BIOREMEDIATION OF HEAVY METAL POLLUTED WATERS

by

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ABSTRACT

Microorganisms have the potential to remove heavy metals from polluted waters and effluents and may be used in clean-up processes. Microbial associations were enriched for and adapted to grow in nutrient solutions containing various concentrations of different metals. As immobilised cells are known to be more stable and more efficient in metal uptake than are corresponding planktonic or free-living cells the attachment of the microbial associations was investigated using a model stream and it was found that biofilm development was better on rough surfaces such as ground glass and polystyrene than on smooth surfaces such as unetched glass plates and glass beads. When comparing metal-uptake by planktonic and attached microorganisms, attached populations were found to have a greater metal-uptake capacity. The uptake of individual metals from various metal combinations was tested with various proportions of pregrown metal-adapted microbial populations as inoculum and it was found that a particular metal was taken up more readily by microbial associations which had previously been exposed to that metal. Lead (Pb^{2+}) appeared to be taken up more readily than copper (Cu^{2+}) or cadmium (Cd^{2+}) while Cd^{2+} was more actively removed than Cu^{2+} from solution. pH also affected metal uptake and the optimum range for Cu^{2+} uptake by the Cu^{2+}-adapted microbial association was found to be between 5.8 and 7.0. Dead microbial biomass was investigated and found to have efficient metal uptake capacity. Living mycelium from an isolated *Aspergillus* species showed poor uptake of Cu^{2+} initially, but when this fungus was pregrown and subsequently killed by moist heat treatment the non-living mycelium was efficient in removal of Pb^{2+} and Cu^{2+} ions. The optimum mycelial biomass concentration for metal uptake was also determined. The mechanism of metal uptake by this *Aspergillus* species was determined, using electron microscopy and EDX techniques, to be metabolism-independent biosorption onto the hyphal surface. Thus the microbial associations and fungal cultures used in this study were shown to have the potential for use in the removal of heavy metals from polluted waters.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations.

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CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Metals are compounds that form cations in solution and whose oxides form hydroxides rather than acids with water: Heavy metals are metals with a specific gravity greater than five (Gadd 1988). Many heavy metals are essential for microbial growth and metabolism in low concentrations, e.g. copper, zinc, manganese, while others have no known biological function, e.g. gold, silver, lead, cadmium. Both groups can be toxic to living cells when present in high concentrations (Gadd 1988).

Aquatic pollution may be defined as the introduction of organisms, substances or energy, resulting from man's activities, into water, which impairs the legitimate uses of the water as a natural resource or as a natural environment (Hawkes 1985). Metal pollution is especially difficult to control and is potentially more hazardous than organic pollution (Anonymous 1990), as metals are not biodegradable, and once they enter the environment their potential toxicity is controlled by biological and environmental factors (Sterritt and Lester 1980; Sahoo *et al.* 1992). Selective extraction of heavy metals from industrial effluents will diminish disposal problems, decrease environmental pollution, and allow for the recycling of valuable heavy metals (Gadd 1988). This needs to be done at the source before the natural aquatic systems become polluted (Sahoo *et al.* 1992).

Microorganisms have efficient mechanisms for the accumulation of heavy metals from aqueous solutions (Gadd 1988), and many functional biological systems have been established. However there are few microbiologically-based metal removal treatments in use as they must compete both economically and effectively with conventional treatments (Simmons *et al.* 1995).

Development of this area of biotechnology is essential for both environmental and economic reasons but is dependent on adequate support from government and industry (Gadd 1992).

1.2 HEAVY METALS CONTAMINATING SOUTH AFRICAN WATERS AND CONVENTIONAL PROCESSES USED FOR CLEAN-UP

1.2.1 Introduction

Many heavy metals are known to cause pollution problems in South Africa, e.g. lead, iron, cadmium, chromium, copper, mercury, nickel, manganese, zinc, and vanadium (Saayman 1992). These heavy metals are found in various industrial effluents, which can cause water pollution problems if they are deposited in the natural water system, without prior treatment (Brown 1991; CSIR 1991).

The maximum concentration of heavy metals allowed in waste waters or effluents released into South African waters are (mg l^{-1}):
cadmium 0.05; chromium 0.05; copper 1.0 and lead 0.1 (Neytzell-De Wilde 1992). Waste waters containing heavy metal pollutants are generally treated by various conventional processes, which are discussed below.

1.2.2 Cadmium (Cd)

Cadmium is toxic to all living organisms and exerts its toxic effects by complexing to SH groups of proteins and amino acids, and by inhibiting cellular respiration (Beveridge *et al.* 1982). It affects the logarithmic growth rates of microorganisms, inhibits fungal spore formation, and induces abnormal microbial morphologies (Babich and Stotzky 1978). Cadmium is employed in various processes: electroplating; pigment production; the production of plastic stabilisers for polyvinyl chloride plastics; and in battery production (Moore and Ramamoorthy 1984). It is found associated with inorganic ligands containing oxygen and sulphur elements, and undergoes multiple hydrolysis at pH values encountered in the environment (Moore and Ramamoorthy 1984).

1.2.3 Chromium (Cr)

Chromium is one of the least toxic of the trace elements. Mammalian bodies can tolerate up to 100-200X their normal total content of Cr without adverse effects (Moore and Ramamoorthy 1984). The three major uses of chromium are in the metallurgical industries (chrome electroplating and production of ferrochrome alloys); the refractory industries (manufacture of refractory bricks, mortars and ramming gun mixes); and in the chemical industries (used in pigments, mordants and dyes in the textile industry, and as tanning agents in the leather industry) (Moore and Ramamoorthy 1984). The principal chromium emissions into surface waters are from metal-finishing processes such as electroplating, pickling and bright dipping (Moore and Ramamoorthy 1984). Cr^{6+} compounds are highly water soluble and toxic, while the Cr^{3+} compounds are less soluble and less toxic (Deleo and Ehrlich 1994).

1.2.4 Lead (Pb)

Lead has two oxidation states (+2 and +4) (Huang *et al.* 1988). Except for the nitrate and acetate salts, lead(+2) salts are insoluble in water (Moore and Ramamoorthy 1984). Lead is used mainly in storage batteries and metal products, (ammunition and casting materials); in various chemicals (tetraethyllead - an antiknock agent in gasoline); and in pigments (anticorrosive and highway traffic safety paints) (Moore and Ramamoorthy 1984). Atmospheric fallout is considered the most important source of lead in marine and fresh waters (Moore and Ramamoorthy 1984). The behaviour of lead in natural waters is a combination of the establishment of precipitation equilibria, and complexing with inorganic and organic ligands (Moore and Ramamoorthy 1984). Lead forms moderately strong chelates with organic ligands containing S, N, and O atoms (Moore and Ramamoorthy 1984).

1.2.5 Copper (Cu)

Copper is widely distributed in nature in the free state and complexed with sulphides, sulphates, arsenides, nitrates, chlorides and carbonates, with the Cu^{2+} ion being the most

common (Moore and Ramamoorthy 1984). Copper is found in industrial and domestic wastes, and is used in metal plating industries, and in mining and mineral leaching (Manaban 1991). Smelter activities and refining processes also contribute to copper contamination in the environment (Trevors and Cotter 1990). Copper is also used as a chemical control agent for microorganisms, eg. the fungicide Bordeaux mixture (Baker 1974). Copper sulphate is used as a feed supplement for pigs and poultry which results in high levels of copper in the faecal wastes, which adds to environmental pollution (Davis 1974). Speciation of copper in natural water systems is determined by the physicochemical and hydrodynamic characteristics, and the biological state of the water (Moore and Ramamoorthy 1984).

1.2.6 Conventional Methods of Metal Removal from Solution

Processes used to date to remove heavy metals from solution include: filtration, chemical precipitation, ion exchange, adsorption and electrolytic treatment (Janson *et al.* 1982; Deans and Dixon 1992; Wood 1992). Some of these methods are expensive, not only to set up but also to operate, and they also have limitations (Deans and Dixon 1992; Wood 1992). With the anticipated stricter statutory limits of the concentrations of metals allowed in released effluents, these methods are becoming increasingly expensive and inefficient (Sahoo *et al.* 1992).

1.2.6.1 Filtration

This involves filtering of effluents to remove the precipitated metals and other pollutants. This method is low cost but is only effective for removing particulate and other insoluble matter, and trace concentrations of metals are not removed (Deans and Dixon 1992).

1.2.6.2 Chemical precipitation

This is achieved by the addition of an alkali to raise the pH so that the soluble metal ions form insoluble metal hydroxides (Wood 1992). Chemical precipitation is fairly low cost and can remove large quantities of metal ions quickly (Deans and Dixon 1992). A major

disadvantage, however, is that it generates toxic sludge thereby merely transferring the pollution problem from water to land disposal (Wood 1992). Trace concentrations of metal ions are also not removed (Deans and Dixon 1992).

1.2.6.3 Ion exchange

Metal ions in the polluting waste water are exchanged for sodium, hydrogen or other ions attached to an insoluble resin (Wood 1992). This is a cost effective procedure that can reduce the contaminating metal ion concentration to the mg l^{-1} level, but it has the disadvantage that in the presence of large quantities of competing ions, eg. Na^+, and Ca^{2+}, the ion exchange process becomes ineffective (Deans and Dixon 1992). This is actually a volume reducing process as the metals can be stripped off the resin and collected for further treatment (Wood 1992).

1.2.6.4 Adsorption

Activated carbon is used widely to remove noble metals from solution (Deans and Dixon 1992; Wood 1992), but this method is inefficient where low concentrations of metal are present (Deans and Dixon 1992). The carbon is regenerated by burning off the impurities, but this can lead to metal ions being released into the atmosphere causing other pollution problems (Wood 1992).

1.2.6.5 Electrolytic treatment

These processes use electrical energy to cause a chemical change within an electrolytic cell, consisting of an anode and a cathode immersed in an electrolyte or metal solution and connected externally by an electrical circuit (Wood 1992). The metal ions are attracted to and adhere to the electrode and are thereby removed from solution. This process is expensive in both capital outlay and in running costs as the systems used are sophisticated and require a high level of technical expertise to operate effectively (Deans and Dixon 1992). It is, therefore, usually only used for the recovery of precious metals that have a high resale value (Wood 1992).

1.2.7 Conclusion

As microorganisms are capable of removing low concentrations of heavy metals from solution, biological applications may offer solutions to the various problems experienced with conventional physicochemical metal extraction methods. As the latter techniques for metal recovery are generally less effective when the metal content of the aqueous solutions is low (Sahoo *et al.* 1992), microbiologically related technologies may provide an alternative, or adjunct, to conventional techniques of metal recovery and removal (Gadd 1988), eg. they could be used as a concentration stage, so that microbiological recovery from very dilute solutions is a preliminary step preceding conventional treatments (De Rome and Gadd 1991). Microbiological processes have also been shown to effectively remove metals in the presence of both organic compounds and high concentrations of dissolved salts (Brown 1991), and may be more economical and effective than existing treatments (Gadd 1990). Thus, microorganisms offer a great potential for use in cleaning up polluted industrial waste waters.

