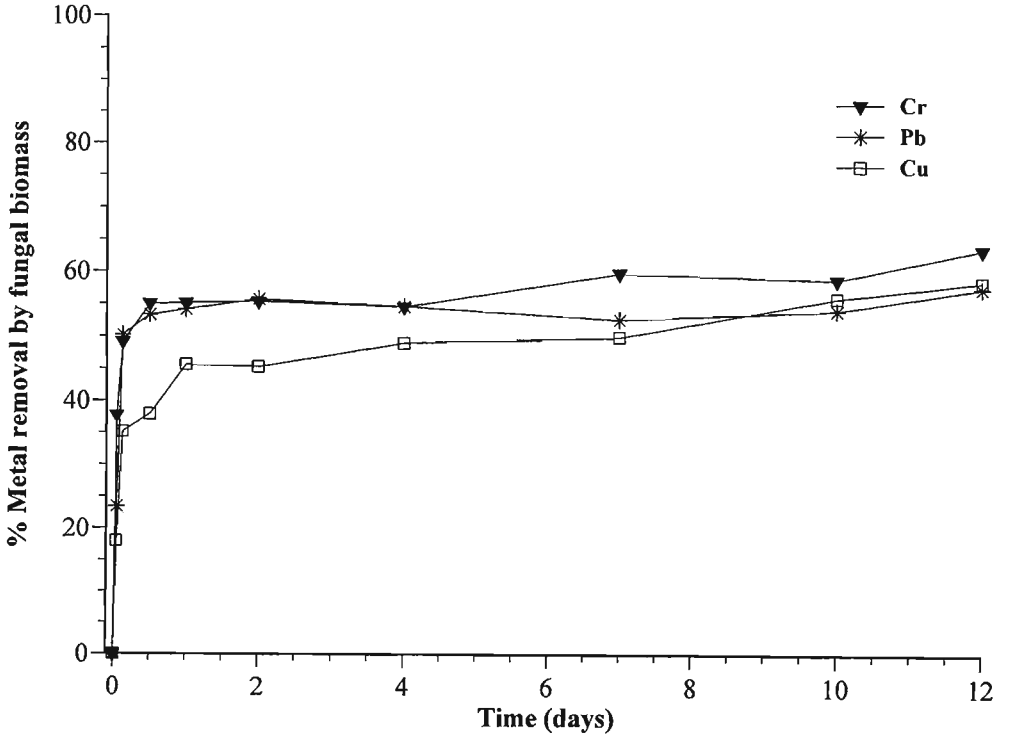
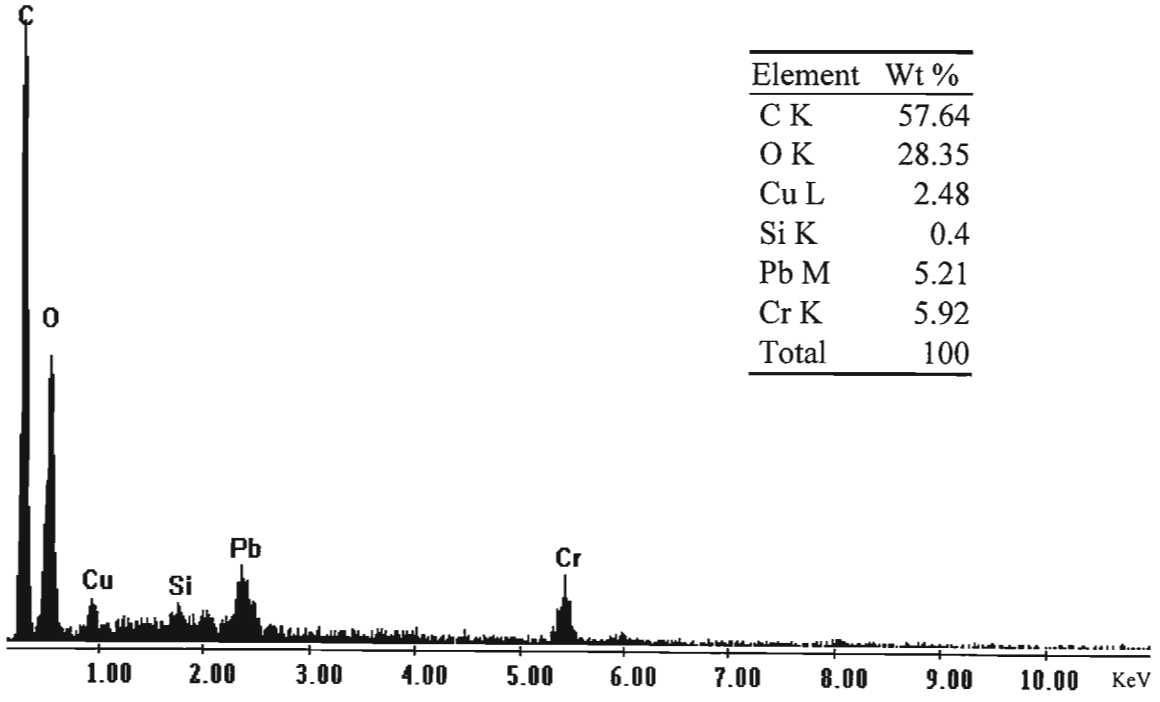


Figure 4.1 Percentage metal ion removal by fungal biofilms at different times.

As shown in Figure 4.1, initially chromium and lead ions were more readily accumulated by the fungal biofilms than the copper ions and thus higher percentage removals of these

metals were achieved by the system. For example, after 3 hours of treatment approximately 50% of both Cr^{+3} and Pb^{+2} and 35% of Cu^{+2} were removed by the system. This might be due to the higher affinity of the metal binding ligands present on the cell wall of fungi to which chromium and lead ions were more readily adsorbed than for copper ions, which resulted in the copper ions remaining in solution during the initial period of contact. However, as contact time increased the removal rates of chromium and lead slowed down until there was no significant removal of either metal by the fungal biofilms, possibly as a result of saturation of the cell surface attachment sites for these metals. On the other hand, the removal rate of copper from the system increased slightly with increased contact time. At the end of the experiment (Day 12) the relative metal removal by the fungal biofilms was 63% for Cr^{+3}, 57% for Pb^{+2} and 58% for Cu^{+2}. The results clearly show that the binding of the metal ions to the fungal cells occurred mainly during the first few hours of contact during which time large amounts of the metal ions were bound to the biofilms.



Element	Wt %
C K	57.64
O K	28.35
Cu L	2.48
Si K	0.4
Pb M	5.21
Cr K	5.92
Total	100

Figure 4.2 EDX quantification of the metal ions present in the fungal biofilm at the end of the experiment (Day 12).

EDX spectral analysis of the metal ions showed a similar trend to the results obtained with AAS, in which the adsorption of Cr^{+3} and Pb^{+2} on the fungal biomass was relatively higher than that of Cu^{+2} ions.

4.3.2.2 Bacteria

As with other microbes, multiplicities of potential metal accumulation sites are found on the cell walls of bacteria (Brierley, 1990). Different bacterial species vary in their responses to metal ions (Beveridge and Fyfe, 1985). After investigating the metal binding ability of the cell walls of various bacterial species, these authors observed that the Gram-positive *Bacillus subtilis* and *Bacillus licheniformis* bound larger quantities of several metals than the envelopes of the Gram-negative bacterium *Escherichia coli*. According to Brierley (1990) the teichoic and teichuronic acids in the cell walls of Gram-positive bacteria are attached to the peptidoglycan network, which gives the surfaces a dense negative charge. The negatively charged carboxyl groups interact electrostatically with the metal cations and are the main agents for the uptake of heavy metals by these organisms (Da Costa and Duta, 2001).

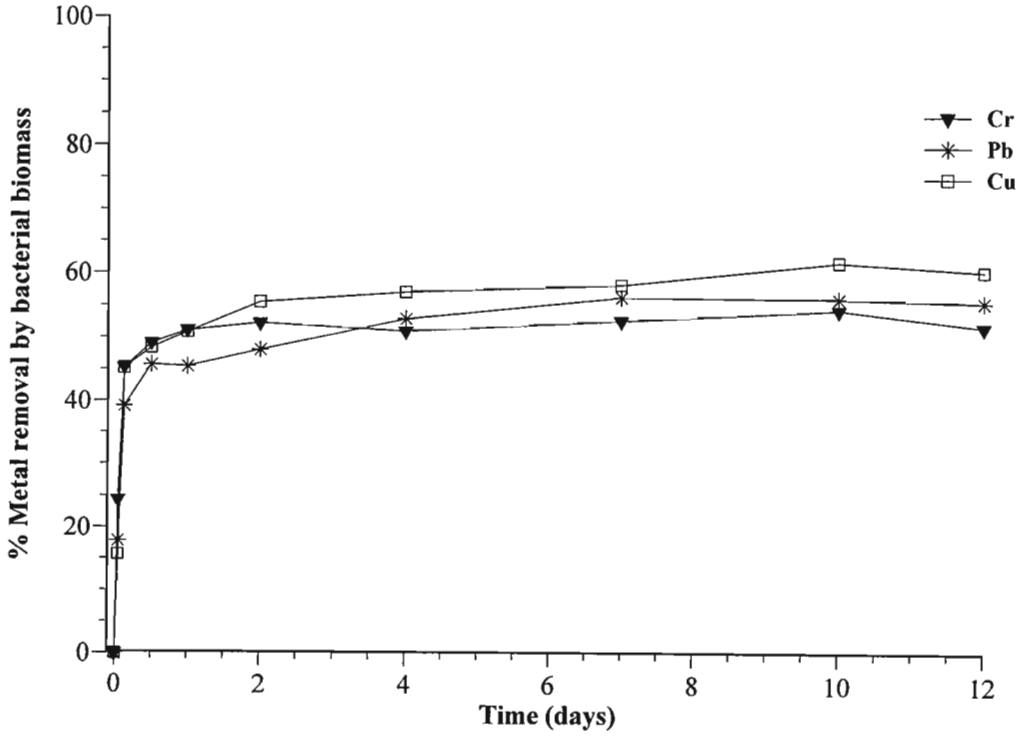
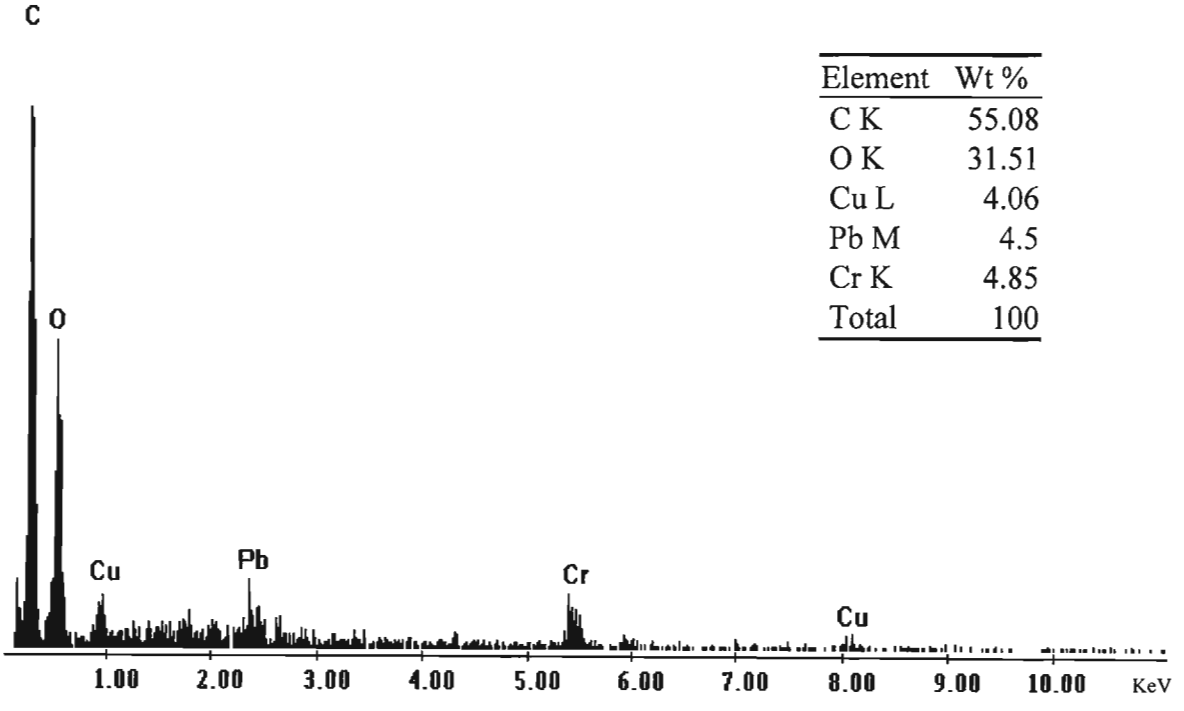


Figure 4.3 Percentage metal ions removed by bacterial biofilms at different times.

As indicated in figure 4.3, the metal ion species were readily adsorbed to the cell wall of the bacteria and the residual concentrations of the three metal ions decreased significantly, with approximately 40 - 45% of all three metals removed from the synthetic effluent within 3 hours of start up. At the end of the experiment (Day 12) 54%, 55% and 62% removal was achieved for Cr^{+3}, Pb^{+2} and Cu^{+2} ions, respectively. Comparing these results with those obtained with the fungal biofilms, copper was removed best by the bacterial biofilms, whereas chromium ions were most readily accumulated by the fungal biofilms. EDX spectral analysis of the metal ions **(Figure 4.4)** confirmed this, since the peak heights and weight percentage copper ions removed by the bacterial biofilms, were greater than those observed for the fungal biofilms.



Element	Wt %
C K	55.08
O K	31.51
Cu L	4.06
Pb M	4.5
Cr K	4.85
Total	100

Figure 4.4 EDX quantification of the metal ions present in the bacterial biofilm at the end of the experiment (Day 12).