1.3 ACCUMULATION OF HEAVY METALS BY MICROORGANISMS

1.3.1 Introduction

Microorganisms have the ability to accumulate heavy metal ions, a process that is important industrially. Industrial effluents and waste waters can be detoxified and valuable heavy metals recovered (Gadd 1988). This process can also lead to a reduction in environmental pollution, since toxic heavy metals are removed from effluents and waste waters before these are pumped into the natural water systems (Gadd 1988).

Microorganisms suitable for heavy metal removal should be carefully selected and tested, as it has been found that bioaccumulating properties are more strain - than species - related (Baldry and Dean 1980; Wnorowski 1991). The metal uptake processes of some microorganisms are non-specific for a particular metal ion and thus these microorganisms may be used in various effluents for general metal recovery (Macaskie and Dean 1984b). Heavy metal accumulation by microorganisms has been known to occur under both aerobic and anaerobic conditions, depending on the microorganisms present and the prevailing environmental conditions (Francis 1990).

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). Knowledge of the chemical or physiological reactions that occur during metal uptake would enable better control of specific process parameters, which could lead to an increase in the rate, quantity and specificity of metal accumulated (Shumate and Strandberg 1985).

1.3.2 Mechanisms of Microbial Metal Accumulation

Microorganisms use a variety of mechanisms for the accumulation of heavy metals:

1. **Metabolism-independent biosorption** - adsorption of metals to cell walls and other cellular constituents (Gadd 1988).

2. **Metabolism-dependent intracellular accumulation** - transport of metals across the cell membrane; internal compartmentalisation (Gadd 1988).

3. **Extracellular precipitation, binding and complex formation** - by excreted metabolites (Gadd 1988); also a metabolism-dependent mechanism

4. **Transformations of metal ions** into other forms (Gadd and Griffiths 1978; Summers and Silver 1978).

In a given microbial system, several mechanisms may operate simultaneously, or in sequence (Gadd 1988).

1.3.2.1 Metabolism-independent biosorption

Adsorption involves the accumulation or concentration of substances, the adsorbate, at the surface of the interface, the adsorbent (Gadd 1988). This is generally a more rapid process than the metabolism-dependent mechanisms (Gadd 1988) and is reversible, and independent of temperature and energy metabolism (Kelly *et al.* 1979). Biosorption may be considered a solid-liquid contact process comprising a metal uptake phase and a desorption phase (Gadd 1992).

Three main types of adsorption have been recognised:

1. Electrical attraction - exchange adsorption: attraction of positively-charged ions to negatively-charged ligands in the cell material (Gadd 1988).

2. Physical or ideal adsorption: adsorbed molecules have translational movement within the interface; involves van der Waals forces (Gadd 1988).

3. Chemical or activated adsorption: chemical attraction between the adsorbate and adsorbent (Gadd 1988).

Most microbial metal-adsorption phenomena involve all three types of adsorption (Gadd 1988) and may occur under conditions that would normally be toxic to living organisms (Darnall *et al.* 1986).

Avery and Tobin (1993) investigated the applicability of applying the 'hard and soft' principle of adsorption to biological systems. This principle predicts that the bonds formed between hard metals (eg. Sr^{2+}, Mn^{2+}) and hard ligands are predominantly ionic, while those between soft metals (eg. Cd^{2+}, Tl^{+}) and soft ligands are more covalent (Pearson 1963). Borderline metals include Zn^{2+} and Cu^{2+}. Niebor and Richardson (1980) refined this classification and stated that hard metals (usually non toxic, and often essential for growth) bind preferentially to oxygen-containing (hard) ligands, while soft metals (displaying greater toxicity) form more stable bonds with N- or S-containing ligands. The capacity of the cell surface for covalent bonding relative to ionic bonding increases with metal softness. This suggests that the capacity of the cells for covalent bonding may be the principal limiting factor that determines the maximum level of metal uptake (Avery and Tobin 1993).

The composition of the cell walls of microorganisms is highly species dependent and differs considerably among Gram negative and Gram positive bacteria, yeasts, filamentous fungi and algae (Shumate and Strandberg 1985). Many constituents in the walls have the potential to complex metal ions, with certain metals being preferentially complexed (Shumate and Strandberg 1985). This complexation of metal ions by charged cell wall constituents may be likened to an ion-exchange type reaction, which is affected by the chemical environment and the presence of other metals or ligands (Shumate and Strandberg 1985). Anionic ligands responsible for cation adsorption include the phosphoryl, carbonyl, sulphydryl and hydroxyl groups of membrane proteins and lipids and bacterial peptidoglycans and associated polymers (Kelly et al. 1979; Tobin et al. 1994). Bacterial extracellular polymers may also be important in the retention of certain metals within the biomass; for example, bacterial extracellular polymers are strongly implicated in the removal of soluble metal ions such as Cu^{2+} and Cd^{2+}, by floc-associated microorganisms (Rudd et al. 1984; Gadd 1988). Capsulated cells of *Klebsiella aerogenes* can retain greater concentrations of metals than an equivalent number of non-capsulated cells (Rudd et al. 1983). Uptake of Pb^{2+} by an *Azotobacter* sp. was better than that by *Micrococcus luteus* due to the surrounding capsular material of the former (Tornabene and Edwards 1972).

Biosorption makes up the most significant proportion of the total uptake of metals by bacteria (Gadd 1990). The walls of bacteria are highly electronegative (Beveridge 1986), and are known to accumulate various heavy metals to varying degrees. A two-step mechanism for metal binding has been proposed: initially a stoichiometric interaction occurs between the metal ions and active sites within the wall, resulting in a nucleation site for the deposition of more metal ions from solution; this subsequently grows within the intramolecular spaces of the wall until it is physically constrained by the wall meshwork (Beveridge and Murray 1980; Beveridge and Fyfe 1985; Beveridge 1986).

Gram positive bacterial walls are efficient metal chelators (Beveridge *et al.* 1982; Beveridge 1989) and have various components in their cell walls which are responsible for metal biosorption. For example, *Bacillus subtilis* has two wall components: a polymeric network of peptidoglycan (a repeating dimer of N-acetylglucosamine and N-acetylmuramic acid), and joined to this peptidoglycan longer, more flexible chains of teichoic acid (linear polymers of glycerol phosphate) (Beveridge 1986). Metals are bound primarily by the COO⁻ groups of the glutamic acid residues of the peptidoglycan moiety of these cell walls (Beveridge *et al.* 1982; Beveridge and Fyfe 1985; Beveridge 1986). *Bacillus licheniformis* has an additional component in the cell wall called teichuronic acid (Beveridge 1986) which has been shown to bind copper to a greater extent than do the teichoic acids (Baldry and Dean 1981). However, McLean *et al.* (1994) showed that the cell walls of *B. licheniformis* were not suitable for repeated use as a heavy metal biosorbent, as teichoic and teichuronic acids were susceptible to hydrolysis by acidic and basic solutions used in metal recovery. *Sporosarcina ureae* has a wall that consists of two structurally independent constituents: peptidoglycan and a proteinaceous surface array (Beveridge 1979). When grown under heavy metal stress, *S. ureae* extrudes large quantities of the surface array protein which effectively binds and precipitates the metals from solution (Beveridge and Fyfe 1985; Beveridge 1986). The cation-binding behaviour of Gram positive bacteria, such as *B. megaterium* and *Streptococcus mutans*, is likened to that of commercial carboxylic ion-exchange resins, with an affinity series of $H^+ >> La^{3+} >> Cd^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > K^+ > Na^+$ (Marquis *et al.* 1976).

In contrast, Gram negative bacteria have envelopes that consist of two chemically distinct membrane bilayers that sandwich a thin peptidoglycan layer in the periplasmic space (Beveridge and Koval 1981). The outer membrane is chemically linked to the peptidoglycan by a low molecular weight polypeptide known as the lipoprotein (Beveridge 1986). The lipopolysaccharide on the outer cell surface, and the surface-exposed protein are the first components to react with soluble metal ions (Beveridge 1986). Gram negative bacteria do not have teichoic acids (Baldry and Dean 1981), and do not bind as much metal from solution as Gram positive cell walls (Beveridge and Fyfe 1985). In studies involving purified cell envelopes of *Escherichia coli* K-12 it was found that most metal deposition occurred at the polar head group regions of the phospholipid constituent of the membranes (Beveridge 1986), or along the peptidoglycan layer (Beveridge and Koval 1981). A two-step deposition mechanism is also believed to operate in these walls, but only a few intramolecular spaces are available in which metal aggregates can grow (Beveridge 1984; 1986). Cabral (1992) found that different metals had different affinities for *Pseudomonas syringae* cells. The divalent ions tested were bound in three distinct groups, i.e. Ca^{2+} and Mg^{2+}; Cd^{2+}, Ni^{2+} and Zn^{2+}; and Cu^{2+} and Hg^{2+}. However, Ca^{2+} and Mg^{2+} did not bind significantly (Cabral 1992). *Zoogloea ramigera* 115 is a Gram negative bacterium that forms zoogloeae or finger-like projections due to production of an extracellular anionic polysaccharide to which metals bind at specific sites (Kuhn and Pfister 1990).

Shuttleworth and Unz (1993) used *Thiothrix* strain A1, a filamentous bacterium, to show that the adsorption of metal ions involved primarily an ion-exchange type of reaction. Desorption of metal ions from the cells was enhanced by increasing the ionic strength and by adding competing ions and chelating agents, which showed that most of the metals were loosely associated (Shuttleworth and Unz 1993). Approximately 20% of the metal was not removed by EDTA suggesting that it may have diffused into the cell (Shuttleworth and Unz 1993).

Heavy metals are accumulated rapidly and reversibly by algae in 5-10 minutes (Gadd 1988; 1990). Metal biosorption is unaffected by metabolic inhibitors, by modest ranges of temperature (0-30°C), uncouplers or light/dark cycles (Trevors *et al.* 1986). It is,

however, affected by the pH of the growth solution and the presence of other divalent cations (Sakaguchi *et al.* 1979). For example, Ca^{2+}, Mg^{2+}, Na^+, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, but not K^+ were found to retard Cd^{2+} binding by *Chlorella regularis* (Sakaguchi *et al.* 1979). Many potential metal-binding sites occur in algal cell walls and extracellular matrices, including polysaccharides, cellulose, uronic acids and proteins (Trevors *et al.* 1986). *Chlorella vulgaris* and *Scenedesmus quadricauda* were both found to strongly bind Ag^+, Cu^{2+}, Cd^{2+} and Zn^{2+} on their cell surfaces (Harris and Ramelow 1990). Crist *et al.* (1992) found that metals were adsorbed onto the surfaces of algae, through binding to the carboxylate and sulphate anions, and were released with a change in pH. Various macroalgal species were found to have varying ion exchange and metal sorption capabilities, due to structural and chemical variations of cell surface components, and cell morphologies (Pirszel *et al.* 1995). Unicellular algae and cyanobacteria have higher surface/volume ratios than macroalgae which may account for their higher ion exchange capacity (Pirszel *et al.* 1995).

The metal removal capacity of fungal biomass is comparable with that of other conventional solids such as activated carbon and soils (Huang *et al.* 1988). For many filamentous fungi general biosorption accounts for most of the total metal uptake (Gadd 1988). Some fungi, for example, *Rhizopus arrhizus, Mucor mucedo*, the yeast *Saccharomyces cerevisiae* and various *Penicillium* species are known to adsorb metals such as copper, lead, zinc, cadmium and mercury (Saxton and Jones 1990).

The first and most important step of the adsorption mechanism is adsorption of the metal ion onto the cell wall surface by means of a complexation reaction between the metal ion and the amine groups of the linked amino-sugar units of chitin (Saxton and Jones 1990). Metal accumulation may continue by nucleation and growth on adjacent sites, and, in some instances, there may be precipitation of the metal hydroxide within the cell wall following hydrolysis (Saxton and Jones 1990). This is a fairly rapid process that occurs within about 30 minutes after exposure to the metal-bearing solution (Saxton and Jones 1990).

Filamentous fungi possess extracellular polysaccharides, slime layers or mucilage, which have high biosorptive capacities, and large amounts of heavy metals are usually rapidly bound (Gadd 1988).

The biomass concentration can be critical in metal uptake (Gadd 1988): at a given equilibrium concentration of metal, fungal cells adsorb more metal ions at low rather than at high cell densities. Possible explanations for this include electrostatic interactions, interference between binding sites and reduced mixing at high cell densities (Gadd 1988). Particulate material such as copper, lead and zinc sulphides, zinc dust and ferric hydroxide, may also be adsorbed by fungal biomass (Wainwright and Grayston 1989). For example, *Trichoderma harzianum* and *Rhizoctonia solani* have been found to accumulate zinc and lead in large quantities (Lokesha and Somasherk 1989).

A diverse range of yeasts has been shown to have a large metal accumulation potential, especially with respect to Co^{2+} and Cu^{2+} (Norris and Kelly 1979). Only extracellular association takes place between live yeast and Cd^{2+} and Pb^{2+} ions, and between dead yeast and Cu^{2+} ions (Huang *et al.* 1990). *Saccharomyces cerevisiae* adsorbs metals in the following order: $Cu^{2+} > Cd^{2+} = Pb^{2+} = Zn^{2+} > Co^{2+}$ (Huang *et al.* 1988).

Luef *et al.* (1991) used *Aspergillus niger*, *Penicillium chrysogenum* and *Claviceps paspali* waste mycelium to adsorb zinc, and found that alkali treatment improved the biosorbent capacity of the fungal mycelium to chelate various metals. Mitani and Misic (1991) determined that a *Penicillium* sp. isolated from soil can remove considerable amounts of copper from solution, which were adsorbed onto the cell surface.

1.3.2.2 Metabolism-dependent intracellular accumulation

Metabolism-dependent intracellular accumulation is a much slower process than adsorption, but greater amounts of heavy metals may be accumulated (Gadd and Griffiths 1978; Gadd 1990). Many metals are taken up by this process, for example, cadmium, silver, zinc, copper, chromium, nickel, uranium, lead, mercury, palladium and gold (Shumate and Strandberg 1985). Specific mechanisms whereby these metals are taken into the cells are essentially unknown and in many cases uptake is under genetic control, i.e. plasmid-linked (Shumate and Strandberg 1985).

The mechanisms of heavy metal transport appear to rely on the electrochemical proton gradient across the cell membrane, the pH gradient and the membrane potential, each of which can drive transport of ionised solutes across cell membranes (Gadd 1990). The rate of uptake is also influenced by the physiological state of the cells and the nature and composition of the growth medium (Gadd 1988).

Transport of metal ions through membranes requires combination with a carrier (specific small molecules or proteins) or the presence of special channels in the cell membranes (Wood and Wang 1983). Alternatively, some non-essential, toxic metals may be taken up via divalent cation transport systems, which evolved to enable the selective concentration of essential trace metals (Kelly *et al.* 1979). Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+} transport systems may be implicated in this type of uptake (Kelly *et al.* 1979). Highly specific transport systems for Mn^{2+} ions have been found in several bacterial species such as *E. coli, B. subtilis*, and *Lactobacillus plantarum* (Archibald and Duong 1984; Gadd 1988). Cadmium ions were found to be specific inhibitors of these high affinity Mn^{2+} transport systems; Cd^{2+} may enter cells via this system (Gadd 1988). Research has shown that intracellular accumulation of Cd^{2+} occurs in the yeast-like cells and mycelium of *A. pullulans*, and it appears that cadmium competes for calcium transport sites, indicating that Cd^{2+} uptake occurred via an existent calcium uptake system (Mowll and Gadd 1984). Metals may also be taken up via monovalent cation transport systems (Kelly *et al.* 1979). Four kinetically and genetically distinct potassium (K^+) transport systems have been observed, for which other alkali metal ions can be competitive substrates; the relative affinities being determined by the similarity of the radius of the metal ion to that of K^+ (Kelly *et al.* 1979). Metals may also be taken up via undefined systems, i.e. toxicity effects can lead to alteration of membrane permeability, leading to diffusion of the metals into the cells, rather than utilisation of cation transport systems (Kelly *et al.* 1979); for example Pb^{2+} diffuses into *Saccharomyces cerevisiae* cells reaching a similar concentration to that of Co^{2+} and Cd^{2+} but does not require an energy source for uptake (Kelly *et al.* 1979; Shumate and Strandberg 1985; Gadd 1990). It has been postulated that Cu^{2+} ion uptake by live yeast may be controlled by a diffusion process through the cell wall, or regulated by intracellular metabolic processes (Huang *et al.* 1990). The energy-dependent uptake of chromate, via the sulphate permease system, was observed in *Neurospora crassa* and is an example of metal uptake via anion transport pathways (Kelly *et al.* 1979).

At elevated metal concentrations, intracellular precipitation may occur after uptake, and is a means of detoxification by compartmentalisation (Gadd and Griffiths 1978).

Algae show a slow phase of metal uptake due to the simultaneous effects of growth and surface adsorption (Gadd 1988). Active uptake of metals is sensitive to lack of light, the presence of uncouplers and metallic inhibitors, and is temperature-dependent (Skowronski 1986). After active uptake, however, intracellular concentrations may be several thousand times greater within cells than in the external medium (Gadd 1988).

In fungi, including yeasts, differences in uptake of heavy metals are due to strain variations, adsorption effects, metal toxicity, influence of surface potential and existence of more than one transport mechanism for metal ions (Norris and Kelly 1977; White and Gadd 1987). With certain fungi greater amounts of metals can be taken up by intracellular transport than by biosorption (Norris and Kelly 1977).

1.3.2.3 Extracellular precipitation, binding and complex formation

Extracellular precipitation, binding and complex formation may also be metabolism-dependent (Gadd 1988). Sometimes crystallisation of metals occurs on microbial surfaces but it is often difficult to detect whether metabolism is involved (Gadd 1988).

Complexing agents are chemically significant and have the potential to change the physical state of metals in solution, thereby altering the accumulation, toxicity, and ability of metal ions to cross the cell membrane (Birch and Bachofen 1990). Complexes are species formed by the association of two or more simpler species, each capable of independent existence (Rossotti and Rossotti 1961). 'Complexation capacity' depends on several parameters, including pH, metal and ligand concentration, ionic strength and redox conditions (Birch and Bachofen 1990).

There are distinct modes of action of complexing agents in biological systems: these may complex metals in solution, in extracellular polymers on the cell surface, or in subcellular compartments in the cell (Wood and Wang 1983). Many metal complexes formed often

cannot pass through the cellular membrane, due to their size or charge, or because the complexes are insoluble and precipitate (Birch and Bachofen 1990).

Various groups of microbial metal-complexing agents have been investigated:

1) By-products of microbial metabolism and degradation, e.g. simple organic compounds or macromolecular humic and fulvic acids (Birch and Bachofen 1990). The ability of heavy metals to bind to organic substances plays an important ecological role as it affects the uptake and toxicity of the metals to the microorganisms (Birch and Bachofen 1990). Chelated heavy metals are generally less toxic when complexed with organics (Babich and Stotzky 1983a). For organic complexation to be important, the ligands must exhibit a high affinity for the metal or they must be present in very high concentrations (Birch and Bachofen 1990). The following decrease in binding capacity of ligands for metal ions has been found: DTPA > EDTA > NTA > humic acids = tricarboxylic acids > dicarboxylic acids > monocarboxylic acids (Birch and Bachofen 1990). Microorganisms are also involved in sulphide formation, which can lead to metal removal from solution and/or high accumulation within cells, or on cell surfaces (Kelly *et al.* 1979; Gadd 1988). Precipitation of metal sulphides, such as mercury and copper sulphides, may initially involve some metal accumulation on the cell surface (Higham *et al.* 1985). Barnes *et al.* (1991) used the Sulphate Reducing Bacteria process to precipitate insoluble heavy metal sulphides from an effluent; H_2S was generated by the microorganisms (Barnes *et al.* 1991).

Some fungi excrete substances such as oxalic and citric acid, which can lead to the extracellular precipitation of metal complexes (Gadd 1986). A *Penicillium* sp. produced citric acid to protect itself against high zinc concentrations; the citric acid complexed with the zinc thereby reducing the toxicity of the latter (Schinner and Burgstaller 1989). The production of oxalic acid can result in the precipitation of metals as insoluble oxalate crystals around the cell walls (Gadd 1986).

2) Microbial exudates induced by the presence or absence of specific metal ions, e.g. siderophores and toxic metal binding proteins (Birch and Bachofen 1990). Microbial exudates may exhibit exceptionally high metal affinities, even though they may only be

present in small quantities (Birch and Bachofen 1990). They are produced for the efficient uptake of essential metal ions or form part of a complex mechanism to protect cells from toxic elements (Gadd 1986; Birch and Bachofen 1990). Metallothioneins are low molecular mass cysteine-rich compounds produced in response to the presence of toxic metals such as cadmium, copper, zinc and mercury (Gadd 1986; Birch and Bachofen 1990). Iron-dependent microorganisms release iron binding molecules, called siderophores, which complex Fe^{3+} (Gadd 1988). Cyanobacteria and algae can also produce iron-chelating siderophores (Gadd 1988). These siderophores are also able to bind other metals, such as gallium, nickel, uranium and copper (Gadd 1990).