The factors responsible for the variation in metal ion adsorption could be intrinsical properties of the metals themselves, composition of the aquatic environment such as dissolved oxygen, pH etc, or may be differences in the nature of the microbial species present (Dong *et al.*, 2003b). Since the composition of the medium, following biofilm development, was maintained unchanged throughout the experiment, this could not be the cause of the variation. However, differences in the nature of the biofilms could provide a possible explanation for the observed variation in uptake of the different metal ions from the effluent solution. As mentioned previously bacteria and fungi differ with respect to the chemistry of their cell walls and this might account for the preferential metal ion adsorption displayed by the biofilms from the different bioreactor compartments. Consequently, the negatively charged groups in the cell walls of the bacteria might more actively accumulate copper ions from the solution whereas the negatively charged ligands on the cell walls of fungi might preferentially adsorb chromium ions. However, the actual cause of this preferential adsorption was not elucidated and further investigation is necessary.

4.3.2.3 Bacteria and Fungi

Mixed microbial cultures have been successfully used in the treatment of a wide range of organically and inorganically contaminated industrial wastes, which often require a complex microbial population to deal with them (Salmon and Bull, 1984). Some resistant species that can accumulate metals may exert a protective effect on sensitive species in the same system by removing the toxic metals from the system (Sterritt and Lester, 1980). Comparison of the adsorption of the three metal ion species in the three-bioreactor compartments at various time intervals showed that the mixed microbial biofilms removed a high percentage of all the metal ion species from the system. For example, 3 hours after start up time, approximately 50% of all three metal ions had been removed by this biofilm. At the end of the experiment (Day 12) the percentage removal of the metal ion species were approximately 64%, 58% and 70% for Cr^{+3}, Pb^{+2}, and Cu^{+2}, respectively. From these results it can be inferred that the use of biofilms containing an array of microorganisms allows large amounts of mixed metal ions to be removed from polluted wastewaters.

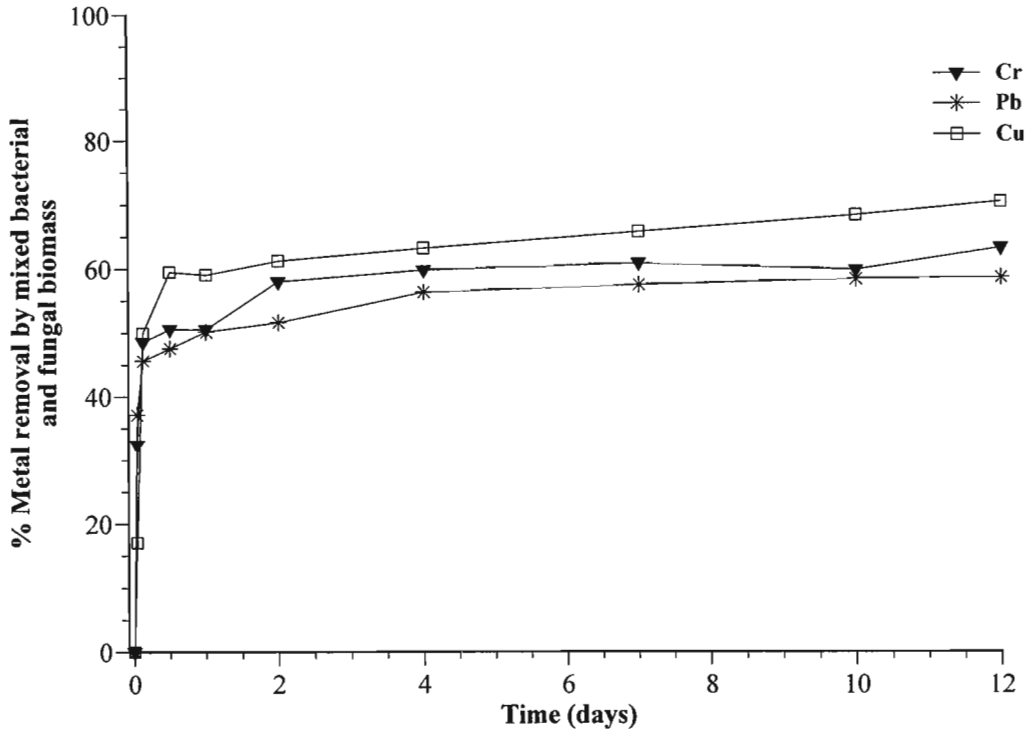
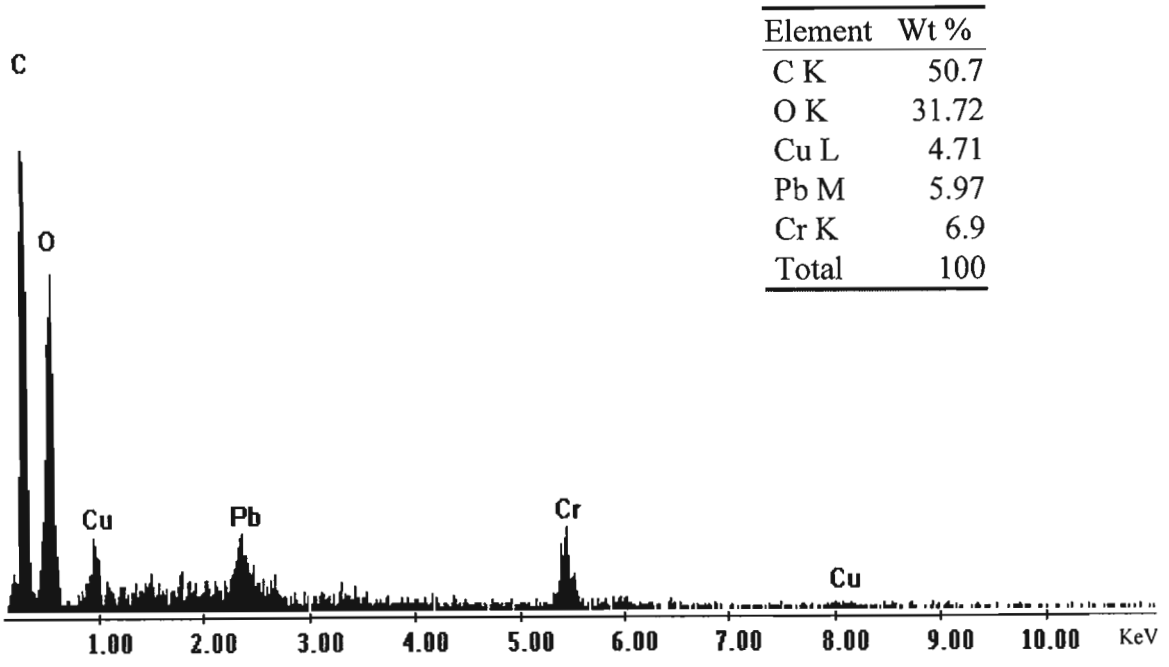


Figure 4.5 Percentage metal ions removed by mixed bacterial and fungal biofilm at different times.

The corresponding EDX spectral analysis of the mixed bacterial and fungal biofilm shows the amount of metals associated with the biofilm. Surprisingly, the EDX spectral peaks for copper ions were smaller than the chromium and lead peaks in all the different biofilms tested. By contrast AAS analysis showed that copper removal by the bacterial and mixed, bacterial and fungal biofilms was higher than the removal rates of chromium and lead. Since no standards were used in the EDX quantification of the metals, the AAS results were considered to be more accurate. Hence, it is considered that copper ions were the most actively removed from the system by the mixed microbial biofilms. Desorption studies on the biofilms described in the next chapter clearly support this contention.



Element	Wt %
C K	50.7
O K	31.72
Cu L	4.71
Pb M	5.97
Cr K	6.9
Total	100

Figure 4.6 EDX quantification of metal ions present in a mixed microbial biofilm at the end of the experiment (Day 12).

With increasing time of contact with the synthetic effluent the microbial biofilms, in all the three bioreactor compartments played a significant role in removal of the metal ion species from the system. Relatively high percentage removals of all three metals were achieved during the initial stage of contact of effluent and biofilms and the removal efficiencies decreased over the later stages of the 12 day investigation suggesting possible saturation of the metal binding sites on the surface of the biofilm microorganisms.

Comparison of the amounts of the metal ions adsorbed by the biofilms showed that mixed bacterial and fungi population acted synergistically in the removal of copper ions. Hence, the mixed biofilms removed more copper ions from the bioreactors than did the biofilms comprised almost exclusively of bacterial or fungal biomass. Comparing the latter two biofilms, greater amount of copper was removed by the bacterium-dominated biofilms. Chromium ions on the other hand, were more actively removed by the predominantly fungal biofilms. The amount of lead ions taken up, however, was essentially the same in all

the treatments over the entire duration of the experiment, indicating that all the biofilms tested readily accumulated lead ions from solution. Similar conclusions were reached by Nakajima and Sakaguchi (1986) after investigating the selective accumulation of heavy metals by various microorganisms. They, found that mercury and lead ions were readily accumulated by almost all the species of microorganisms tested, including bacteria, fungi, actinomycetes and yeasts.

However, analysis of variance of the mean concentrations of the three metal ions adsorbed on the various biofilms showed there was no significant difference among the biofilms for the removal of the metal ions from the synthetic effluent. This suggested that even though various microorganisms may accumulate certain metal ions more efficiently than others; and that specific microbial biofilms have different preferences for binding different metal ions, immobilized microbial systems are generally effective for the removal of heavy metals from metal contaminated effluents.

4.4 Conclusions

Different microorganisms have different preferences for the metal ions they will most actively adsorb. Hence, the fungus-dominated biofilms established in this study selectively accumulate chromium ions whereas the mainly bacterial biofilms more readily accumulated the copper ions from the mixed metal contaminated effluent solution, while lead ions were accumulated to almost the same extent by all three types of biofilms tested. Interactions between bacterial and fungal components enhanced the mechanical stability of the biofilms so that large amounts of all three metal ion species were removed by this biofilm. The removal of copper in particular was enhanced when compared to its removal rate by biofilms comprising largely fungal or bacterial biomass.

CHAPTER FIVE

THE INTRODUCTION OF TWO-STAGE HUMIC ACID COLUMNS INTO A COMPARTMENTALISED RBC BIOREACTOR FOR EFFICIENT REMOVAL OF METAL IONS

5.1 Introduction

Many industries discharge aqueous effluents containing high levels of heavy metals into the environment, with resultant contamination of surface and ground waters, which become serious environmental problems (Nagase *et al.*, 1997). There are a number of physical and chemical methods for removing metals from industrial wastes; however, these methods have been reported to be commercially impractical due to their high operating costs and the difficulty in treating the solid waste subsequently generated (Environmental Management Division of Hong Kong, 1991). As indicated in the previous chapters, although microbial systems work well for the removal of metal ions in low concentrations, they cannot treat successfully the high levels of metal ions that are found in seriously contaminated areas and certain industrial effluents. Thus, the continuing demand for a pollutant-free environment is exceeding the ability of all these traditional waste treatment methods to produce high quality effluent at reasonable costs (Kinner *et al.*, 1983). Hence, it is important to look for effective alternative and/or combined technologies for the removal of metals from heavily contaminated effluents before biological treatment commences.

Considerable research has been devoted to understand the respective role of various adsorbents in removing trace metals from contaminated environments (Dong *et al.*, 2003a). Among the possible adsorbents, humic acid has received prominent consideration because of its high heavy metal complexing ability and consequent reduction of their toxic effects (Kochany and Smith, 2001; Itabashi *et al.*, 2001). Humic substances are natural products formed through the chemical and biological humification of plant and animal matter brought about by the activities of microorganisms (Kochany and Smith, 2001). Thus, the introduction of a series of columns containing humic acid to biological treatment systems would have the advantage of complexing the heavy metals thereby reducing their

concentrations and hence toxicity to the immobilized mixed microbial population comprising the biofilms. In addition, the efficiency and economy of the treatment process could be substantially improved by the removal of a high percentage of the contaminating metal ions.

After removal of a large proportion of the metal ions by the humic acid, a multistage RBC bioreactor, arranged as a series of compartments separated by baffles was used for more efficient and economical treatment of the metal-ion contaminated effluent. As the wastewater passes from one compartment to the next stage within the bioreactor, it undergoes an increasing degree of purification by the biofilm attached to the RBC discs. Hence, the concentrations of the pollutants are decreased at each subsequent stage of the treatment (Bishop and Kinner, 1986).

The aim of the present experiment was to determine the combined effect of a series of bench-scale columns containing liquid humic acid and a three stage RBC bioreactor for the removal of chromium, lead and copper ions from a mixed metal contaminating effluent.

5.2 Experimental Procedure

5.2.1 Effect of Humic Acid on the Concentration of Single Metal Ion Species in Solution

Flask experiments were carried out under batch culture condition to determine the ability of humic acid to remove metals from a solution containing single metal ion species. Liquid humic acid (1, 2, 5 and 10 ml) were transferred separately to 1000 ml Erlenmeyer flasks containing aliquots of single metal ion species taken from concentrated stock solutions. The volume in each flask was made up to 1000 ml with distilled water to give a final concentration of 200 mg l^{-1} for each metal ion. The flasks were covered with Parafilm to prevent evaporative lost and after 24 h aliquots (10 ml) were decanted into clean centrifuge tubes and centrifuged for ten minutes at 3030 x g. The supernatants were then carefully

transferred to clean McCartney bottles for metal analysis by flame atomic absorption spectroscopy **(Section 2.7.1)**.

5.2.2 Effect of Humic Acid on the Concentration of Mixed Metal Ions Species in Solution

Batch laboratory experiments were carried out to determine the efficacy of humate for removal of mixed metal ion species from solution. One, two, five and ten ml volumes of liquid humic acid were transferred to four different Erlenmeyer flasks into which mixed aliquots of Cr^{+3}, Pb^{+2} and Cu^{+2}, taken from the respective concentrated stock solutions, were added. The final volume of each flask was made up to 1000 ml with distilled water to obtain a final concentration of 200 mg l^{-1} of each metal ion and the flasks were covered with Parafilm to prevent evaporation. After 24 h, samples were removed from each flask and transferred to clean tubes, centrifuged at 3030 x g for ten minutes and the supernatants carefully transferred to clean McCartney bottles for metal analysis by flame atomic absorption spectroscopy (Section 2.7.1).