A common response to metal toxicity in fungi is the synthesis of intracellular metal-binding proteins (Butt and Ecker 1987). In some fungi and yeasts these metallothioneins have been found to bind exclusively to Cu^{2+} (Gadd 1986), but Cd^{2+} and Zn^{2+}, for example, did not induce metallothionein synthesis in *S. cerevisiae* (Butt and Ecker 1987). However, in other yeasts Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and K^+ ions are taken in and located in the vacuole and may be bound to low molecular weight polyphosphates (Gadd 1988).

The synthesis of low-molecular weight cysteine-rich Cd^{2+} binding proteins was observed in *Pseudomonas putida* (Higham *et al.* 1985).

Escherichia coli cells were found to be more resistant to Cd^{2+} than were *S. aureus* or *B. subtilis* cells and this could be due to one or more of the proteins found in *E. coli,* which function like metallothioneins and can bind free Cd^{2+} in the cytoplasm (Laddaga and Silver 1985).

Algae are also able to synthesise phytochelatins in response to heavy metal exposure (Gekeler *et al.* 1988). The production of metal binding proteins has been reported in many algae, eg. *Chlorella ellipsoide*, *Dunaliella bioculata* and *Scenedesmus acutiformis* (Reddy and Prasad 1990). These proteins contain large amounts of glutamic acid, cysteine and glycine (Reddy and Prasad 1990). An alga, *Anabaena* sp. strain 7120, was found to produce a low molecular weight iron chelator - a siderophore known specifically as schizokinen (Clarke *et al.* 1987). Although this substance is synthesised in response to

iron starvation it also complexes with copper ions in the external environment thereby moderating copper toxicity (Clarke *et al.* 1987).

3) Substances excreted or derived from microbial biomass (Gadd and De Rome 1988). These compounds may provide a more defined and efficient biosorptive system than whole biomass (Gadd and De Rome 1988). Fungus-produced products, such as mannans, glucans, phosphomannans, chitin, chitosan and melanin (biological pigments), are especially important (Bell and Wheeler 1986; Gadd 1988; Gadd and De Rome 1988) since waste fungal biomass is available in considerable quantities and at relatively low cost (Gadd 1988). These substances, which are located in and/or exterior to the cell wall, have a high affinity for Cu^{2+} ions and can act as more efficient biosorptive agents in comparison with whole cells (Gadd and De Rome 1988; Gadd and White 1989a). Phosphorylated derivatives of chitin and chitosan were also found to be more efficient biosorption agents than their non-phosphorylated counterparts (Gadd 1990). Greater than stoichiometric amounts of metals have been found biosorbed to cell surfaces and precipitation of metal complexes on cell surfaces may be a possible explanation (Shumate and Strandberg 1985). Aiken *et al.* (1979) found evidence that Pb^{2+} accumulation by a *Citrobacter* sp. involved the formation of firmly bound $PbHPO_4$ on the cell surface. This mechanism was attributed to the activity of a cell-bound phosphatase (Macaskie and Dean 1984a;b). This enzyme functions in resting and immobilised cells liberating inorganic phosphate from an organic phosphate donor molecule, and precipitates metal phosphate on the cell surface (Macaskie and Dean 1984a;b). Any metal having an insoluble phosphate could be similarly precipitated (Macaskie and Dean 1984a;b); eg. cadmium phosphate was found on the cell surface of another *Citrobacter* sp. (Macaskie *et al.* 1987a).

Growth of *Alcaligenes denitrificans* provoked the precipitation of cadmium carbonate, as the culture was alkalised, but the amount of cadmium adsorbed on these cells was low (Remacle *et al.* 1992). *Bacillus subtilis* is able to retain several heavy metals as silicate minerals or oxyhydroxides (Urratia and Beveridge 1993).

Romeo and Gnassia-Barelli (1980) showed that substances produced by *Citrosphaera elongata* and excreted into the medium reduce the amount of Cd^{2+} and Cu^{2+} taken up by

this alga. It was shown with *Plectonema boryanum* and *Anabaena cylindrica* that the amount of copper-complexing material released by the cells increased when they were stressed with copper (Jaroim and Pearson 1984). This shows that at least certain cyanobacterial species can respond to toxic Cu^{2+} concentrations by complexing the metal thereby reducing the free metal ion concentration (Jaroim and Pearson 1984).

Natural lipid mixtures may provide an environment suitable for nucleation of lead through a general complexing phenomenon (Tornabene and Peterson 1975). The strong affinity of Pb^{2+} ions for any anionic groups, in combination with electronic and steric factors, form the basis of this theory (Tornabene and Peterson 1975). It was observed that more than 99% of a lead solution was retained at the surface of *Micrococcus luteus* by this mechanism (Tornabene and Edwards 1972).

Cells differ in the complexing agents they produce, and in the types of complexes they form with metals, and different strains of the same species may also exhibit different binding properties (Birch and Bachofen 1990).

1.3.2.4 Metal transformations

Microbes participate in transformations of several metals, which have important short term effects (Summers and Silver 1978). Metals can be enzymatically transformed by oxidation, reduction, methylation, demethylation, etc. into chemical forms that are either less toxic or more volatile than the original compound (Wood and Wang 1983; Gadd 1988). However, some metal transformations may result in the formation of compounds more toxic than the original metal (Summers and Silver 1978). For example, mercuric reductase reduces the mercuric ion (Hg^{2+}) to elemental mercury (Hg^{o}) which is more toxic but also more volatile, and can be released from an aqueous environment (Wood and Wang 1983; Williams and Silver 1984). Chromate reductase reduces Cr^{6+} to Cr^{3+}, transforming chromates (CrO_4^{2-}) and dichromates ($Cr_2O_7^{2-}$) from a toxic to a nontoxic state (Williams and Silver 1984).

Transformations of metals, involving either the generation or expenditure of energy, are carried out by many apparently unrelated groups of microorganisms most commonly found in clean and in industrially polluted environments (Summers and Silver 1978). Metal transformations are thus not limited to the unusual chemoautotrophs growing in unfavourable environments (Summers and Silver 1978). Genetic determinants of these transformation mechanisms are frequently found on extrachromosomal plasmids and transposons and are associated with detoxifying enzymes (Gadd 1988).

Cadmium, lead and tin are toxic in the cationic state and, like mercury, they are more toxic when methylated (Summers and Silver 1978). A heavy-metal tolerant strain of *Pseudomonas* was found to convert Cd to a volatile form in the presence of cobalamin (Summers and Silver 1978). It was suggested that the volatile cadmium compound was methylcadmium, but it was not positively identified (Summers and Silver 1978). The formation of tetramethyl-lead by sediment microorganisms has been observed occasionally (Summers and Silver 1978). It has been proposed that lead is likely to be methylated *in vivo* by methylated cobalamin (Summers and Silver 1978).

Because of the difficulties in capturing volatilised metals, little research and development effort have been devoted to developing commercial processes employing this microbial mechanism of metal resistance and removal in the past (Brierley *et al.* 1989). Recently, however, bacterially mediated ionic mercury reduction to volatile Hg^o has been shown to play an important role in the geochemical cycling of mercury, and this process together with the degradation of methylmercury has been shown to reduce the concentration of methylmercury in the environment (Barkay *et al.* 1992).

1.3.3 Immobilised Cell Systems

The form of biomass to be used in metal accumulation processes is important (Gadd 1988). Microbial biosorption has lacked commercial application in the past, due largely to the way in which it was applied, i.e. single or homogenised cell suspensions that required subsequent mechanical separation from solution (Wood 1992).

Immobilised or pelleted biomass show great potential (Gadd 1988). Its advantages include: mechanical strength and durability, a better capacity for reusing the biomass, easy separation of cells from the reaction mixture, high biomass loading, minimal clogging in continuous flow systems, control of particle size, and high flow rates (Gadd 1988; Tobin *et al.* 1994). Immobilisation also protects the cells from toxic metal effects (Wilkinson *et al.* 1989). For industrial use, a dead pelletised or immobilised biosorbent that exhibits efficient metal uptake and rapid settling characteristics may be the answer.

Immobilisation via entrapment is usually within alginate or polyacrylamide gels (Gadd 1988). Polyacrylamide gels have low mechanical strength and the addition of hardening or chemical coupling agents can result in toxicity, weakening of electrostatic attraction and changes in pH (Macaskie and Dean 1987; Macaskie *et al.* 1987b). Many successful immobilisation procedures have been undertaken; for example, *Zoogloea ramigera* 115, a Gram negative bacterium, was immobilised in Ca-alginate beads for the adsorption of cadmium (Duxbury 1985; Kuhn and Pfister 1990). Polyacrylamide immobilised *Saccharomyces cerevisiae* can remove Cu^{2+}, Co^{2+} and Cd^{2+} from synthetic metal solutions (Brady and Duncan 1994). This biomass can be reused after removing the metal ions with EDTA (Brady and Duncan 1994). Chitosan is a low-cost and abundant waste by-product of fishery operations and provides an algal support material (Mallick and Rai 1994). Both chitosan and carrageenan- immobilised algae possess high efficiency for anion and cation uptake (Mallick and Rai 1994). Microbial mats consisting of cyanobacteria and held together by slimy secretions produced by various microbial groups, were capable of removing metals such as Cd^{2+}, Pb^{2+}, Cr^{2+}, Se^{2+} and As^{2+}, from contaminated waters (Bender *et al.* 1995). Metals were taken up by deposition of metal compounds outside the cell surfaces as well as chemical modification of the aqueous environment surrounding the mats (Bender *et al.* 1995).

The use of biofilms supported on inert matrices is a way of overcoming the problems associated with entrapment and is thus commonly employed (Macaskie and Dean 1987).

Most of the copper (92%) was removed from a solution by wall growth of a ten-membered bacterial community in continuous culture suggesting that solid supports would be the most

useful in a stirred tank reactor (Dunn and Bull 1983). *Enterobacter aerogenes* grew on activated carbon particles and developed a biofilm due to excreted bacterial polysaccharide (Scott and Karanjkar 1992). This biofilm rapidly adsorbed Cd^{2+}, initially onto its external surface, followed by a slower uptake, possibly by metabolism-dependent means (Scott and Karanjkar 1992). This biosorbent could be reused as the polysaccharide producers showed a regenerative capacity (Scott and Karanjkar 1992).

Many filamentous microorganisms rapidly form pellets in submerged liquid culture and may provide a cheap alternative process for removing trace concentrations of metal ions from solution (Wood 1992). Living, non-growing biomass may also be used either as pelleted mycelium (for filamentous fungi), or as immobilised preparations, for yeasts and other unicellular microorganisms (De Rome and Gadd 1991). Zhou and Kiff (1991) immobilised *Rhizopus arrhizus* in reticulated foam particles to increase its mechanical strength and resistance to chemicals in the environment, so that it could be used in industrial application (Zhou and Kiff 1991). This process, however, could deal only with low concentrations of metallic effluents and would thus be best used in combination with a conventional process (Zhou and Kiff 1991).

It has also been found that immobilised *Chlorella* cells accumulate more mercury than free cell systems (Wilkinson *et al.* 1989). Immobilising algae in copolymers may lead to a whole new class of biosorbents (Harris and Ramelow 1990). These would function like traditional ion-exchange or chelating resins, with the advantage that interfering ions such as the alkali and alkaline-earth elements, would be effectively removed because they do not bind to the algae (Harris and Ramelow 1990). Future industrial processes might involve the use of pre-grown immobilised organisms as a filter through which metal-containing effluent could be passed (Gadd 1992).

1.3.4 Dead versus Living Cells for Metal Accumulation

Both living and dead cells have been found to accumulate heavy metals and both systems have various advantages and disadvantages (Gadd 1988). Living cells have a variety of uptake or accumulation mechanisms and can be manipulated morphologically,

physiologically and genetically with considerable ease (Gadd 1990). Additionally the possibility exists for the removal of other pollutants from the effluents (Gadd 1990). With immobilised living cells each cell may become saturated, but the system as a whole is self-replenishing due to continued growth; also two or more organisms may be employed in a synergistic way (Macaskie 1990). When using living material, a microbial strain is required which can take up metals, grow in high metal concentrations with no lag and no marked increase in the doubling time (Baldry and Dean 1980). Metal-uptake by growing batch cultures has been found to be maximal during the lag phase or early stage of growth, and then to decline as the culture reaches stationary phase (Gadd 1988). In commercial application these properties would be important as the process would be of limited use if it was slow or had to be restricted to very low metal concentrations (Baldry and Dean 1980).

For metal removal and recovery, dead fungal biomass seems to offer several advantages: it may be obtained cheaply from several industrial sources; it is not subject to metal toxicity or adverse operating conditions; it acts rapidly as it functions as an ion-exchanger; it needs no nutrient supply; and surface-bound metals may be recovered by relatively simple non-destructive treatments (Gadd 1986; 1988; 1990). The disadvantages include its sensitivity to pH and it is subject to metal speciation (Macaskie 1990). The method employed to inactivate the biomass (heat or solvent treatment) is also likely to modify the surface properties of the biomass and thus affect its metal-binding properties (Brown 1991). If organisms are degraded before use, they will not be subject to the regulatory requirements governing the release of genetically manipulated organisms (Brown 1991). However, many workers have found little difference in metal uptake between living and processed biomass (see Siegel *et al.* 1990, for examples).

As indicated above waste mycelial biomass, eg. *Rhizopus, Mucor, Penicillium* and *Aspergillus*, from various fermentation industries can be used as good metal biosorbents (Fourest and Roux 1992). The efficiency of these systems depends on many factors such as the metal binding capacity of the biosorbents and the physical and chemical conditions in the effluent (Fourest and Roux 1992). Dried cells of *Zoogloea ramigera* have a greater ability to adsorb metal ions than do their living counterparts because their optimum

conditions for fermentation are different from their requirements for optimum metal adsorption, especially for Cr^{6+} (Sag and Kutsal 1989).

Processes employing biomass that is viable, but grown prior to contacting the metal solution, are not limited by metal toxicity as are those using growing biomass; nor do they require nutrient addition and the biomass is more compact and can be recycled many times without being degraded (Brown 1991). For example, a novel hybrid bioprocess employing a *Citrobacter* sp. has been described that involves a pre-growth phase in the absence of heavy metals (Macaskie 1990).

1.3.5 Recovery of Metal Ions

Recovery of metal ions from the biomass is very important for two reasons: it allows the metals to be recycled to the industry thus avoiding the need for their disposal; and, it allows the biosorbent to be reused (Brown 1991).

Whether a metal is accumulated predominantly by surface retention, or intracellularly, will affect the economics of any recovery process applied (Norris and Kelly 1979). Metabolism-independent biosorption is often reversible by simple non-destructive physical\chemical treatments (Norris and Kelly 1979; Gadd 1990). For instance, manipulations of the pH of desorbing solutions may be a good method for selective removal of metal ions, while carbonates and\or bicarbonates have the potential for non-destructive recovery (Gadd 1990). Metals can be eluted from biosorbents using dilute mineral acids, but under some conditions alkali solutions (e.g. sodium carbonate) and EDTA can also be used (Brown 1991). Since intracellular accumulation may be irreversible destructive recovery, such as incineration or dissolution in acids or alkalis, may be necessary (Norris and Kelly 1979; Gadd 1990).

With the alga *Chlorella vulgaris* the biosorption and elution of Au^{3+}, Hg^{2+} and Ag^+ is very different to that of other metals as these metal ions are not released back into solution by lowering the pH of the reaction mixture (Darnall *et al.* 1986). However, exposure of the metal bound algae to high concentrations of sulphur-containing ligands, such as

thiourea or mercaptoethanol, is effective at certain pHs in stripping these metals from the algal surface (Darnall *et al.* 1986). Thus, some metal ions can be selectively recovered from the algae using an appropriate elution scheme (Darnall *et al.* 1986).

1.3.6 Conclusion

With continued pollution of the environment by potentially toxic heavy metals, it is important that administrative authorities and responsible industries take steps to rectify the situation - perhaps fuller exploitation of microbially-based technologies (Gadd 1988) will provide some solutions.

The potential for development of such microbial-based pollution control processes depends on many factors such as the capacity, efficiency and the selectivity of the biosorbent; ease of metal recovery; competitiveness with existing physical and chemical treatments; economics; and immunity from interference by other components of the effluents or operating conditions (Gadd 1988). Microbial accumulation of heavy metals will probably never replace existing processes but it may act as a polishing system to existing processes that are not completely efficient (Gadd 1988). For both economic and environmental reasons microorganisms, due to their unique features of metal biosorption and resistance, have the potential for use in metal recovery from solutions.

1.4 RESISTANCE OF MICROORGANISMS TO HEAVY METALS

1.4.1 Introduction

Twenty-six of the elements essential to life are required in intermediate to trace amounts, with an overabundance of any of these causing a build-up to an intracellularly toxic level, often resulting in death (Wood and Wang 1983). Some microbial cells are resistant to one or many of the heavy metals to varying degrees (Williams and Silver 1984), which is particularly relevant to microbial ecology (Gadd and Griffiths 1978). Metals exert toxicity through several mechanisms: they can bind to a variety of organic ligands, which causes denaturation of proteins, including enzymes; disruption of cell membranes; and, decomposition of essential metabolites (Bowen 1966).

Some metals are intrinsically more toxic than others (Darnall *et al.* 1986). Since metals act primarily because of their affinity for chelating agents, an assessment of the degree of affinity may indicate the comparative toxicity of a metal (Sterritt and Lester 1980). A general trend in metal toxicity is as follows: Fe^{3+}, Hg^{2+} > Cu^{2+}, Al^{3+} > Ni^{2+}, Pb^{2+} > Co^{2+}, Zn^{2+} > Fe^{2+}, Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Li^+ > Na^+ > K^+, but this rating alone has limited value in the estimation of toxicity (Sterritt and Lester 1980), as many other factors affect the toxicity of metals.

Microbial resistance to toxic heavy metals has arisen in two distinct patterns: 1) some microorganisms have inherited the ability to resist high concentrations of toxic elements through their evolution under extreme conditions; for example, a *Pseudomonas putida* strain initially resistant to low concentrations of cadmium (0.25 mM Cd^{2+}) easily adapted to grow at a higher concentration of cadmium (3 mM Cd^{2+}) (Higham *et al.* 1985); and 2) some acquired a transferred resistance to the polluted environment, such as the acquisition of extrachromosomal DNA. Studies have shown that, generally, Gram negative bacteria (with *Pseudomonas* species predominating (Seyfried 1980)), are more metal tolerant than Gram positive bacteria (Duxbury and Brickwell 1983).

There is evidence that species diversity may be reduced in metal polluted habitats (Gadd and Griffiths 1978). This may be due not only to adaptive changes, but also to other environmental changes associated with the metal pollution (Gadd 1986). In other words, altered pH and nutrient status may be important in altering species composition (Gadd 1986). Resistant species that can accumulate metals may exert a protective effect on sensitive species in the same system by removing the metal from the system (Sterritt and Lester 1980), for example, mercury is bacteriostatic and thus the mercury resistant bacteria would detoxify the medium enabling the sensitive cells to eventually grow (Summers 1984). With cadmium, however, resistance usually involves an efflux system, and thus sensitive bacteria will not be protected in the presence of resistant bacteria (Summers 1984).

Two main types of resistance have been recognised: 1) Non-specific resistance arising from differences in the physiological state of the organism, such as impermeability, or detoxification by chelation (Sterritt and Lester 1980); and 2) inheritable specific resistance for particular heavy metals, coded for by genes located on plasmids.

1.4.2 Non-specific Resistance

'Gratuitous' (Gadd and Griffiths 1978) resistance mechanisms are based on the presence or production of materials that protect cells from metal toxicity but are not synthesised specifically for that purpose (Duxbury 1985). These mechanisms tend to be population-density-dependent because the amount of such materials is related to the number of individuals present - the larger the population size, the more chance there is for some cells to become tolerant, and *vice versa* (Duxbury 1985).

Microorganisms can produce substances that bind or chelate metals and, therefore, reduce their toxicity; for example, citric acid produced by many yeasts and fungi can chelate with many metal ions (Gadd and Griffiths 1978). Intracellular organic substances can also determine metal tolerance; mercury-tolerant *Aspergillus niger*, for example, contains a pool of intracellular sulphydryl compounds which complex with mercury and alleviate its toxic effects (Ashworth and Amin 1964). Certain mutants of the yeast *Saccharomyces cerevisiae*

demonstrate copper and cadmium resistance due to the production of a metallothionein which causes metal detoxification by chelating copper and cadmium (Tohoyama *et al.* 1995).

Precipitation of insoluble metal complexes can also occur through the activities of membrane associated sulphate reductases (Wood and Wang 1983). In the sulphate reducer *Desulfovibrio desulfuricans* H_2S is produced in high concentrations and its production is unaffected by the addition of high concentrations of heavy metals (Temple and Le Roux 1964). Biosynthesis of oxidising agents such as oxygen or hydrogen peroxide can also lead to the precipitation of insoluble metal complexes (Wood and Wang 1983). Resistance often depends on the metabolic activity of the cell (Wood and Wang 1983).

Ahmed *et al.* (1992) found that in *Pseudomonas testosteroni* the chemical speciation of a metal complex can change during the growth of the organism owing to the consumption of a nutrient that functions as a ligand, eg. speciation of the major Cu(II) compound changes from $[Cu(NH_3)_3(H_2O)_3]^{2+}$ to $[Cu(NH_3)_2(H_2O)_4]^{2+}$ during growth because of the uptake of ammonia by the cells. These changes can affect the bioavailability and toxicity of the metal ions (Ahmed *et al.* 1992).