5.2.3 Effect of Contact Time with Humic Acid on the Removal of Metal Ion Species from Solution

Flask experiment was carried out in batch culture using the humic acid as a metal complexing agent to investigate the effect of contact time on removal of mixed metal ion species from solution. Ten ml of liquid humic acid was transferred to a 2 *l* Erlenmeyer flask and after adding an appropriate aliquot of each metal ion; the flasks were thoroughly mixed with a magnetic stirrer to homogenize the mixture. Finally, the volume was made up to 1000 ml with distilled water to obtain a final concentration of 200 mg l^{-1} of each metal ion species. The experiment was performed in triplicate and samples were withdrawn from each flask after 1, 2, 4, 6, 12, 24, 48, 72, 96 and 120 hours of contact between the metals and the humic acid. The samples were placed in clean centrifuge tubes and centrifuged at 3030 x g for ten minutes to remove colloidal particles that could give rise to erroneous results. The samples were diluted as necessary to remain within the calibration range of the

instrument and metal concentrations were determined by flame atomic absorption spectroscopy **(Section 2.7.1)**.

5.2.4 Establishment of Mature Biofilm in the Multistage RBC Bioreactor

The multistage laboratory-scale RBC was operated as described in **Section 2.6.1** with polyester batting as support matrix. Each bioreactor compartment was inoculated with 260 ml of the second stage enrichment culture, and synthetic effluent containing the three metal ions was added to each compartment to give a total working volume of 1300 ml. The pH of the medium was kept between 5.0-5.5 to favour growth of the microbial populations established during the enrichment procedure.

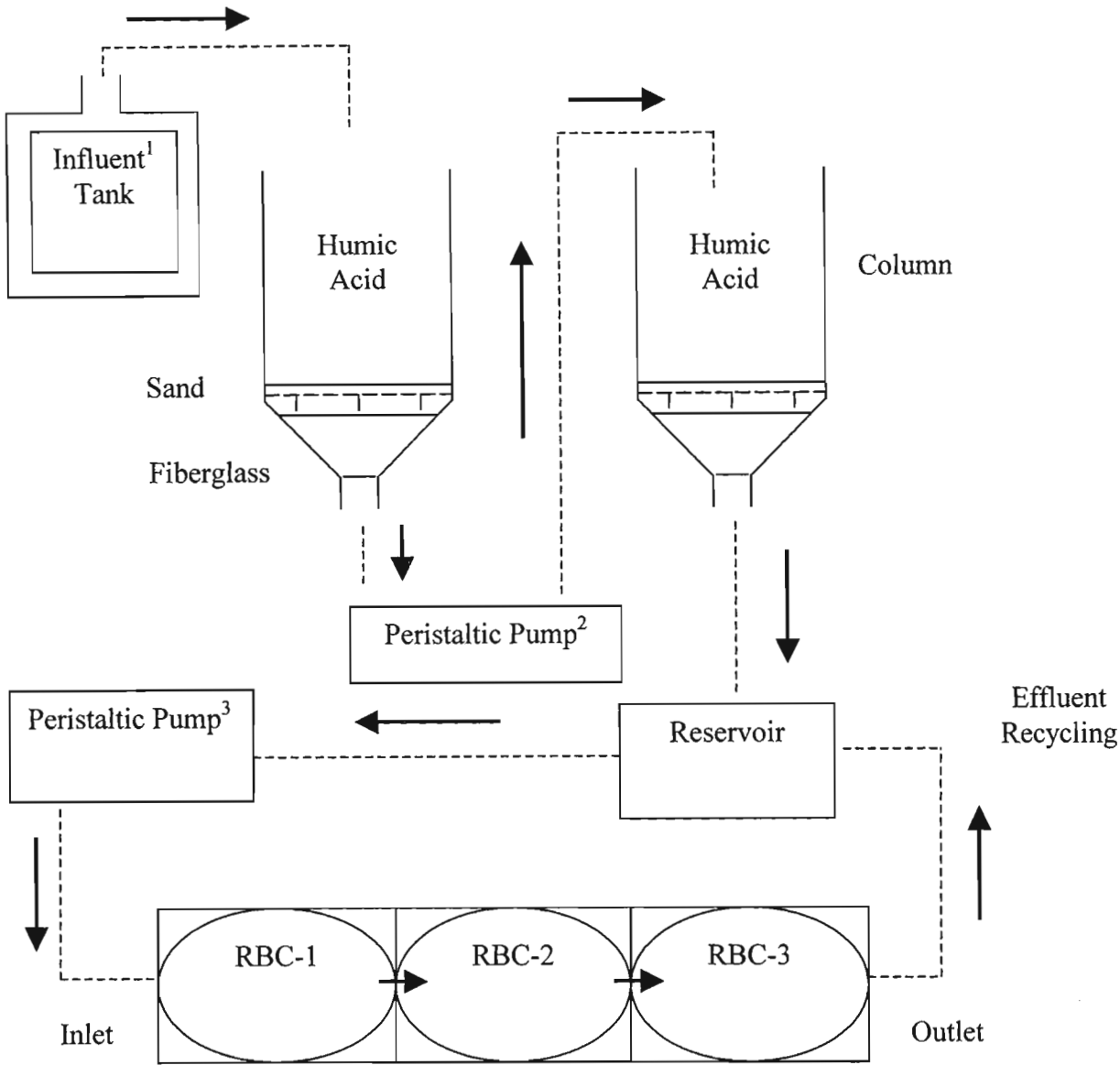
During the development of the biofilms synthetic effluent was pumped into the bioreactor continuously in a cascade mode from one compartment to the next, via silicon tubing. Biofilm samples were taken at various times during the development process and at the end of the experiment (week 7) from all three bioreactor compartments for ESEM analysis. After mature biofilms had established the bioreactor compartments were drained and washed with deionised distilled water for 24 hours to remove any metals adsorbed by the biofilms during their development.

5.2.5 Removal of Metal Ion Species Using a Series of Humic Acid Containing Columns and Subsequent Passage Through a Biofilm-Containing Multi-Compartment RBC

In this experiment a Watson Marlow pump was used to pass an effluent solution containing mixed Cr^{+3}, Pb^{+2} and Cu^{+2} ions through two in-line columns that contain liquid humic acid. Aliquots from the concentrated stock solutions of the three metal ions were transferred to the columns and liquid humate (10 ml) was added to attain maximum metal complexation **(Section 5.3.2)**. The volume of each column was made up to 1000 ml with distilled water to obtain a final concentration of 200 mg l^{-1} of each metal ion. The base of the columns was packed with fibreglass and acid washed sand to facilitate easy through-flow of the effluent.

The flow rate was set at 0.7 ml min^{-1} to give a retention time of 24 h in each column. After passing through the columns, the effluent with its residual metal concentrations was drained into a reservoir and then pumped to the RBC compartments at 0.9 ml min^{-1} for further treatment **(Figure 5.1)**. At each stage of the treatment process a sample (2.5 ml) was taken for AAS analysis. Samples were diluted with distilled water as required to remain within the range of the standard solution.

The multistage RBC bioreactor compartments were set in a cascade mode to remove the largest possible amounts of metal ions from the effluent. Samples (2.5 ml) were taken before pumping the effluent into the bioreactor to determine the initial metal concentration in the humic acid pre-treated synthetic effluent and thereafter on a daily bases for a period of one week. The samples were filtered using nitrocellulose filter membranes and stored at 4 ^{0}C in clean McCartney bottles until analysed by flame atomic absorption spectroscopy **(Section 2.7.1)**.



→ Flow direction

1 Initial Cr^{+3}, Pb^{+2} and Cu^{+2} concentrations were 200mg l^{-1}
2 Peristaltic pump operating at flow rate 0.7 mL min^{-1}
3 Peristaltic pump operating at flow rate 0.9 mL min^{-1}

Figure 5.1 Schematic representation of the treatment system showing influent solution, two columns in series containing humic acid, peristaltic pumps, reservoir, three RBC reactor compartments and effluent solution.

5.2.6 Desorption of the Adsorbed Metal Ions

In an attempt to recover bound metal ions from the biofilms, the RBC bioreactor compartments were drained and refilled with fresh, 0.1 M HCl metal desorbent, for 24 h in each desorption cycle, over a period of 72 hours. After each desorption cycle effluent samples (2.5 ml) were taken from each bioreactor compartment and filtered through nitrocellulose filter membranes and collected in clean McCarthy bottles. When necessary samples were diluted with distilled water to fall within the calibration range and metal analysis was performed using flame atomic absorption spectroscopy. Percentage metal recovery was determined in terms of total metal accumulation by the biofilms as follows (Atkinson *et al.*, 1998).

$$\text{Desorption efficiency} = \frac{\text{Quantity desorbed}}{\text{Quantity adsorbed}} \times 100$$

5.2.7 Dry Weight Analysis of the Biofilms

Prior to biofilm development and start-up of the metal ion adsorption process, the RBC discs with the attached polyester batting were air-dried overnight and then oven-dried for 24 h at 100 ^{0}C, and the weight of each disc recorded. Following biofilm development and completion of the adsorption process, the biofilms were washed repeatedly with 0.1M HCl to desorb the attached metal ions and finally, after washing with distilled water, the bioreactor compartments were drained and the immobilised cells air-dried overnight. The RBC discs with the biofilms attached to the polyester batting were then oven dried at 100 ^{0}C for 24 h. The weight of each disc with its attached biofilm was recorded and the difference between the final and initial weights was considered to represent biomass production, in grams, per disc and the amount of biomass (milligrams) produced in each bioreactor compartment per square centimetre of disc surface was calculated. The results obtained were used to estimate the amount of metal, in micrograms, adsorbed per gram of biomass in each of the three bioreactor compartments.

5.3 Results and Discussion

5.3.1 Effect of Humic Acid on the Concentration of a Single Metal Ion Species in Liquid Wastes

According to Itabashi *et al.* (2001) the availability of heavy metals in natural waters is largely dependent on the complexing ability of organic ligands such as humic and fulvic acids, hence, the determination of the amount of the organic ligand in a solution determines the concentration and the availability of metal ion species in that solution. To evaluate the complexing ability of humic acid for different metal ion species, liquid humic acid at different concentrations was added to different flasks containing single metal ion species and its efficacy in removing the ions was determined.

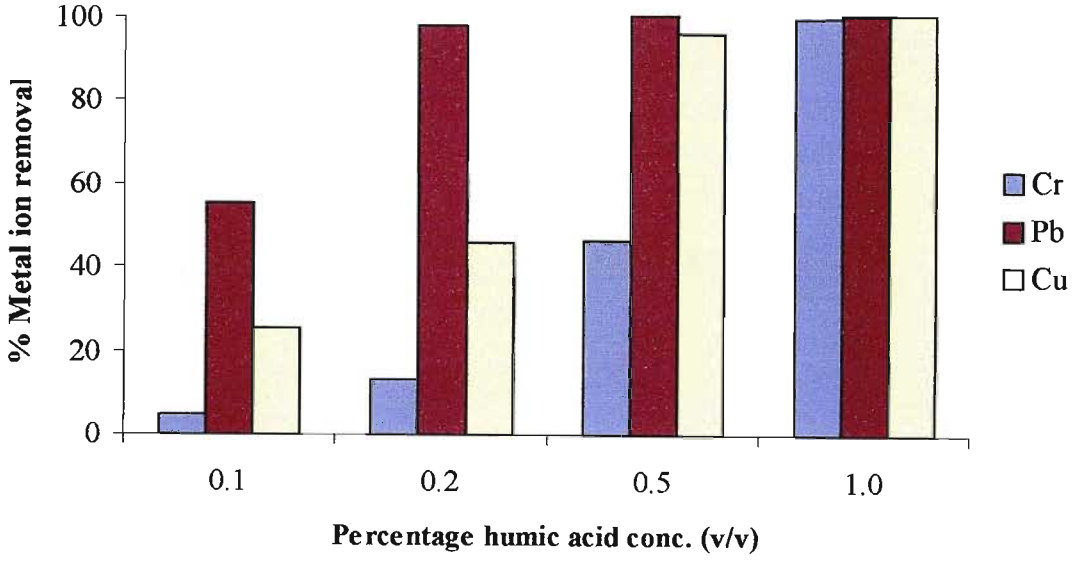


Figure 5.2 The effect of increasing concentrations of humic acid on the removal of single metal ion species from solution after 24 h treatment.

Figure 5.2 shows that after addition of 0.1% (v/v) humic acid to one litre of effluent containing a single metal ion species, more than 50% of the lead ions, about 25% of the copper and only about 5% of the chromium ions were removed from solution. As the

concentration of the humic acid in the solution was increased, so too did the percentage removal of the metals from the solution. The results indicated that humic acid has a high binding capacity for lead ions, less capacity for binding to copper ions and only poor chromium binding ability. The formation of such organo-metallic complexes reduced the bioavailability of the metal ions in the solution. The removal of chromate in significance required higher concentration of the complexing agent (humic acid), which might be due to the poor binding of the chromium ions with the humic acid **(Section 5.3.3)**.

5.3.2 Effect of Humic Acid on the Concentration of Mixed Metal Species in Liquid Wastes

The effect of humic acid on multiple metal ion species in solution was also evaluated by exposing an effluent containing equal concentrations of chromium, lead and copper ions to different humic acid concentrations for a period of 24 hours. Humic acid contains various functional groups including, carboxyl, phenol, hydroxyl and carbonyl structures of various types, and can form water-soluble and insoluble complexes (Francis, 1990). Each of these groups is capable of bonding with a cation by sharing a pair of electrons (Douglas *et al.*, 1992). Since all three metals tested competed strongly for binding sites on the surface humic acid a higher concentration of the acid was required to remove the metal ions from solution. The maximum metal complexing ability of the liquid humic acid was achieved at a humate concentration of 10 ml per litre of mixed metals containing solution. Above this value the metals become totally homogenized with the humate substances, making filtration and metal analysis difficult.