1.4.3 Inheritable Specific Resistance

These mechanisms of genetic resistance involve genetic determinants that are found on small plasmids and transposons (Williams and Silver 1984). Plasmids are autonomous genetic elements of DNA that encode for a variety of functions in many different species of bacteria (Trevors *et al.* 1985), and are often known as Resistance Transfer Factors (RTFs) which are found linked to antibiotic resistance (Sterritt and Lester 1980). These can make bacteria resistant to 1000X the concentration of the metal normally causing inhibition (Sterritt and Lester 1980). Plasmid-mediated metal resistance may be ecologically important since resistance can be rapidly transferred from resistant to sensitive bacteria (Gadd and Griffiths 1978). Thus populations in natural habitats could adapt genetically to survive conditions of metal toxicity faster than by the processes of spontaneous mutation and natural selection (Silver *et al.* 1976).

Metal ion resistance systems have been found on plasmids of a vast eubacterial group, and genes initially found on plasmids are often, subsequently found on chromosomes of related bacteria (Ji and Silver 1995). Resistance has also been found to be chromosomally determined, such as the resistance of a *Bacillus* sp. to Cd^{2+} and Hg^{2+} (Collins and Stotzky 1989). Plasmid-mediated resistance to many metals has been studied and, in addition to mercury resistance determinants, highly specific resistance systems for arsenic, (and antimony) copper, cadmium (and zinc in Gram positive bacteria), tellurite, silver and perhaps other toxic metal ions are known (Ji and Silver 1995).

Plasmid-encoded genes may provide organisms with 4 basic mechanisms of resistance:

1. Transformation of the metals by enzymes such as oxidases, reductases, alkylating and dealkylating lyases (See Section 1.3.2.4).

2. Alteration of the site of inhibition (Trevors *et al.* 1985).

3. Impermeability of the cell wall to metals. Blocking cellular uptake by altering the metal uptake pathway: the mechanism of chromosomally determined resistance to arsenate, cadmium and chromate (Trevors *et al.* 1985).

4. Metal efflux and bypass mechanisms which keep toxic heavy metal concentrations low in the cell interior (Wood and Wang 1983; Trevors *et al.* 1985), exemplified by resistance to arsenic, cadmium and chromate in Gram negative and Gram positive bacteria (Silver *et al.* 1989a,b). Resistance to Zn^{2+}, Ni^{2+}, Co^{2+} and Cd^{2+} in Gram negative bacteria is based on the action of proton-cation antiports which are members of a newly-recognised protein family (Nies and Silver 1995).

These resistance mechanisms not only protect the organisms in harsh environments but also play an important role in the cycling of toxic metals in the biosphere (Williams and Silver 1984).

1.4.4 Specific Metal-Resistant Bacteria

Chromium Resistant Bacteria

A *Pseudomonas aeruginosa* strain, known to be resistant to arsenic, mercury, silver, and tellurium, was found to contain a plasmid that also conferred resistance to boron and chromium (Summers and Jacoby 1978).

Cadmium Resistant Bacteria

Resistance to high concentrations of cadmium salts has only been found as a stable, inheritable phenotype on the plasmids of the pathogenic bacterium *Staphylococcus aureus* (Belliveau *et al.* 1987). No plasmids were found in a Cd^{2+}-resistant *Alcaligenes* sp. (McEntee *et al.* 1986). This resistance, and reduced metal uptake, are constitutive, and are not dependent on prior exposure to cadmium, although the biochemical basis is unknown (Summers and Silver 1978). Cadmium is prevented from accumulating intracellularly as it is expelled from the cell by a separate energy-dependent system (Summers 1984; Belliveau *et al.* 1987; Trevors 1989). Conversely, in Gram negative bacteria, Cd^{2+}-resistance is due to the metal complexing with metallothionein proteins rather than being expelled via an efflux system (Laddaga and Silver 1985). In a cadmium resistant strain of *S. cerevisiae*, Cd-binding proteins with high affinity for Cd^{2+} were found in the cytosol (Joho *et al.* 1985). Cadmium entering the cytoplasm can be chelated by polyphosphate during the early growth stages, with binding proteins playing a role during the later stages of growth (Rayner and Sadler 1989).

Copper Resistant Bacteria

The mechanisms of copper resistance in bacteria are efficient, diverse and plasmid-encoded (Belliveau *et al.* 1987). Microbial production of organic acids and chelating agents may provide protection from Cu^{2+} (Belliveau *et al.* 1987). Bitton and Freihofer (1978) showed that encapsulated *K. aerogenes* were more resistant to copper and cadmium than were strains with no capsules. An energy-dependent influx system was found in *Penicillium ochre-chloron* (Gadd and White 1985).

Lead Resistant Bacteria

Micrococcus luteus and an *Azotobacter* sp. are both capable of immobilising Pb^{2+}, without producing any toxic effects on growth, as 99% of the metal was found in the cell wall and membrane (Tornabene and Edwards 1972). Some bacteria, such as *S. aureus* contain plasmids that confer resistance to several metals, including lead (Trevors *et al.* 1985). There is, however, no evidence to show that lead resistance involves transformation (Summers and Silver 1978). Bacterial resistance mechanisms to lead require further investigations (Belliveau *et al.* 1987).

1.4.5 Conclusion

Heavy metal resistant microorganisms are important in natural environments, as they enable the survival and continuation of microbial populations exposed to toxic metal concentrations. They may also be important in the accumulation of heavy metals from industrial effluents and in decreasing metal contamination in natural water systems as they would have the potential to survive and function in these harsh conditions.

1.5 ECOLOGICAL CONSIDERATIONS

1.5.1 Introduction

Few studies have evaluated the effects of heavy metals on the activities of natural heterogeneous microbial populations (Babich and Stotzky 1985). The effects of pollutants on microorganisms and their biochemical activities need to be more completely understood, so that quality control criteria can be established (Babich and Stotzky 1985). The *in situ* response of a microorganism to a pollutant is dependent on many biotic and abiotic factors (Babich and Stotzky 1980).

Environments contaminated with heavy metals affect microorganisms in two general ways; they cause a reduction in the number and species diversity of the microbiota, and cause the development of metal-resistant heterotrophs as the dominant members of the microbial population (Tyler 1981; Duxbury 1985).

The effects of toxic heavy metals on any microorganism-mediated processes may adversely affect the continued homeostatic functioning of the biosphere (Babich and Stotzky 1985). For example, phytoplankton represent the basic food resource, and inhibition of photosynthesis would thus have severe repercussions on the entire natural ecosystem (Babich and Stotzky 1985).

The concentration of available, or potentially available, heavy metals, and not their total chemical concentration appears to be the primary determinant in their toxicity towards microorganism-mediated ecological processes (Babich and Stotzky 1985). Studies to establish the minimum concentrations of heavy metals inhibitory to microbial growth must allow for the effects of organics and inorganics on heavy metal toxicity (Babich and Stotzky 1980).

1.5.2 Environmental Factors affecting Metal Toxicity

Metal toxicity is dependent on the physicochemical characteristics of the environment (Babich and Stotzky 1979), as these determine the chemical speciation forms and, therefore, the bioavailability (and toxicity) of the heavy metals to the indigenous microbiota (Babich and Stotzky 1985; Hughes and Poole 1991).

The availability of metal ions for transport into cells is restricted by their natural abundance and solubility in water (Wood and Wang 1983). Solubility is influenced by pH, temperature, oxidation-reduction potential (E_h), the presence of competing ions, and the presence of surface-active substances such as particulates and macromolecules, including proteins, humic acids, clays and other organics (Babich and Stotzky 1979; Wood and Wang 1983). High pH and E_h favour higher oxidation states for trace metals, which results in low solubility and availability (Wood and Wang 1983). Of all environmental factors pH was found to have the most significant effect on the toxicity of the metals (Kuyucak and Volesky 1988). The nutritional state of the cells may also affect heavy metal toxicity (Brynhildsen et al. 1988).

1.5.2.1 pH

pH is the measure of the availability of H^+ ions in a system (Babich and Stotzky 1980). It can change the metabolic state of the cell, for example, by altering the activity of functional groups in the biomass, which affects the competition of metallic ions for the cellular binding sites (Kuyucak and Volesky 1988). At low pH it was found that the ligands of the cell wall are closely associated with the hydronium ion (H_3O^+). This restricts the access of the metal ions to the ligands due to repulsive forces; the lower the pH the stronger the repulsive forces (Zhou and Kiff 1991). With increasing pH more of the negative charge bearing ligands would be exposed resulting in the attraction of metal ions onto the cell surface. Metal uptake may also be inhibited by proton competition at low pH (Wong et al. 1993), and the negatively charged sites, such as caboxylates and phosphates are greatly reduced in number and, therefore, fewer metal cations are adsorbed. pH also affects the extent of complexation of metals with organic and inorganic

constituents (Babich and Stotzky 1985), i.e. it affects metal chemistry in water (Zhou and Kiff 1991). At neutral pH adsorption is enhanced by a proportional increase in the number of ionised acidic groups, making microbial biofilms more efficient at neutral pH (Ferris *et al.* 1989).

Every metal is accumulated by microbial cells at a specific pH (Wnorowski 1991). Generally, at an acid pH the metals exist as ionic cations that are freely available; and at an alkaline pH the ionic cations precipitate as insoluble hydroxides, oxides or carbonates, decreasing their availability (Gadd and Griffiths 1978; Gadd 1986). The pH at which precipitation occurs varies between the different metals and among the oxidation states of the same element (Gadd and Griffiths 1978), with different metals being found in different ionic forms depending on the pH. Lead exists as the divalent cation Pb^{2+} below pH 5 and as the pH increases the Pb^{2+} is converted to the monohydroxylated species $PbOH^+$, with all the lead present existing as $PbOH^+$ at pH 8-9 (Hahne and Kroontje 1973) (Fig. 1.1)
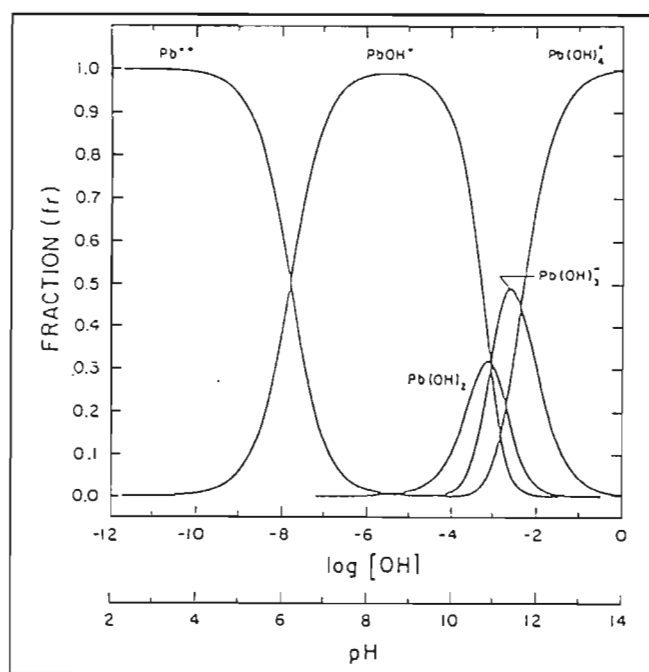
Figure 1.1 Distribution of molecular and ionic species of Pb^{2+} at different pH values (Hahne and Kroontje 1973).