It is apparent from Figure 5.3 that as the humate concentrations in the system increased from 0.1% to 1% (v/v) the complexation of the metals ions also increased. Thus, the residual metal ion concentration in the effluent decreased as a function of the level of complexation with the humic acid. Of the three metal ion species in the effluent, about 68% of the chromium, 70% of the lead, and 53% of the copper ions were removed in

24 hours following the addition of 1%(v/v) humic acid. The percentage metal removed was calculated a follows: $\frac{\text{Initial metal concentration - Final metal concentration}}{\text{Initial metal concentration}} \times 100$

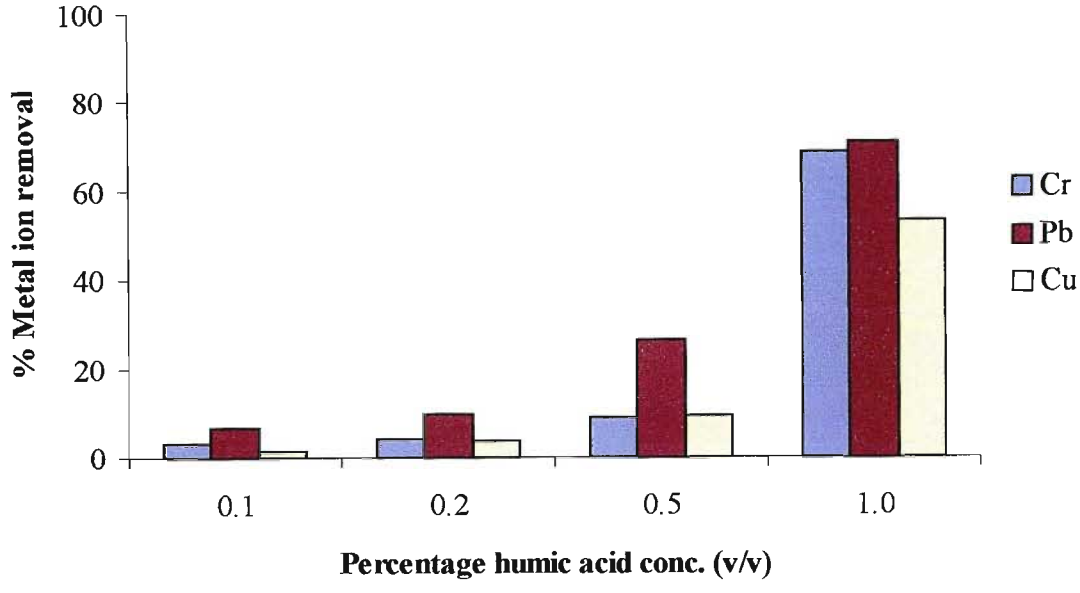


Figure 5.3 The effect of increasing humic acid concentration on the removal of metal ion species from a mixed metal containing effluent.

5.3.3 Time Dependency Studies on Humic Acid for the Removal of Soluble Metal Ion Species from Liquid Effluent

The effect of contact time between humic acid and soluble mixed metal ions was evaluated at different time intervals. Humate substances have different affinities towards various metal ions (Sanjay *et al.*, 1996); hence, as shown in Figure 5.4, during the first few hours of contact with the mixture of metal ions, the humic acid showed relatively higher affinities for the lead and copper ions than for the chromium ions. For example, after one-hour contact time, lead and copper removal was 88% and 61% respectively. However, with increased contact time the removal capacity for both these metals decreased so that at the end of the experiment (120 h) only 48% of the lead ions were removed, while the copper

concentration had returned to its original value. These findings are at variance with the results obtained by Sanjay *et al.* (1996) who, after treating Cr^{+3}, Pb^{+2} and Cu^{+2} with humic acid, concluded that increased contact time did not have any significant effect on the removal of lead and copper ions from solution, whereas chromium ions required longer treatment periods to reach the maximum removal rate. The chromium removal rate increased from 40% after the first hour of contact to 81% after 120 hours of contact between the metal ions and humic acid. As shown in Figure 5.4 as contact time increased, the chromium ions complexed more strongly with the various humate functional groups, resulting in desorption of the previously bound lead and copper ions into the solution, with concomitant increase in the percentage removal of chromium by the system. The fact that chromium removal efficiency increased on longer contact time and chromium was less effectively removed than lead and copper for a given humic acid concentration, suggesting that removal of chromium was possibly the rate-limiting step, requiring a higher concentration of the complexing agent (Sanjay *et al.*, 1996).

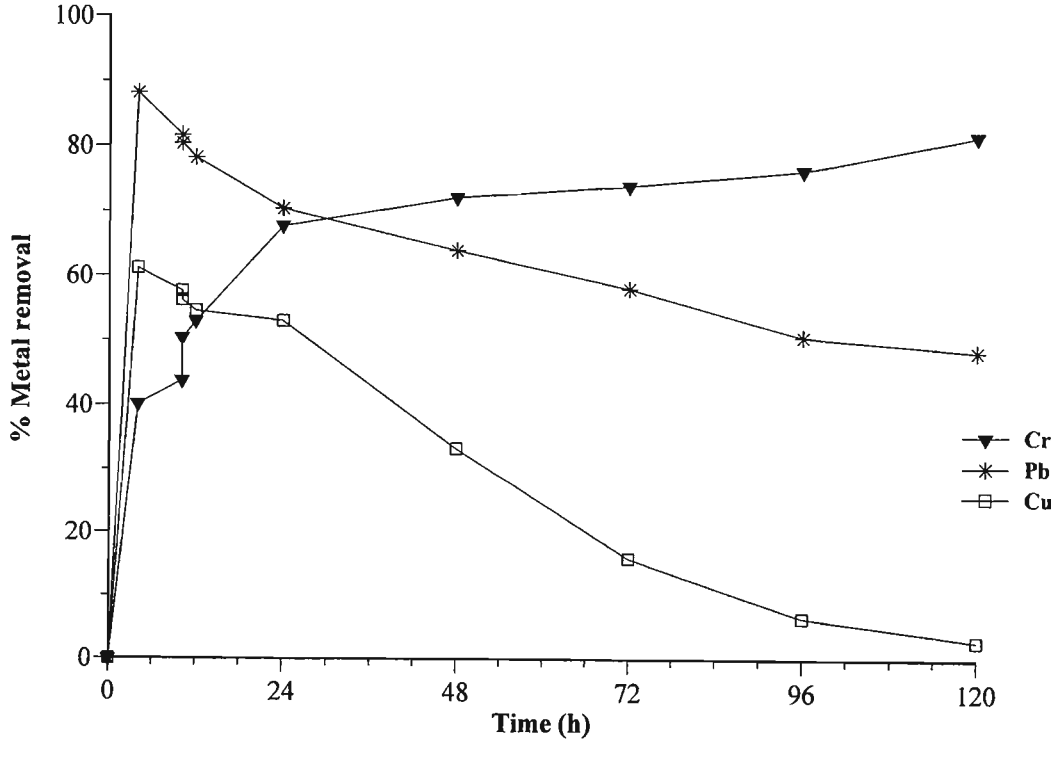


Figure 5.4 Percentage removals of metal ions after various contact times with humic acid, due to complexation.

The actual binding of metal ions to humic substances and the formation of complexes are not clearly understood. However, according to Petrovic *et al.* (1999) different reaction mechanisms are involved in interactions of metal ions with humic acid and what type of reaction predominates will depend on the type of metal ions present and the pH of the solution. Hence, the desorption of lead and copper from the system on one hand, and the increased removal rate chromium ions with time on the other hand, could arise either because of a change in pH of the solution or through variation in the mechanisms of the reactions involved in the complex formation. Consequently, the pH of each flask was measured before and after sampling (data not shown) and interestingly, no significant change was detected in any of the treatments with the pH ranging between 4.0 - 4.15. This implied that desorption of both lead and copper was not due to change in pH.

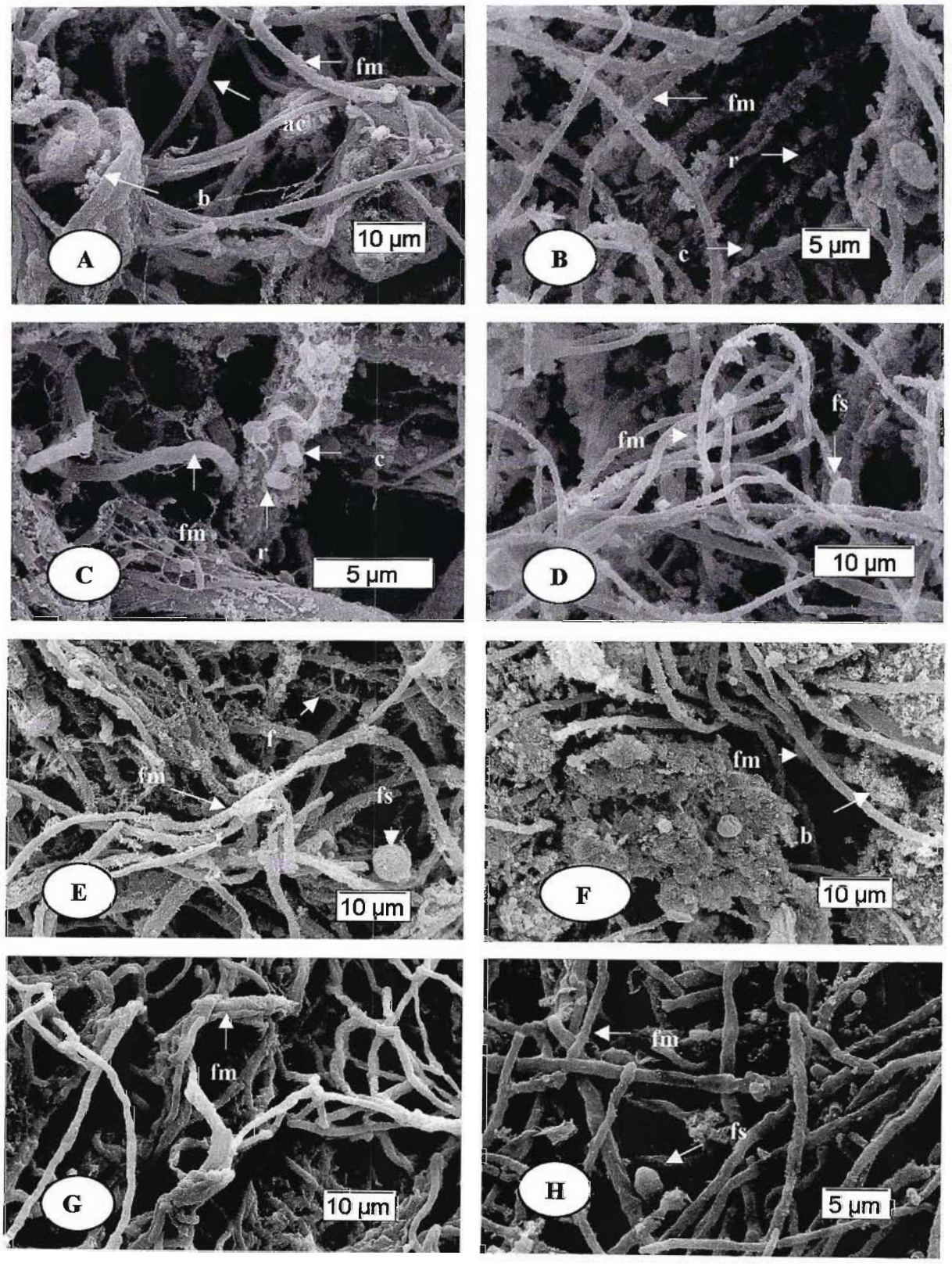
The reaction mechanisms involved in the interaction of metal ions with humic acid, include chelation, inner and outer sphere complex formation, adsorption and co-precipitation (Petrovic *et al.*, 1999). From this it can be inferred that the desorption of the lead and copper ions from the system and concomitant increased removal of chromium ions with time, could be the result of difference in the mechanisms of the three metal ion species interaction with the humic acid, resulting in change in the equilibrium of the reaction. Generally an increase in time of contact of the metal ions with humic acid favoured the removal of chromium from the solution. However, during the initial stage the humic acid had a relatively higher affinity for the lead and copper ions than for the chromium ions. According to Douglas *et al.* (1992) chemical reactions are seldom complete; instead, after sufficient time has elapsed they reach a state of chemical equilibrium in which the concentration of reactants and products is a constant ratio. After determining the residual concentrations of each metal ion species and calculating the percentage removal of the metals by humic acid after different time intervals in triplicate flasks, mean values were computed. The values obtained were similar to those determined by AAS after 24 hours of treatment. It was thus assumed that equilibrium was achieved at this time when approximately 68% chromium, 70% lead and 53% copper ions had been removed by the humic acid.

5.3.1 Development of Biofilms on the RBC Discs

The ESEM investigation of the biofilms removed from the three bioreactor compartments after different periods of time revealed that the biofilms comprised a variety of microbial cells, predominantly fungal mycelium but with numerous rod-and coccus-shaped bacterial cells, some filamentous organisms, actinomycetes and some fungal spores also present. The presence of a diverse microbial population was considered to be an advantage for the removal of heavy metals from the synthetic effluent. Micrograph **Plate 5.1 B, C and F** illustrate the presence of fungal mycelia and clusters of bacteria cells; including rods and cocci, in biofilm samples removed from Bioreactor compartments 1, 2 and 3 respectively. Low magnification micrographs illustrate the presence of fungal mycelium, some bacterial clusters and actinomycetes in biofilm removed from Bioreactor 1 **(Plate 5.1 A)**. **Plate 5.1 D** shows the presence of numerous fungal hyphae and spores in 5-week-old biofilm removed from Bioreactor 2. Biofilm removed at the same time from Bioreactor 3 also showed the presence of some filamentous organisms, fungal mycelia and spores **(Plate 5.1 E)**.

Throughout the duration of the experiment the distribution, biomass growth and composition of the biofilm was very similar in all three bioreactor compartments. Micrographs of biofilms **(Plate 5.1 G and H)** removed from Bioreactors 1 and 2 respectively, at the end of the experiment (week 7), clearly illustrate this and show a predominance of fungi biomass.