At a pH below 8 in a pure aqueous solution cadmium exists predominantly as the free divalent ion, which is available for adsorption. The formation of $CdOH^+$ begins at pH 7.5 and that of $Cd(OH)_2$ at pH 9 (Hahne and Kroontje 1973) (Fig. 1.2).
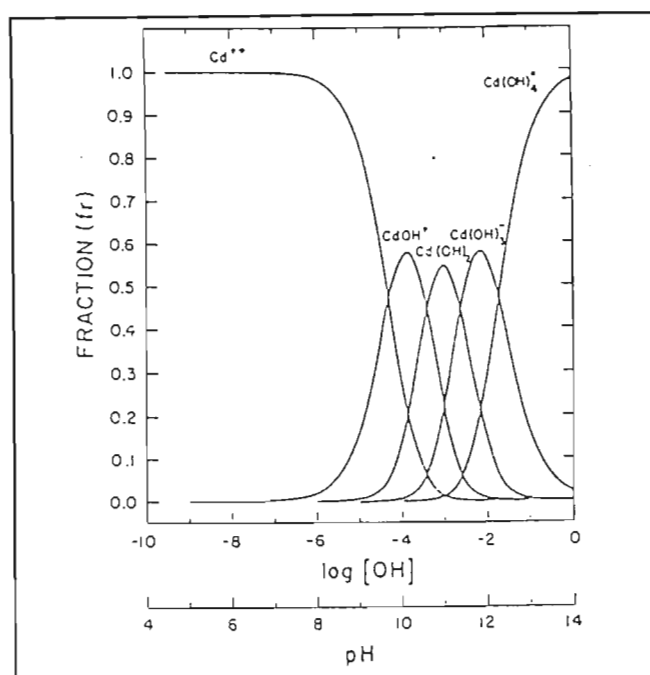
Figure 1.2 Distribution of molecular and ionic species of Cd^{2+} at different pH values (Hahne and Kroontje 1973).

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). As each metal usually binds to a different ligand in the cell, the toxicity may vary with pH, because the affinity of a metal for a ligand depends on pH (Collins and Stotzky 1989). A low external pH generally decreases the rate and the extent of metal biosorption in fungi (Gadd 1990), eg. *Mucor mucedo* mycelia could bind Cu^{2+} ions if the solution pH was not less than 4 (Wales and Sagar 1990). A rapid decrease in accumulation occurred at extreme pH's, especially toward the acid side (Brady and Duncan 1994). Using waste filamentous fungi as biosorbents the maximum Pb^{2+} uptake occurred at pH 5 as the Pb^{2+} uptake was less inhibited by protons (Fourest and Roux 1992). With dead *Zoogloea ramigera* cells the pH of the biosorption media affected the rate of adsorption onto the

cells, eg. the optimum pH for Cr^{6+} adsorption was 2, while that for Cd^{2+} was pH 6 (Sag and Kutsal 1989). The optimum pH will vary with each microorganisms and with changes in environmental conditions.

1.5.2.2 Oxidation-Reduction potential (E_h)

E_h is a measure of the availability of electrons (Babich and Stotzky 1980). Reducing environments have a negative E_h, while oxidising environments have a positive E_h (Collins and Stotzky 1989). Heavy metals deposited in an environment with a negative E_h may combine with S^{2-} ions to form insoluble sulphide salts which are then unavailable for uptake by microorganisms (Collins and Stotzky 1989). E_h also affects the valence state of the metal, for example, chromium can exist as Cr^{3+} or as the more toxic, Cr^{6+} form, depending on the E_h of the environment (Babich and Stotzky 1980; Collins and Stotzky 1989). It has been found that the total metal adsorption by whole cells of *Chlorella fusca* did not increase under oxygen reduced conditions, as the cells were able to compensate for these small changes in redox potential (Wehrheim and Wettern 1994a).

1.5.2.3 Ion interactions

The biological activity of heavy metal ions can be markedly affected by the presence of other ions (Babich and Stotzky 1985). In natural environments, 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 reduce metal toxicity by precipitation; for example, hydroxyl, phosphate, thiosulphate, chloride, carbonate and bicarbonate ions form precipitates with metal ions depending on their concentration and the pH of the solution (Gadd and Griffiths 1978; Babich and Stotzky 1983b). These inorganic anions can also form soluble coordination complexes with metal cations and these have different toxicities to the various microorganisms (Babich and Stotzky 1980). In general, the extent of metal-uptake inhibition caused by inorganic and organic anions follows the order EDTA $> > SO_4^{2-} > Cl^- > PO_4^{3-} >$ glutamate $> CO_3^{2-}$, with EDTA forming considerably stronger complexes than the other anions (Tobin *et al.* 1987). Chloride complexes are found in all natural soil and water environments and are

the most mobile and persistent of the complexing agents with regard to heavy metals (Hahne and Kroontje 1973). The extent of metal-uptake inhibition is related to the metal-ligand binding strength: in Cd^{2+} and Pb^{2+} systems the effects are generally consistent with a mechanism of direct competition between the biomass and anions in solution (Tobin *et al.* 1987).

Cations can reduce metal toxicity, perhaps by competing for uptake systems (Collins and Stotzky 1989). There is a need to understand how combinations of metals affect the physiological, biochemical, and ecological processes of the various organisms (Ting *et al.* 1991). Antagonistic interactions may result from competition between the metals for common cell surface binding sites (Babich and Stotzky 1980). The rate of nickel uptake by both *S. cerevisiae* strains and *C. utilis* was inhibited more strongly by Mg^{2+} than by Zn^{2+} (Norris and Kelly 1979). Another antagonistic interaction involves the sorption of one heavy metal to the amorphous complex of another heavy metal (Babich and Stotzky 1980), for example, Cu^{2+} ions adsorbed to the negatively charged sites on the micelles of amorphous $Fe(OH)_3$, making copper unavailable for uptake by the alga (Steeman and Kamp-Nielson 1970). Although cobalt uptake, and the simultaneous uptake of another cation such as Ni^{2+}, Mn^{2+} or Zn^{2+} has been reported to be mutually inhibitory, this pattern of mutually exclusive uptake among metals does not operate with all metal cation combinations (Norris and Kelly 1979). A synergistic interaction may result from the adsorption of two metals to the cell surface, with the adsorption of one metal increasing the permeability of the membrane for the second (Babich and Stotzky 1980). More research is required in this area (Ting *et al.* 1991).

1.5.2.4 Organic constituents

Organics can lead to a decrease in toxicity of heavy metals in 2 possible ways:
1) The organic matter reacts with the metal ions by either covalent or coordination bonding, which results in the formation of compounds which are less toxic than the metal ions themselves (Babich and Stotzky 1980; Bagy *et al.* 1991);
and/or 2) the ions adsorb onto the surface of colloidal particles which renders them less toxic (Bagy *et al.* 1991).

Binding to environmental constituents usually reduces toxicity, but sometimes toxic action still results even when there are no free metallic ions; for instance, some metal complexes, such as methyl mercury, are more toxic than the Hg^{2+} ions (Gadd and Griffiths 1978).

Humic acids are especially important in the aquatic environment and may result in the complete disappearance of toxic effects of some heavy metals (Gadd and Griffiths 1978). Humic compounds are a complex assemblage of aliphatic and aromatic constituents (Dissanayake 1983). They function as natural chelators and retain large quantities of metals by cation exchange and surface adsorption, depending on the environmental conditions and the types of heavy metals encountered (Dissanayake 1983).

Attractive forces between metal ions and organic material range from weak, which leaves the ions easily replaceable (physical adsorption), to strong, making them indistinguishable from chemical bonds, as in metal chelation by organic materials (Forstner 1981).

Higher concentrations of organics would perhaps help in protection of microorganisms against toxic heavy metals, but could also lead to eutrophication of the water (Albright and Wilson 1974). Other compounds which chelate metals (such as citrate, glutamate and EDTA) have significant affects on microbial responses to heavy metals when included in the growth media (Gadd and Griffiths 1978).

1.5.2.5 Temperature

Temperature can also affect the toxicity of heavy metals to microorganisms by altering the physiological state of the cells, rather than by affecting the chemical speciation or availability of the metals (Collins and Stotzky 1989). Generally, at temperatures optimum for growth, the toxicity of heavy metals increases (Collins and Stotzky 1989). For dead cells of *Zoogloea ramigera* the optimum temperature for metal uptake was found to be 25°C as adsorption reactions are normally exothermic and, therefore, the extent of metal adsorption generally increases with a decrease in temperature (Sag and Kutsal 1989).

1.5.2.6 Nutritional state of microorganisms

The nutritional state of an organism can influence its sensitivity to heavy metals (Brynhildsen *et al.* 1988). Most microorganisms live in energy-limited environments and, therefore, it is important to study metal stress at low energy and nutrient concentrations to mimic the conditions found in nature (Brynhildsen *et al.* 1988). For example, the uptake of cadmium by growing *Citrobacter* cells was observed to rise sharply during the mid-exponential phase of growth in a nutrient-rich medium, and then to decline concomitantly with a fall in pH of the growth medium (Macaskie and Dean 1984a). This would not be observed under nutrient limited conditions.

Fungi accumulate considerable amounts of Pb^{2+} and Cd^{2+} from their growth medium (Duddridge and Wainwright 1980). It was found that a change from a high to a low carbon and nitrogen medium did not affect the amount of Pb^{2+} and Cd^{2+} accumulated by the aquatic fungi, *Pythium* sp, *Dictyuchus* sp and *Scytalidium lignicola* (Duddridge and Wainwright 1980). This suggests that these fungi are very versatile and can accumulate heavy metals from fresh waters, which are either nutrient poor or enriched with organic effluents (Duddridge and Wainwright 1980). In a phosphate-rich medium, the bacterium *Pseudomonas fluorescens* precipitates lead, while in a phosphate-deficient medium lead was concentrated within the microorganism (Al-Aoukaty *et al.* 1991). Thus, variation in the concentration of nutrients may promote either intracellular or extracellular bioaccumulation of lead (Al-Aoukaty *et al.* 1991), which would be important in metal recovery systems.