Plate 5.1 Scanning electron micrographs illustrating the development of the consortia of microbial species on the surface of the RBC discs from the different bioreactor compartments at various times. A, low magnification of a mature biofilm removed at week 5 showing the presence of fungal mycelium (fm), clusters of bacteria cells (b) and some actinomycetes (ac); B and C, high magnification of the biofilms removed from Bioreactor 1 and Bioreactor 2 respectively at week 6, revealing the presence of rods (r), and cocci (c) together with fungal mycelium (fm); D, low magnification of mature biofilm removed from Bioreactor 2 illustrating the presence of masses of fungal mycelium (fm) and some spores (fs) removed at week 5; E, shows the presence of some filamentous organisms (f), fungal mycelium (fm) and spores (fs) on mature biofilm removed at week 5 from Bioreactor 3; F, clusters of bacterial cells (b) and fungal mycelium (fm) and, possibly, metal deposits on the surface of the biofilm removed at week 6 from Bioreactor 3; G, low magnification of biofilm removed from Bioreactor 1 at the end of the experiment (week-7) comprising predominantly fungal mycelia (fm); H, high magnification of the biofilm removed from Bioreactor 2 at week 7 comprising mainly fungal mycelia and some spores.


fm
ac
b
10 µm
A
fm
r
c
5 µm
B
c
fm
r
5 µm
C
fm
fs
10 µm
D
f
fm
fs
10 µm
E
fm
b
10 µm
F
fm
10 µm
G
fm
fs
5 µm
H

5.3.2 Complexation of Metals by Humic Acid and their Bioaccumulation in the RBC Compartments

Humic acid, although effective in removal of large amounts of metal ions from wastewaters does not meet the WHO guidelines nor satisfy other worldwide regulations for permissible concentrations of heavy metals in water streams. According to the WHO guidelines the acceptable concentrations of Cr^{+3}, Pb^{+2} and Cu^{+2} in drinking water are 50 μg l^{-1}, 10 μg l^{-1} and 20 mg l^{-1}, respectively (Muhammad *et al.*, 1997). Furthermore, in the city of Durban, South Africa, no trade effluent is acceptable for discharge into water streams unless it complies with the regulations regarding metal ion concentrations. The bylaws state that effluent shall not contain concentrations of these metals in excess of 5 mg l^{-1} for small operations (Durban Metro, 2004). Thus, after removing the bulk of metal ions with humic acid, the application of supplementary metal ion removal technology is important for an effective wastewater treatment process. To this end a multistage RBC bioreactor, operated in continuous mode, was set-up to remove the residual low concentration of metal ions from the effluent **(Figure 5.1)**.

Table 5.1 Overall metal removal from a synthetic effluent solution by a combination of humic acid precipitation and RBC biofilm activity

Metals	Conc. (mg l^{-1})		% Metal Removal by Humic acid in:		% Metal Removal in the three RBC Compartments	Overall% Metal Removal
	Initial	Final	Column 1	Column 2		
Cr^{+3}	175.98	2.44	68.07	18.32	12.22	98.61
Pb^{+2}	177.89	3.41	70.35	16.43	11.30	98.08
Cu^{+2}	189.58	18.61	52.73	20.30	17.05	90.20

The percentage chromium removed by the combined system over the seven-day period was approximately 99%. Complexation with humic acid in Columns 1 and 2 accounted for approximately 68% and 18% Cr^{+3} removal respectively, after a retention time of 24 h in each column. A further 12% of the Cr^{+3} was removed in the three RBC compartments. Figure 5.5 illustrates the contribution of Bioreactor compartments 1, 2 and 3 to the removal of the Cr^{+3}, Pb^{+2} and Cu^{+2} ions in the RBC bioreactor. For chromium Bioreactor

compartment 1 was responsible for approximately 48% removal while bioreactor compartments 2 and 3 removed 27% and 25%, respectively, of the total chromium removed in the RBC.

Overall lead removal by the system was approximately 98%. Lead was removed predominantly by humic acid complexation where Column 1 accounted for more than 70% of the total lead removed and Column 2 for 16%. The three RBC compartments accounted for only 11% of the total lead removed, of which Bioreactor compartment 1 removed 44% and the other two compartments removed approximately 28% each **(Figure 5.5)**. Even though, Bioreactor compartment 1 removed substantially large amounts of chromium and lead from effluent, nevertheless both Bioreactor compartments 2 and 3 also contributed significantly to the removal of Cr^{+3} and Pb^{+2} ions from the metal contaminated synthetic effluent over the entire duration of the experiment.

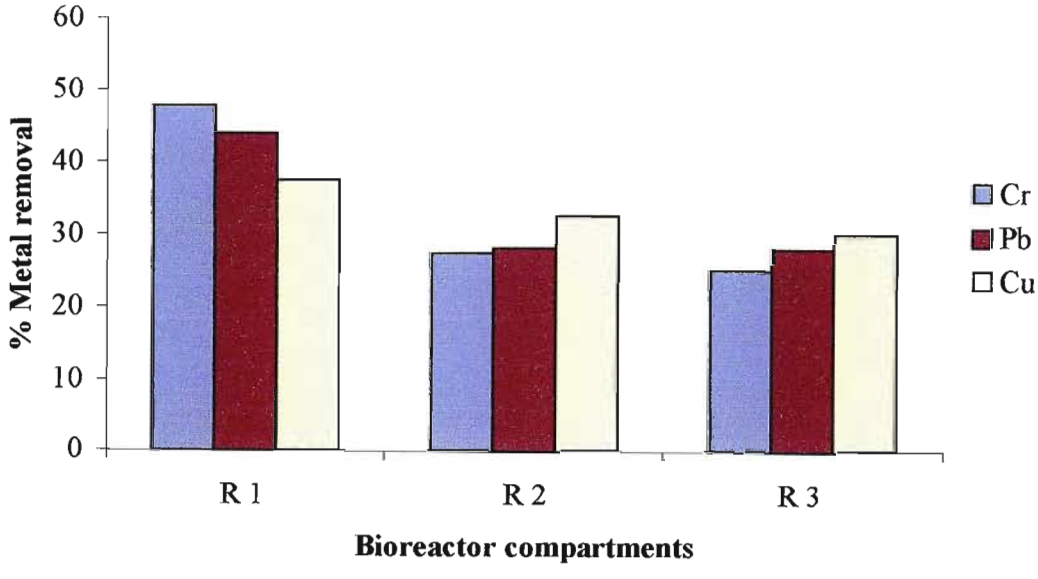


Figure 5.5 Percentage metals removed from the metal contaminated effluent over five days by the biofilms in the three RBC compartments.

The overall percentage copper removed by the whole system was approximately 90%. Of this complexation with humic acid was the predominant factor accounting for approximately 53% of the total copper removal in Column 1 and 20% in Column 2. The three RBC compartments played a more significant role in the removal of copper ions than they did during the removal of both chromium and lead; approximately 17% of the total copper removal occurred in the three compartments **(Table 5.1)**. Approximately the same amount of copper was removed in each bioreactor compartment, viz. Bioreactor compartment 1 accounted 37% removal while Bioreactors 2 and 3 removed 33% and 30% of the Cu^{+2} respectively. Comparing the removal capacity of the three RBC compartments for all three metal ions, showed that copper was removed mainly in Bioreactor compartments 2 and 3, which may have been due to the removal of large amounts of the competing ions, chromium and lead in the humic acid columns and in Bioreactor compartment 1. In Chapter 3 it was suggested that the presence of competing metal ions suppressed the uptake of copper from the effluent solution. Thus the relatively low concentrations of both chromium and lead ions encountered in the RBC compartments (due to their earlier complexation with the humic acid) may have resulted in an increased removal rate of copper ions in the RBC bioreactors. Similar high values for copper accumulation were reported by Costley and Wallis (2001) after investigating the metal removal ability of biofilms over a 12-week period while growing in a metal contaminated effluent solution containing Cu^{+2}, Zn^{+2} and Cd^{+2}.

5.3.3 Desorption of Adsorbed Metal Ions

Guy *et al.* (1975) and Gardea-Torresdey *et al.* (1998) suggested that bound metal ions could be removed from the microbial biomass by lowering the pH of the solution and it was presumed that under such condition protons would displace the adsorbed metal ions, thereby resulting in recovery of the bound metals. Numerous investigators have used HCl as a striping agent for the recovery of metals bound to various substances. Heavy metals adsorbed onto immobilized cells in a bioreactor were recovered by eluting with 0.1 M HCl. After treatment the biofilms could be used for at least five adsorption-desorption cycles without loss of their metal binding capacity, however, some unrecoverable metals might be trapped intracellularly (Wong *et al.*, 1993).

Costley and Wallis (2001) found that repeated acid washing of the biofilms caused decolourisation in the biomass. As a result, the colour of the biofilm changed from deep blue to a light green shade and finally to a brown colour, which suggested significant desorption of metals from the biomass. Similarly, in the present investigation, the colour of the biofilms changed from light brown to deep brown as the metal impregnated biomass was repeatedly washed with 0.1 M HCl. This visual effect confirmed significant desorption of the adsorbed metals had occurred. AAS analysis also confirmed that large amount of the bound metals had been desorbed from biomass. The total percentage recovery of chromium, lead and copper was computed with respect to the total amount of metals adsorbed and it was found that 75% of the Cr^{+3}, 92% of the Pb^{+2} and 86% of the Cu^{+2} ions were recovered from the three RBC bioreactor compartments after repeated exposure of the biofilms to 0.1 M HCl. All three bioreactor compartments showed a similar pattern of desorption of the metal ions **(Figure 5.6 B-D)**. Atkinson *et al.* 1998 suggested that the desorption efficiency of the desorbing agent is directly proportional to the amount of metal bound to the biomass.

Table 5.2 Comparison of the overall mean concentrations of the metal ions desorbed from the three bioreactor compartments in the three acid treatment cycles

Cycles of treatment with HCl	Cr^{+3} conc. mg l^{-1}	Pb^{+2} conc. mg l^{-1}	Cu^{+2} conc. mg l^{-1}
Cycle-1	3.70	51.60	52.75
Cycle-2	16.27	12.50	12.85
Cycle-3	21.64	1.00	5.03
Total desorbed metal ions	41.61	65.1	70.63
LSD (5% level)	3.37	6.63	4.87
P-values	<0.001*	<0.001*	<0.001*

* Highly significant difference at P value <0.05

Analysis of variance of the mean metal ion concentrations desorbed from the biofilms in all three bioreactor compartments showed significant differences (P<0.001) between the concentrations of each metal ion desorbed following each acid treatment cycle **(Table 5.2)**.

Comparison of the recoveries of each metal ion species after the three treatment cycles revealed that large amounts of the total adsorbed lead and copper ions were desorbed from the biofilms following the first cycle of acid washing. The respective desorption rates of lead and copper after the first wash were approximately 73% and 76%, respectively **(Figure 5.6 A)**. However, the recovery of chromium ions following the first cycle of acid treatment was only 9% of the total adsorbed Cr^{+3} ions. This could be due to either intracellular-uptake of the chromium ions by the biofilm microorganisms or the chromium ions resisted displacement by the H^{+} group of dilute hydrochloric acid, resulting in a low recovery rate of chromium ions from the biomass. Guy *et al.* (1975) found that lowering the pH of a system destabilized the adsorbed metals, which resulted in desorption of a large proportion of the metal ions into the solution. Thus, as the biofilms were repeatedly treated with HCl the desorption rate of chromium ions increased, indicating that application of more concentrated HCl could possibly achieve complete removal of the adsorbed chromium from the biomass after a single treatment only. Percentage desorption of the metals after the second washing cycle was 38% of the total Cr^{+3}, 25% of the total Pb^{+2} and 17% of the total adsorbed Cu^{+2}, and the relative desorption of Cr^{+3}, Pb^{+2} and Cu^{+2} after the third cycle was 54%, 2% and 7%, respectively. Gardea-Torresdey *et al.* (1998) also suggested that the usage of stronger striping agents like EDTA could facilitate the recovery of high percentage of adsorbed chromium and cadmium. However, this could only be applied to dead cells because the biofilms might be damaged and could no longer be used for cyclic adsorption-desorption cycles. Furthermore, HCl is cheaper than EDTA (Wong *et al.*, 1993); hence, 0.1 M HCl was used for eluting the biofilms to recover the metals from the bioreactors in the present investigation.

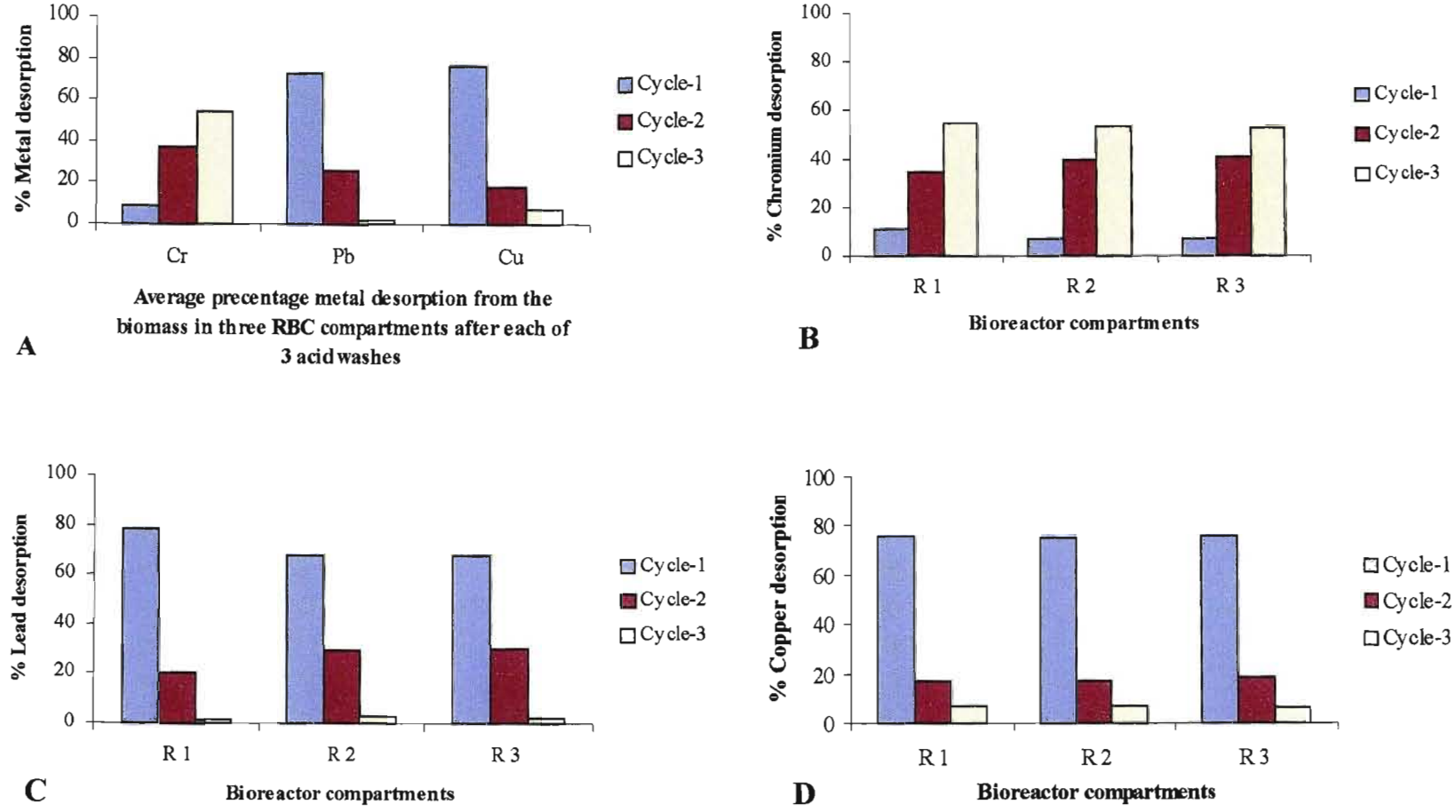


Figure 5.6 Percentage metal desorption in the three RBC bioreactor compartments after elution of the biofilms with 0.1M HCl in three cycles. A, total metal desorption; B, chromium desorption; C, lead desorption; D, copper desorption.

N.B: R 1, R 2 and R 3 = Bioreactor compartments 1, 2 and 3.

5.3.4 Biomass Quantification

The amount of biomass produced in each bioreactor compartment and the amount of metals associated with the biofilms was determined. It was found that significant amounts of biomass were produced in each bioreactor compartment over the entire course of the experiment. RBC discs removed from the same bioreactor compartment carried similar amounts of biomass determined by dry weight **(Appendix 3)**, hence, disc position relative to the influent pipe within a specific bioreactor compartment had an insignificant effect on the development of the biomass. In addition to this, the surfaces of each disc within a compartment were compared for the development of biomass and in most instances it was found that the downstream side of the discs produced slightly higher amounts of biomass (dry weight) than the upstream side, however the differences were insignificant **(Appendix 3)**.

The average biomass yields per disc (biofilm removed from both surfaces) in Bioreactor compartments 1, 2 and 3 were 4.31 g, 3.65 g and 2.93 g, respectively. There was significant difference in total biomass within the three bioreactor compartments; significantly more biomass developed in Bioreactor compartment 1 than the other two compartments. This variation occurred because the influent solution containing the nutrient source voermolas was pumped into the bioreactor and circulated from compartment 1 into compartment 2 and then into compartment 3. Compartment 1 thus, received higher concentration of voermolas, which resulted in the development of larger quantities of biomass. Hence, the average biomass dry weight per cm^2 of a disc surface area in bioreactor compartments 1, 2, and 3 was 17.55 mg, 14.86 mg and 11.93 mg, respectively. Similar variations were reported by Sudo *et al.* (1977) who, in a four compartment RBC, obtained a yield of approximately 7.0 mg of biomass per cm^2 of disc surface in the first two compartments and only about 2.0 mg of biomass per cm^2 of disc surface from the third and fourth compartments when treating domestic wastewaters.

Table 5.3 Amounts of biofilm-associated metal ions in the three RBC compartments

Metal Ion species	Metals (μg) per milligram of biomass		
	Compartment 1	Compartment 2	Compartment 3
Cr^{+3}	3.21	2.16	2.47
Pb^{+2}	2.70	2.10	2.51
Cu^{+2}	3.74	3.84	4.41

Even though there were significant differences in biomass production between the three bioreactor compartments. However, there was only small variation between the compartments in terms of metal ion accumulation **(Table 5.3)**. The biomass in compartment 1 accumulated relatively higher levels of chromium and lead ions than did the biomass in the other two compartments, while compartment 3 accumulated larger amounts of copper. Thus, when equating the amount of metals accumulated with the amount of biomass produced in each compartment, it became clear that the amounts of metal ions associated with the biomass in compartments 3 was higher than those within compartment 1 and 2. These findings support the results obtained by Sakaguchi (1979), who found that decreased metal uptake is usually associated with increasing biomass concentration. Similarly, Tsekova and Petrov (2002) confirmed that the amount of heavy metal adsorbed per unit weight of biomass was higher at lower biomass concentrations than at high biomass concentrations.

5.4 Conclusions

Combining humic acid precipitation and microbial adsorption for treatment of metal ion contaminated effluents improved the economy of the treatment process because much larger quantities of the heavy metals could be removed in a shorter period of time than was possible when using the individual treatments separately. The humic acid efficiently removed chromium and lead ions from the effluent, even though initially a higher affinity towards lead and copper ion was observed. Similarly, both chromium and lead were removed most efficiently in the first compartment of the RBC bioreactor. The high percentage removal of chromium and lead ions during the initial stages of the treatment

process reduced the number of competing metal ions in the effluent with the result that copper was successfully removed in the second and third bioreactor compartments. Hence, the results indicated that introduction of humic acid into a compartmentalised RBC bioreactor improves the potential of the treatment system in large-scale by reducing the toxicity and competing effects of the metal ions to the microbial biofilms.

A large proportion of the bound metal ions could be desorbed from biomass by eluting the metal impregnated biofilms repeatedly with 0.1 M HCl. Lead and copper ions were easily recovered following a single treatment cycle; however, chromium was desorbed only after repeated treatment of the biofilms with dilute acid. The use of concentrated HCl might facilitate the removal rate of chromium, but care should be taken not to affect sustainability of the metal recovery ability of the biofilm by killing the cells. Killed cells would still remove soluble metal ions but the biofilm could not regenerate and hence, the economy of the process would be negatively impacted on.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUDING REMARKS

The application of immobilised microorganisms for biological wastewater treatment is becoming more popular because of the benefits offered by the biofilms; these include the active building-up of biomass in bioreactor compartments and its maintenance through attachment to solid surfaces. Many previous investigators used half-strength nutrient broth for establishment of biofilms and determination of the ability of the biofilms to remove metal ions from solution. However, half-strength nutrient broth was found to precipitate the metal ions causing a significant reduction in their availability to the biofilm microflora. Hence, in this study voermolas was used in the preparation of the metal containing synthetic effluent used to evaluate the metal removal capacity of the biofilms that developed in the RBC bioreactor compartments. Accordingly, 0.1% (v/v) voermolas, which was shown to complex minimally with the metals, while concomitantly providing the necessary carbon and energy source for microbial cell growth, was used throughout this investigation when formulating the synthetic effluent. Consequently, biofilms comprised of a variety of microbial cells (predominantly fungal biomass but with some rod-and coccus-shaped bacterial cells present) could be established to tolerate and grow in the presence of higher concentrations of Cr^{+3}, Pb^{+2} and Cu^{+2}. Such metal-adapted biofilm microflora were capable of removing larger amounts of the metal ion species from contaminated effluents than were their planktonic counterparts thus further enhancing the efficiency of the reactors.

Successful biological treatment of heavy metal polluted wastewater depends on the development and maintenance of an active and appropriate microbial biofilm on a suitable support matrix. Various materials, including gravel, polyester batting and sand were used as support matrices for the establishment of biofilms in the separate RBC bioreactor compartments. Of these polyester batting was most quickly colonized by the microorganisms during the biofilm establishment process. This suggested that porous support matrices are most suitable for microbial immobilization because they usually provide more surface area for microbial loading and the pores also protect the cells from

the share forces arising through turbulence in bioreactor compartments when operating at high flow rates. Likewise surface roughness of the support matrix as occurs with gravel and sand allowed an increased rate of microbial colonisation by protecting the microorganisms from such fluid dynamic shear forces. However, both gravel and sand added too much extra-weight to the RBC discs, which in turn caused stress damage to the motor during operation. Thus, polyester batting would provide a better matrix for microbial attachment in industrial scale RBC bioreactors than would the other two support matrices by providing a porous hydrophilic surface for quick colonisation and a larger surface area for microbial adhesion and subsequent biofilm formation. However irrespective of the attachment matrix present the predominantly fungal biofilms showed the same order of affinity for the metals tested, viz. $Cr^{+3} > Pb^{+2} > Cu^{+2}$. EDX spectral analyses also showed that the metal ions accumulated at different times were associated with the biofilms. Other advantages of polyester batting are that it is relatively cheap and readily available.

Mixed microbial cultures have been successfully used in the treatment of a wide range of contaminants in liquid industrial wastes. In such systems some resistant species that can accumulate various toxic metal ions may exert a protective effect on sensitive species in the mixed population by removing the metals from solution. Different and varied microbial populations were established in the three RBC bioreactor compartments using polyester batting as support matrix and the metal removal abilities of the biofilms determined. Interactions between bacterial and fungal components enhanced the mechanical stability of the biofilm so that comparatively larger amounts of Cr^{+3}, Pb^{+2} and Cu^{+2} ions were removed by this biofilm over time. The removal of copper in particular was enhanced in the mixed microbial biofilm when compared to its removal rate by biofilms comprising largely fungal or bacterial biomass. The amount of lead ions taken up, however, was essentially the same in all the treatments over the entire duration of the experiment, indicating that all the biofilms tested readily accumulated lead ions from solution.

The types of microorganisms in the microbial populations present in the inoculum were found to influence metal removal from mixed metal ion contaminated solutions. This suggests that different microorganisms have different preferences for the metal ions they

will most actively adsorb. Comparing the metal removal ability of the biofilms established in this study showed that the fungus-dominated biofilm selectively accumulated chromium ions whereas the mainly bacterial biofilms more readily removed the copper ions from the mixed metal contaminated effluent solution. Since bacteria and fungi differ with respect to the chemistry of their cell walls, this might account for the preferential adsorption of specific metal ions displayed by the biofilms used to treat the mixed metal ion contaminated effluent solution. However, the actual mechanism governing this preferential adsorption was not elucidated and further investigation is necessary. In addition, further studies on specific isolates of bacterial and fungal strains and their efficacy for the removal of specific metal ion species should be undertaken.

The rate of influx of heavy metals into the environment is exceeding the capacity of all traditional wastewater treatment methods to produce high quality effluent at reasonable cost. Although microbial systems work well for the removal of metal ions in low concentrations, they cannot treat successfully seriously contaminated industrial effluents, as the microbial surfaces rapidly become saturated in the presence of high concentrations of metal ions. Hence, a series of bench-scale columns that contained humic acid was used to pretreat an artificial effluent before it was introduced into a three stage RBC bioreactor, and the combined effect of this dual system for the removal of Cr^{+3}, Pb^{+2} and Cu^{+2} from a mixed metal contaminated effluent was determined. After 24 hours contact the humic acid displayed a higher affinity for chromium and lead ions than for copper ions. Similarly, both chromium and lead were removed most efficiently in the first compartment of the RBC bioreactor. The high percentage removal of chromium and lead ions during the initial stages of the treatment process reduced the number of competing metal ions in the effluent with the result that copper could be successfully removed in the second and third bioreactor compartments. Hence, the introduction of humic acid into a compartmentalised RBC bioreactor improved the economy of the treatment process by removing larger quantities of the heavy metals in a shorter period of time than was possible when using the individual treatments separately.

A large proportion of the heavy metals adsorbed onto the immobilized cells in the bioreactor compartments could be recovered, without losing the metal binding capacity of the biofilms by eluting with 0.1 M HCl. Lead and copper ions were easily recovered following a single acid treatment cycle; whereas, chromium was desorbed only after repeated treatment of the biofilms with dilute acid. The use of more concentrated HCl or EDTA might accelerate the rate of chromium removal, however, care should be taken not to affect the sustainability of the metal recovery ability of the biofilm by killing the cells. This could impact negatively on the economy of the treatment process by reducing the number of adsorption-desorption cycles that the biofilm could withstand.

A major problem encountered during this research was the evaporative loss of liquid from the system. This caused the effluent to become more and more concentrated which resulted in erroneous AAS results. To counteract this loss of liquid, diluted voermolas was pumped into the RBC bioreactor compartments at a very low flow rate (0.2 ml min^{-1}) to replenish the loss of water from the system while concomitantly serving as a source of carbon and energy for the biofilm microflora. In future studies undertaken to remove metal ions using microbial biofilms, it will be necessary to introduce control mechanisms in the wastewater laboratory at the University of KwaZulu-Natal.

REFERENCES

Abel, P.D. 1989. *Water Pollution Biology*, Ellis Horwood Limited, England.

Aiken, H., Govers, H. and Van't Riet, J. 1985. Detoxification of mercury, cadmium and lead in *Klebsiella aerogenes* NTCT418 growing in continuous culture. *Applied and Environmental Microbiology* **50**, 1262-1267.

Andrews, J.F. 1971. Kinetic models of biological waste treatment process. In Canale, R.P. (ed). *Biological Waste Treatment*, Interscience Publishers, New York, 5-34.

Ashida, J. and Nakamura, H. 1959. Role of sulphur metabolism in copper resistance of yeast. *Plant Cell Physiology* **1**, 71-79.

Atkinson, B.W., Bux, F. and Kasan, H.C. 1998. Waste activated sludge remediation of metal plating effluents. *Water SA* **24**, 4, 355-359.

Babich, H. and Stotzky, G. 1977a. Effect of cadmium on fungi and on interactions between fungi and bacteria in soil: influence of clay minerals and pH. *Applied and Environmental Microbiology* **33**, 5, 1059-1066.

Babich, H. and Stotzky, G. 1977b. Sensitivity of various bacteria, including actinomycetes, and fungi to cadmium and the influence of pH on sensitivity. *Applied and Environmental Microbiology* **33**, 5, 681-695.

Babich, H. and Stotzky, G. 1980. Environmental factors that influence the toxicity of heavy metals and gaseous pollutants to microorganisms. *CRC Critical Reviews in Microbiology* **8**, 99-145.

Bader, J.L., Gonzalez, G. and Goodell, P.C. 1999. Chromium-resistant bacterial population from a site heavily contaminated with hexavalent chromium. *Water, Air, and Soil Pollution* **109**, 263-276.

Barnes, D., Bliss, P.J., Gould, B.W. and Vatlentine, H.R. 1981. *Water and Wastewater Engineering Systems*, Pitman Books Ltd, London.

Bercovitz, K. and Laufer, D. 1992. Health effect of metals. In Venet J.P. (ed). *Impact of Heavy Metals on the Environment*, Elsevier Amsterdam, 1-13.

Beveridge, T.J., Forsberg, C.W. and Doyle, R.J. 1982. Major sites of metal binding in *Bacillus licheniformis*. *Journal of Bacteriology* **150**, 1438-1448.

Beveridge, T. J. and Fyfe, W.S. 1985. Metal fixation by bacterial cell walls. *Canadian Journal of Earth Science* **22**,1893-1898.

Beveridge, T.J., Hughes, M.N., Leung, K.T., Poole, R.K., Savvaidis, I., Silver, S. and Trevors, J.T. 1997. Metal-microbe interactions: contemporary approaches. In Poole, R.K. *Advances in Microbial Physiology* **38**, 192-205.

Beveridge, T.J. and Koval, S. F. 1981. Binding of metals to cell envelopes of *Escherichia-coli* K-12. *Applied and Environmental Microbiology* **42**, 325-335.

Bishop, P.L. and Kinner, N.E. 1986. Aerobic fixed-film processes. In Rehm, H.J. and Reed, G. (eds). *Biotechnology*, Vol. 8: Microbial Degradation, VCH Publishing Weinheim, 113-176.

Bitton, G. 1994. *Wastewater Microbiology*, Wiley-Liss, New York.

Blackman, I.C. and Frank, J.K.1996. Cited by Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G. Banerjee, S. and Farber, J.M. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology* **91**, 725-734.

Blanco, A., Sanz, B., Llama, M.J. and Serra, J.L. 1999. Biosorption of heavy metals to immobilized *Phormidium laminosum* biomass. *Journal of Biotechnology* **69**, 227-240.

Borchardt, J.A. 1971. Biological waste treatment using rotating disc. In Canale, R.P. (ed). *Biological Waste Treatment*. Interscience Publishers, New York, 131-140.

Brierley, J.A. 1990. Production and application of a Bacillus-based product for use in metal biosorption. In Volesky, B. (ed). *Biosorption of Heavy Metals*, CRC Press Boca Raton, 305-311.

Bryers, J.D. and Characklis, W.G. 1990. Biofilms in water and wastewater treatment. In Characklis, W.G. and Marshall, K.C. (eds). *Biofilms*. John Wiley and Sons, Inc, New York, 671-696.

Bryers, J.D. and Hamer, G. 1988. Application of immobilized captured microorganisms in water purification. *Techniques and Aspects of Enzymes and Cells*, Vol. 136, Academic Press Inc, New York, 697-711.

Brynhildsen, L., Lundgren, B.V., Allard, B. and Rosswall, T. 1988. Effect of glucose concentrations on cadmium, copper, mercury and zinc toxicity to a *Klebsiella* sp. *Applied and Environmental Microbiology* **54**, 1689-1693.

Characklis, W.G. 1983. Process analysis in microbial system: biofilms as a case study. In Bazin M. (ed). *Mathematics of Microorganism*. Academic Press, London, 171-234.

Characklis, W.G. 1990. Biofilm processes. In Characklis, W.G. and Marshall, K.C. (eds). *Biofilms*. John Wiley and Sons, Inc, New York, 195-231.

Characklis, W.G. Marshall, K.C. and McFeters, G.A. 1990. The microbial cells. In Characklis, W.G. and Marshall, K.C. (eds). *Biofilms*. John Wiley and Sons, Inc, New York, 131-159.

Collins, Y.E. and Stotzky, G. 1989. Factors affecting the toxicity of heavy metals to microbes. In Beveridge, T.J. and Doyle, R.J. (eds). *Metal Ions and Bacteria*. Wiley, New York, 31-90.

Costley, S.C. 1999. Removal of heavy metals from wastewaters using rotating biological contactors. *PhD thesis,* University of Natal, South Africa.

Costley, S.C. and Wallis, F.M. 1999. Effect of disc rotational speed on heavy metal accumulation by rotating biological contactor (RBC) biofilms. *Letters of Applied Microbiology* **29**, 401-405.

Costley, S.C. and Wallis, F.M. 2000. Effect of flow rate on heavy metal accumulation by rotating biological contactor (RBC) biofilms. *Journal of Industrial Microbiology and Biotechnology* **24**, 244-250.

Costley, S.C. and Wallis, F.M. 2001. Bioremediation of heavy metals in a synthetic wastewater using a rotating biological contractor. *Water Research* **35**, 15, 3715-3723.

Couillard, D. and Shucai, Z. 1992. Bacterial leaching of heavy metal from sewage sludge for agricultural application. *Water, Air, and Soil Pollution* **63**, 67-80.

Da Costa, A.C.A. and Duta, F.P. 2001. Bioaccumulation of copper, zinc, cadmium and lead by Bacillus sp. *Bacillus Cereus*, *Bacillus Sphaericus* and *Bacillus Subtilis*. *Brazilian Journal of Microbiology* **32**: 1-5.

Dart, R.K. and Stratton, R.J. 1980. *Microbiological Aspect of Pollution Control*, 2nd ed. Elsevier Scientific Publishing Company, Amsterdam.

Dong, D., Derry, L.A. and Lion, L.W. 2003a. Pb scavenging from a freshwater lake by Mn oxides in heterogeneous surface coating materials. *Water Research* **37**, 1662-1666.

Dong D., Li Y., Zhang, J. and Hua, X. 2003b. Comparison of the adsorption of lead, cadmium, copper, zinc and barium to freshwater surface coatings. *Chemosphere* **51**, 369-373.

Douglas, A. S., Donald, M.W. and James, F.H. 1992. *Fundamentals of Analytical Chemistry,* 6th ed. Saunders College Publishing, San Diego.

Durban Metro. 2004. Schedule A: sewage disposal bylaws, pollution management. http://durban.gov.za/wastewater/tariff/sewagesbylaws/schedulesea.htm [Accessed on April 2004].

Ebdon, L. 1982. *An introduction to Atomic Absorption Spectroscopy*. Heyden and Son Ltd, London, 42-56.

Engelder, C.L. and Matson, S.A. 1991. Bioremediation of ethanol and petroleum hydrocarbons contaminated groundwater using a rotating biological contactor, *45th Purdue University Industrial Water Conference Proceedings*, Lewis Publishers Inc, Chelsea, 286-294.

Environmental Management Division of Hong Kong. 1991. *A Study on Industrial Waste Exchange and Recycling for the Electroplating Industry.* Hong Kong Productivity Council, Hong Kong.

Ewers, U. and Schlipkoter, H. 1991. Lead. In Merian, E. (ed). *Metal and Their Compound in the Environment*. VCH, Weinheim, 971-1014.

Fletcher, M. 1979. The attachment of bacteria to surfaces in aquatic environments. In Ellwood, D.C. and Melling, J. (eds). Adhesion of Microorganisms to Surfaces. *Society for General Microbiology Publishers*, London, 87-108.

Francis, A.J. 1990. Microbial dissolution and stabilization of toxic metals and radionuclides in mixed wastes. *Experientia* **46**, 840-851.

Freitas dos Santos, L.M. and Livingston, A.G. 1995. Membrane-attached biofilms for VOC wastewater treatment: novel in-situ biofilm thickness measurement technique. *Biotechnology and Bioengineering* **47**, 82-89.

Gadd, G.M. 1988. Accumulation of metal by microorganisms and algae. In Rehm, H.J. and Reed, G. (ed). *Biotechnology*, Vol. 6b: Special Microbial Processes, VCH Publishing, Weinheim, 401-433.

Gadd, G.M. 1990. Heavy metal accumulation by bacteria and other microorganisms. *Experientia* **46**, 834-840.

Gadd, G.M. 1992. Heavy metal pollutants: environmental and biotechnological aspects. In Lederberg, J. (ed). *Encyclopaedia of Microbiology*, Vol. 2, Academic Press. Inc, New York, 351-360.

Gadd, G.M. and Griffiths, J.A. 1978. Microorganisms and heavy metal toxicity. *Microbial Ecology* **4**, 303-317.

Gardea-Torresdey, J.L., Hernandez, A., Tiemann, K.J., Bibb, J. and Rodriguez, O. 1998. Adsorption of toxic metal ions from solution by inactivated cells of *Larrea Tridentata. Journal of Hazardous Substances Research*, Vol 1. Kansas State University.

Gauglhofer, J. and Bianchi, V. 1991. Chromium. In Merian, E. (ed). *Metal and Their Compound in the Environment*. VCH, Weinheim, 853-878.

Guy, R.D., Chakrabarti, C.L. and Schramm, L.L. 1975. Cited by Petrovic, M., Kastelan-Macan, M., and Horvat, A.J.M. 1999. Interactive sorption of metal ions and humic acids onto mineral particles. *Water, Air, and Soil Pollution* **111**, 41-56.

Hahne, H.C. H. and Kroontje, W. 1973. Significance of pH and chloride concentration on behaviour of heavy metal pollutants. *Journal of Environmental Quality* **2**, 4, 444-445.

Hamilton, W.A. 1987. Biofilms: microbial interaction and metabolic activities. In Fletcher M., Gray, T.R.G. and Jones, J.G. (eds). *Ecology of Microbial Communities*, Oxford University Press, London, 361-385.

Hammer, M.J. 1996. *Water and Wastewater Technology* 3rd ed. Prentice Hall, New- Jersey.

Hsieh, K. M., Lion, L.W and Shuler, M.L. 1985. Bioreactor for the study of defined interaction of toxic metals and biofilms. *Applied and Environmental Microbiology* **50**, 5, 1155-1161.

Hsueh, K.P., Hao O.J. and Wu, Y.C. 1991. Removal of volatile organic compounds in rotating disc contactor: batch and continuous operation. *Research Journal of WPCF* **63**, 1, 67-75.

Hughes, M.N. and Poole, R.K. 1989. *Metals and Micro-organisms*, Chapman and Hall, London.

Hutchins, S.R., Davidson, M.S., Brierley, J.A. and Brierley, C.L. 1986. Microorganisms in reclamation of metals. *Annual Review of Microbiology* **40**, 311-336.

Itabashi, H., Yamazaki, D., Kawamoto, H. and Akaiwa, H. 2001. Evaluation of the complexing ability of humic acid using solvent extraction technique. *Analytical Sciences* **17**, 785-787.

James, G.A., Beaudette, L. and Costerton, J.W. 1995. Interspecies bacterial interaction in biofilms. *Journal of Industrial Microbiology* **15**, 257-262.

Janson, C.E., Kenson, R.E. and Tucker, L.H. 1982. Treatment of heavy metals in wastewaters. *Environmental Progress* **1**, 3, 212-216.

Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G., Banerjee, S. and Farber, J.M. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology* **91**, 725-734.

Kennish, M.J. 1992. *Ecology of Estuaries: Anthropogenic Effect*. CRC Press, Boca Raton, Florida.

Kinner, N.E., Balkwill, D.L. and Bishop, P.L. 1983. Light and electron microscopic studies of microorganisms growing in rotating biological contactor biofilms. *Applied and Environmental Microbiology* **45**, 5, 1659-1669.

Kochany, J. and Smith, W. 2001. Application of humic substance in environmental remediation. *WM'01 Conference*, February 25 – March 1, Tucson, AZ.

Kolot, F.B., 1988. *Immobilized Microbial Systems: Principles, Techniques and Industrial Applications*. Robert E. Krieger Publishing Company, Inc, Florida.

Kuyucak, N. and Volesky, B. 1988. Biosorbents for recovery of metal from industrial solutions. *Biotechnology Letters* **10**, 137-142.

LaPara, T.M., Konopka, A., Nakatsu, C.H. and Alleman, J.E. 2000. Effect of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater. *Journal of Industrial Microbiology and Biotechnology* **24**, 140-145.

Lester, J.N. 1987. Biological treatment. In Lester, J.N. (ed). *Heavy Metals in Wastewater Sludge and Treatment Processes*, Vol. 2: Treatment and Disposal, CRC Press Inc. Baca Raton, Florida, 19-36.

Lester, J.N., Sterritt, R.M., Rudd, T. and Brown, M.J. 1984. Assessment of the role of bacterial extracellular polymers in controlling metal removal in biological wastewater treatment. In Grainger J.M. and Lynch J.M (eds). *Microbiological Methods for Environmental Biotechnology*, Academic Press, London, 197-217.

Lin, S.D. 1987. Rotating biological contactor technology. In Wise, D.L. (ed). *Bio-treatment Systems*, Vol. 2, CRC Press, Florida, 161-208.

MacLeod, R.A., Kuo, S.C. and Gelinas, R. 1967. Metabolic injury of bacteria induced by copper in the plating diluents. *Journal of Bacteriology* **93**, 961-969.

Marshall K.C. and Bitton G. 1980. Microbial adhesion in perspective. In Bitton, G and Marshall K.C. (eds). *Adsorption of Microorganisms to Surfaces*, John Wiley and Sons, New York, 1-6.

McEldowney, S. and Fletcher, M. 1986. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. *Journal of General Microbiology* **132**, 513-523.

McKinney, R.E. 1992. Wastewater treatment municipal. In Lederberg, J. (ed). *Encyclopaedia of Microbiology*, Vol. 4, Academic Press, New York, 363-375.

Milanovich, F., Wilson, D. and Yeh, Y. 1975. The detoxification effect of yellow substance on *Escherichia coli* in media containing copper. *Nature* **235**,460-461.

Misra, T.K. 1992. Heavy metals bacterial resistance. In Lederberg, J. (ed). *Encyclopaedia of Microbiology*, Vol. 2, Academic Press Inc, New York. 361-369.

Mohan, P.M. 1998. Metal toxicities and resistance in fungi, In Varma, A. (ed). *Microbes: For Health, Wealth and Sustainable Environment*, Malhotra Publishing House, New Delhi, 431-465.

Moore, J.W. and Ramamoorthy, S. 1984. *Heavy Metal in Natural Waters Applied: Monitoring and Impact Assessment*. Springer-Verlag, New York.

Mueller, R.F., Characklis, W.G., Jones, W.L. and Sears, J.T. 1992. Characterization of initial events in bacterial surface colonization by two *Pseudomonas* species using image analysis. *Biotechnology Bioengineering* **24**, 59-64.

Muhammad, N., Parr, J., Smith M.D. and Wheatley, A.D. 1997. Removal of heavy metals by slow sand filtration. *Water and Sanitation for All: Partnerships and Innovations 23rd WEDC Conference*, Durban, South Africa, 167-170.

Mullen, M.D., Wolf, D.C., Ferris, F.G., Beveridge, T.J., Flemming, C.A. and Bailey, G.W. 1989. Bacterial sorption of heavy metals. *Applied and Environmental Microbiology* **55**, 12, 3143-3149.

Murray, W.D. and Van Den Berg, L. 1981. Effect of support material on the development of microbial fixed film converting acetic acid to methane. *Journal of Applied Bacteriology* **51**, 257-265.

Muyima, N.Y.O., Momba, M.N.B. and Cloete, T.E. 1997. Biological methods for the treatment of wastewater. In IAWQ, *Microbial Community Analysis*, Great Britain, 1-22.

Nakajima, A. and Sakaguchi, T. 1986. Selective accumulation of heavy metals by microorganisms. *Applied Microbiology and Biotechnology* **24**, 59-64.

Nagase, H., Inthorn, D., Yutake, I., Hirata, K. and Miyamoto, K. 1997. Selective cadmium removal from hard water using NaOH treated cells of the cyanobacterium *Tolypothrix tenius*. *Journal of Fermentation and Bioengineering* **84**: 2, 379-385.

Ohtake, H. and Silver, S. 1994. Bacterial detoxification of toxic chromate. In Chaudry, G.R. (ed). *Biological Degradation and Bioremediation of Toxic Chemicals*, Doiscorides Press, Portland, 403-415.

Olson, B.H. and Panigrahi, A.K. 1991. Bacteria, fungi and blue-green algae, In Merian, E. (ed). *Metals and Their Compounds in the Environment. Occurrence, Analysis and Biological Relevance*, VCH, Weinheim, 419-427.

Petrovic, M., Kastelan-Macan, M. and Horvat, A.J.M. 1999. Interactive sorption of metal ions and humic acids onto mineral particles. *Water, Air, and Soil Pollution* **111**, 41-56.

Phillips, S. 2003. Evaluation of growth media parameters for the cultivation of selected biological control agents. *MSc thesis*, University of Natal, South Africa.

Prahalad, A.K. and Seenayya, G. 1988. Bioavailability of zinc and cadmium and their effect on microbial growth and metal uptake. *Bulletin of Environmental Contamination and Toxicology* **41**, 921-927.

Qureshi, F.M., Badar, U. and Ahmed, N. 2001. Biosorption of copper by a bacterial biofilms on a flexible polyvinyl chloride conduit. *Applied and Environmental Microbiology* **67**, 9, 4349-4352.

Rittmann, B.E. 1982. Comparative performance of biofilms reactor types, *Biotechnology and Bioengineering* **17**, 1341-1370.

Rittmann, B.E., Jackson, D.E. and Storck, S.C. 1988. Potential for treatment of hazardous organic chemical with biological processes. In Wise, D.L. (ed). *Bio-treatment Systems*, Vol. 3, CRC Press, Florida, 15-64.

Sakaguchi, T., Tsuji, T., Nakajima, A. and Horikoshi, T. 1979. Cited by Gadd, G.M. 1988. Accumulation of metal by microorganisms and algae. In Rehm, H.J. and Reed, G. (ed). *Biotechnology*, Vol. 6b: Special Microbial Processes, VCH Publishing, Weinheim, 401-433.

Salmon, I and Bull, A.T. 1984. Mixed-culture fermentation in industrial microbiology. In Klug. M.J. and Reddy, C.A. (eds). *Current Prospectives in Microbial Ecology.* Proceedings of the Third International Symposium of Microbial Ecology. *American Society for Microbiology*, Washington, D.C., 656-662.

Sanjay, H.G., Tiedje, M., Stashick, J.J., Srivastava, K.C., Johnson, H.R. and Walia, D.S. 1996. Development of HUMASORB ^{TM}, a lignite derived humic acid for removal of metal and organic contaminants from groundwater. *US Department of Energy.* http://www.netl.doe.gov/publications/proceedings/96/96em/Em96/Em-p1-12.pdf [Accessed on December 2003]

Shceinberg, H. 1991. Copper. In Merian, E. (ed). *Metal and Their Compound in the Environment.* VCH, Weinheim, 893-908.

Shumate, S.E. and Strandberg, G.W. 1985. Accumulation of metals by microbial cell. In Moo-Young M. Robinson, C.N. and Howell, J.A. (eds). *Comprehensive Biotechnology: The Principles, Application and Regulation of Biotechnology in Industry, Agriculture and Medicine*, Vol. 4, Pergamon Press, New York, 235-247.

Siebel, M.A. and Characklis, W.G. 1991. Observations of binary population biofilms. *Biotechnology and Bioengineering* **37**, 778-789.

Solt, G.S and Shirley, C.B. 1991. *An Engineer's Guide to Water Treatment.* Avebury Technical, Sydney.

Stephenson, T. 1987. Source of heavy metals. In Lester, J.N. (ed). *Heavy Metals in Wastewater Sludge and Treatment Processes*, Vol. 2: Treatment and Disposal, CRC Press Inc. Baca Raton, Florida, 32-59.

Sterritt, R.M. and Lester, J.N. 1980. Interaction of heavy metals with bacteria. *The Science of the Total Environment* **14**, 5-17.

Strandberg, G.W., Shumate II, S.E. and Parrott, J.R. 1981. Microbial cells as biosorbents for heavy metals: accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*, *Applied and Environmental Microbiology* **41**, 1, 237-245.

Sudo, R., Okada. M. and Mori, T. 1977. Cited by Bishop, P.L. and Kinner, N.E. 1986. Aerobic fixed-film processes. In Rehm, H.J. and Reed, G. (eds). *Biotechnology*, Vol. 8: Microbial Degradation, VCH Publishing Weinheim, 113-176.

Tchobanoglous, G. and Burton, F.L. 1997. *Wastewater Engineering: Treatment, Disposal and Reuse*, 3rd ed. Tata McGraw-Hill Publishing Company Limited, New Delhi.

Trevor, J.T. and Cotter, C.M. 1990. Copper toxicity and uptake in microorganisms. *Journal of Industrial Microbiology* **6**, 77-84.

Tsekova, K. and Petrov, G. 2002 Removal of heavy metals from aqueous solution using *Rhizopus delemar* mycelia in free and polyurethane-bound form. *Verlag der Zeitschrift fur Naturforschung* **57**, 629-633.

Vatanyoopaisarn, S., Nazli, A., Dodd, C.R., Rees, C.E. and Waites, W.M. 2000. Effect of flagella on initial attachment of listeria monocytogenes to stainless steel. *Applied and Environmental Microbiology* **66**, 2, 860-863.

Verran, J., Broadbent, J. and Ward, A. 1991. The effect of denature surface on adhesion of *Candida albicans*. *Journal of Applied Bacteriology Symposium Supplements* **74**, 67-78.

Watnick, P. and Kolter, R. 2000. Biofilm, city of microbes. *Journal of Bacteriology* **182**, 10, 2675-2679.

Winkler, M.A. 1983. Application of the principles of fermentation to biotechnology. In Wiseman, A. (ed). *Principles of Biotechnology*. Surrey University Press, Blackie and Son Ltd, 94-142.

Wong, P.K., Lam, K.C. and So, C.M. 1993. Removal and recovery of Cu^{+2} from industrial effluent by immobilized cell of *Pseudomonas putida* II-11. *Applied Microbiology and Biotechnology* **39**, 127- 131.

Wood, A. 1992. Trace metal removal from effluents. *Water and Waste Treatment* **35,** 32-36.

Wood, J.W. and Wang, H. 1983. Microbial resistance to heavy metals. *Environmental Science and Technology* **17,** 12, 582A-590A.

Yan, G. and Viraraghavan, T. 2001. Heavy metal removal in a biosorption column by immobilized *Mucor rouxii* biomass. In Van-Guangyu, V. *Bioresource Technology*, Elsevier Applied Science Publishing Company, New York, 243-249.

Zobell, C.E. 1943. The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology* **46**, 539-556.

APPENDIX 1

Stock solutions containing 10 000 mg l^{-1} of the metal species were prepared as follows:

1 Chromium

$Cr(NO_3)_3 .9H_2O$ molecular weight 400.15

Molecular weight of Cr^{+3} = 51.996 (13%)

76.92 g of $Cr(NO_3)_3 .9H_2O$ were weighed out, and dissolved in one litre of distilled water in a volumetric flask.

2 Lead

$Pb(NO_3)_2$ molecular weight 331.21

Molecular weight of Pb^{+2} = 207.2 (62.6%)

15.97 g $Pb(NO_3)_2$ were weighed out, and dissolved in one litre of distilled water in a volumetric flask.

3 Copper

$CuCl_2.2H_2O$ molecular weight 170.45

Molecular weight of Cu^{+2} = 63.54 (37.3%)

26.81 g of $CuCl_2.2H_2O$ were weighed out, and dissolved in one litre of distilled water in a volumetric flask.

APPENDIX 2

Residual metal ion concentration (mg l^{-1}) in the three-bioreactor compartments over the sixty days duration of the experiment

	Cr^{+3}			Pb^{+2}			Cu^{+2}		
Time	Gravel	Polyester	Sand	Gravel	Polyester	Sand	Gravel	Polyester	Sand
0	182.2	184.7	184.3	192.3	198.1	193.5	198.2	198.3	194.7
30min	168.4	165.4	168.3	177.2	175.6	180.6	191.3	192.4	189.2
1hr	164.1	162.5	164.2	173.5	172.0	176.2	187.6	186.5	188.2
3hr	159.2	155.6	160.2	171.3	169.7	174.3	185.4	183.2	188.0
6hr	152.2	149.2	155.4	169.6	168.5	170.7	180.6	181.2	186.0
12hr	145.3	142.0	145.2	166.4	165.2	171.6	177.4	178.1	184.2
24hr	138.2	137.0	141.5	159.4	161.7	168.7	175.3	175.6	180.5
2-days	135.8	134.8	138.0	147.6	152.8	157.2	173.8	172.3	177.9
4	161.6	156.2	149.1	174.2	181.5	177.0	177.9	186.3	189.6
6	163.0	155.9	151.1	179.8	185.4	174.1	183.1	190.1	191.6
8	125.4	122.7	122.7	141.1	153.9	144.0	153.6	163.6	176.5
10	114.2	117.2	117.2	141.1	147.7	144.7	148.4	159.2	173.2
12	109.9	98.7	103.5	135.2	136.2	138.0	137.0	154.1	159.5
14	102.4	89.3	98.9	130.7	134.1	131.6	144.4	152.9	151.6
16	92.6	77.0	98.1	120.1	120.4	119.9	128.0	147.3	142.8
18	86.7	73.6	94.7	109.0	117.8	118.3	116.7	141.5	120.9
20	84.8	68.4	92.0	106.9	114.0	112.0	115.6	139.2	118.9
22	83.5	66.0	79.8	103.4	108.8	107.9	114.1	134.5	111.3
24	81.6	68.5	79.1	100.1	103.0	108.7	107.5	130.6	108.1
26	79.5	65.7	76.2	96.8	102.8	108.3	104.2	114.3	106.3
28	76.3	63.1	75.4	91.8	100.5	102.8	91.5	109.9	95.7
30	71.8	60.5	73.4	89.3	96.2	98.9	86.8	103.7	94.5
32	68.4	56.6	73.2	84.7	95.9	93.7	86.2	101.3	94.1
34	61.9	54.2	72.5	80.3	92.7	92.2	85.0	92.9	91.8
36	61.8	53.8	67.1	79.3	87.0	90.8	85.8	89.6	88.1
38	62.6	54.6	63.0	78.3	85.1	89.2	80.4	87.9	84.8
40	60.5	53.2	66.4	74.5	84.8	88.4	79.2	85.9	85.4
42	64.0	48.7	60.9	72.6	82.9	89.4	78.5	84.3	84.1
44	53.0	47.8	58.8	65.2	80.1	82.0	75.5	85.1	86.2
46	39.9	46.5	56.3	59.2	80.5	74.2	76.0	78.9	86.1
48	36.2	45.5	50.0	57.8	77.8	71.0	74.9	79.7	83.5
50	34.2	41.9	45.8	56.2	72.2	70.9	73.2	78.5	80.9
52	32.0	36.8	39.3	54.8	68.1	64.6	70.5	77.3	75.8
54	27.8	32.2	36.2	50.5	63.9	61.2	70.3	75.6	74.0
56	23.6	29.5	32.9	37.8	55.6	56.2	53.3	63.2	60.2
58	20.2	26.3	29.5	32.0	51.2	48.2	48.8	59.8	54.6
60	16.3	26.4	28.1	28.5	43.5	41.9	44.4	55.9	52.1
LSD		2.884			3.431			3.833	
CV%		2.0			1.7			1.8	
P. Values		< 0.001*			< 0.001*			< 0.001*	

* Highly significant metal removal at P<0.05 by all biofilms taking time as a factor

APPENDIX 3

Total biomass dry weight associated with the discs in the three compartments of the RBC bioreactor and affect of disc orientation on biomass yield

Bioreactor Compartment	Disc position within the component	Total biomass weight (g) per disc	Biomass yield (g) associated with disc surfaces*	
			Downstream surface	Upstream surface
1	1	4.52	2.41	2.11
	2	4.27	2.26	2.01
	3	4.30	2.20	2.10
	4	4.08	2.08	2.00
	5	4.32	2.25	1.17
	6	4.38	2.22	2.16
2	1	3.86	2.03	1.83
	2	3.84	2.13	1.71
	3	3.62	1.98	1.64
	4	3.61	1.89	1.72
	5	3.46	1.78	1.68
	6	3.63	1.71	1.92
3	1	2.83	1.56	1.27
	2	2.67	1.33	1.34
	3	2.79	1.34	1.45
	4	2.75	1.54	1.21
	5	3.06	1.57	1.49
	6	3.26	1.62	1.63

* Downstream and upstream surfaces refer to the orientation of the disc relative to the direction of the flow of the effluent through the bioreactor compartment.