1.5.3 Conclusion

There is little information about how heavy metals are partitioned and cycled in natural unpolluted environments, nor is it known what potential damaged ecosystems possess to enable them to recover when a pollutant is removed (Duxbury 1985). Chemostats can be employed to reproduce particular aspects of natural ecosystems, or to produce more physiologically realistic cultures for detailed laboratory studies (Duxbury 1985). More research, incorporating environmental conditions, is necessary before these microbial processes can be used efficiently in the cleaning up of metal contaminated natural water systems.

Heavy metals are known to be toxic to living organisms and to cause pollution problems when effluents containing them are discharged into natural water systems. However, it is also known that microbial associations exist which can remove heavy metals from solution and thus have the potential to replace or enhance existing clean-up processes.

Are attached or free-living biomasses more efficient at removing heavy metals from solution? Are living or dead biomasses more capable of removing heavy metals from solution? Would fungal biomasses offer a better system of metal removal than bacterial biomasses? Do mechanisms of metal uptake differ? Which environmental conditions affect these systems? Are adaptation periods beneficial? Do the proportions of adapted microbial associations affect the efficiency of metal removal? Is metal uptake from mixed metal solutions different to uptake from single ion solutions? Can one establish a relationship between biomass and metal-removal capacity? Do the characteristics of an attachment surface influence biofilm formation and subsequent metal removal? Can systems be developed which will answer these questions?

This investigation was thus aimed at enriching for and adapting microbial associations to grow in various metal-containing nutrient solutions. Initially five metals, *viz.* copper, cadmium, lead, chromium and mercury will be included and their uptake by the adapted microbial associations assessed either, indirectly using High Performance Liquid Chromatography and Flame Atomic Absorption Spectrophotometry to determine the concentration of metals in solution before, during and after metal uptake by the microorganisms, or directly by means of energy dispersive X-ray microanalysis, including spot analysis and mapping to determine the amount of metal associated with the biomass itself.

As attached microorganisms are known to be more stable, the attachment of cells to various surfaces will also be investigated and the extent of metal-uptake by these attached cells compared with that of the free-living or planktonic microbial associations.

Various factors known to affect metal uptake by microbial cells, *viz.* metal uptake from combinations of metals; pH of the metal-containing solutions and the nature of the

microbial associations, will be investigated. The latter will be studied using various proportions of pregrown metal-adapted inocula to determine if this has any affect on the uptake of the individual metals.

As fungi are known to be more stable and more efficient at metal uptake than bacteria, their capacity to remove heavy metals from polluted waters will also be investigated, together with determining the most efficient biomass concentration for metal uptake. The metal uptake capacities of living and dead biomass will also be compared.

The mechanism by which the fungal biomass removes heavy metals from solution will be investigated using electron microscopy, both Scanning and Transmission Electron Microscopy, and energy dispersive X-ray microanalysis.

CHAPTER 2: ENRICHMENT FOR AND ADAPTATION OF MICROORGANISMS FOR GROWTH IN HEAVY METAL CONTAINING SOLUTIONS

2.1 INTRODUCTION

Microorganisms are known to be very versatile and adaptable and to have the potential for use in the cleaning-up of heavy metal polluted waters. It has been found that microbial associations are more stable and better able to withstand harsh conditions, than are isolated single species (Gadd 1988). Thus, microbial associations consisting of various bacteria and some yeasts were enriched for and adapted to withstand elevated metal concentrations. These microorganisms need to be able to survive and grow at metal concentrations above those that normally produce toxic conditions.

2.2 MATERIALS AND METHODS

2.2.1 Initial Enrichments

2.2.1.1 First stage enrichments

An initial inoculum was obtained from an industrial effluent from Hammarsdale sewage works in Natal. Nutrient Broth (Biolab) or Nutrient Agar (Oxoid) were both used at half-strength in all the experiments, to provide a carbon/energy source, but at the same time decrease the chances of complexation between the metals and the organic constituents in these media. Liquid medium, consisting of half-strength Nutrient Broth (9 ml), was inoculated with 1 ml industrial effluent in 100 ml Erlenmeyer flasks and heavy metals were added to obtain the following final metal concentrations: copper ($CuCl_2.2H_2O$) - 0.2 mg l^{-1}; cadmium ($CdCl_2$) - 0.08 mg l^{-1}; lead ($Pb(NO_3)_2$) - 0.2 mg l^{-1}; chromium ($Cr(NO_3)_3.9H_2O$) - 0.2 mg l^{-1}; mercury ($HgCl_2$) - 0.1 mg l^{-1}. Glucose (0.5% v/v) was added to some flasks to provide an additional carbon/energy source. The flasks were incubated at 30°C on a rotary shaker (170 rpm). A chromium-containing tannery effluent (containing approximately 4% (w/v) Cr^{3+}) was also used as an inoculum source in

enrichment cultures for chromium-resistant microorganisms. Very low metal ion concentrations were used in these first stage enrichments and therefore further enrichments were carried out to determine if the microorganisms would remain viable at elevated metal concentrations.

2.2.1.2 Second stage enrichments

Cultures were set up in 250 ml Erlenmeyer flasks, with 10 ml of inoculum from the first stage enrichments, various aliquots of concentrated metal salt solutions (APPENDIX A) to obtain final metal concentrations of: Cu^{2+} - 100 mg l^{-1}; Cd^{2+} - 100 mg l^{-1}; Pb^{2+} - 150 mg l^{-1}; Cr^{3+} - 150 mg l^{-1} and Hg^{2+} - 5 mg l^{-1}, and the volume made up to 100 ml with half-strength Nutrient Broth. After incubation at 30°C on a rotary shaker (150 rpm), these cultures were filtered through 0.22μm cellulose acetate membrane filters. The filters were fixed in 3% (v/v) glutaraldehyde, washed twice in 0.05 M cacodylate buffer and dehydrated in an alcohol series (10 minutes each in 30, 50, 70, 80, 90 and 3 times in 100%), critical point dried in a Hitachi HCP-2 CPD and viewed in a Hitachi S-570 SEM after gold-palladium coating. The Gram stain technique was also used to view the microbial associations under the light microscope.

2.2.1.3 Growth of mixed cultures under different oxygen regimes

Microbial associations obtained from the second stage enrichments were cultured under aerated and non-aerated conditions to determine their growth rates and their metal uptake potential under these conditions.

Ten Erlenmeyer flask cultures were set up with 5 ml inoculum from the second stage enrichment cultures, various aliquots of the concentrated heavy metal solutions (APPENDIX A) to obtain final metal concentrations of Cu^{2+} - 100 mg l^{-1}; Cd^{2+} - 100 mg l^{-1}; Pb^{2+} - 150 mg l^{-1}; Cr^{3+} - 150 mg l^{-1}; Hg^{2+} - 6 mg l^{-1}, and the volume made up to 50 ml with half-strength Nutrient Broth.

Five of the flasks were aerated using a sparging system supplied with sterile air by a fish tank pump. The flasks were incubated at 30°C, and absorbance readings (546 nm) were taken daily of samples from each flask, to determine the growth rate of each microbial population.

Liquid medium samples (1.5 ml) for heavy metal analysis were withdrawn on day 1 and day 4, together with samples of uninoculated controls containing only half-strength Nutrient Broth and the corresponding metal concentrations. Cu^{2+}, Pb^{2+} and Cr^{3+} containing samples were analyzed by AAS (see section 2.2.4.2) and Cd^{2+} containing samples by HPLC (see section 2.2.4.1).

The above experiment was repeated at 22°C, with the following alteration:
The volumes of the aerated cultures were doubled, i.e. 10 ml inoculum, double aliquots of the concentrated heavy metal solutions were used, and the volume made up to 100 ml with half-strength Nutrient Broth, to offset problems caused by evaporative losses.

2.2.2 Determination of Metal Concentrations at which the Microbial Associations were Capable of Growth in Liquid Culture

2.2.2.1 Initial increases in metal ion concentrations

McCartney bottles were used to set up five series of cultures with different metal concentrations. The cultures contained inoculum from the second stage enrichment cultures (0.1 ml), metal ion solutions (various aliquots from concentrated metal solutions (APPENDIX A)), and were diluted to a total volume of 10 ml with half-strength Nutrient Broth. The final metal ion concentrations were:

Cu^{2+} - 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg l^{-1}

Cd^{2+} - 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg l^{-1}

Pb^{2+} - 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg l^{-1}

Cr^{3+} - 20, 40, 60, 80, 100, 110, 120, 130, 140, 150 mg l^{-1}

Hg^{2+} - 1, 2, 3, 4, 5, 6, 7, 8 , 9, 10 mg l^{-1}

A visual assessment of growth was made after 24 hours.

2.2.2.2 Effects of increasing concentrations of metal ions on microbial resistance in liquid cultures

Erlenmeyer flasks containing progressively higher metal concentrations were set up to determine if the microorganisms could grow and take up metals at these increasing concentrations. Thus: microorganisms growing in the presence of 100 mg l^{-1} Cu^{2+} were inoculated into nutrient solutions containing 200 and 250 mg l^{-1} Cu^{2+}, and then into 400 mg l^{-1} Cu^{2+}; microorganisms growing at 100 mg l^{-1} Cd^{2+} were inoculated into 250 mg l^{-1}, and then into 500 mg l^{-1} Cd^{2+}; microorganisms growing at 150 mg l^{-1} Cr^{3+} were inoculated into solutions containing 200 and 250 mg l^{-1} Cr^{3+}, and then into 400 mg l^{-1} Cr^{3+}; microorganisms growing in Pb^{2+} solutions of 150 mg l^{-1} were inoculated into solutions containing 500 and 1000 mg l^{-1} Pb^{2+}. No further work was conducted with mercury due to problems encountered with volatilisation and analysis of the residual mercury after microbial uptake.

These cultures were incubated at 30°C on a rotary shaker (200 rpm) and observed after 3 days. The shaker ensured that the metals were kept in constant contact with the microorganisms and that the cultures were aerated to a certain extent.

Absorbance readings (546 nm) of the cultures containing the higher metal concentrations (400 mg l^{-1} Cu^{2+}; 500 mg l^{-1} Cd^{2+}; 400 mg l^{-1} Cr^{3+} and 500 mg l^{-1} Pb^{2+} (1000 mg l^{-1} Pb^{2+} was not used as there was no growth and metal precipitation was excessive)) were taken immediately after inoculation, and after 1, 2, 5, and 6 days. Liquid samples (2 ml) for metal analyses were withdrawn from these cultures immediately after metal addition on day 0 and then on day 2. Uninoculated controls containing corresponding initial metal concentrations in half-strength Nutrient Broth were used to determine the initial metal concentrations in solution. Cd^{2+} samples were analyzed by HPLC (see section 2.2.4.1) and Cu^{2+}, Cr^{3+} and Pb^{2+} samples by AAS (see section 2.2.4.2).

2.2.3 Determination of Metal Concentrations at which the Microbial Associations were Capable of Growth on Solid Media

Solid media were also used to determine at which metal concentrations the microorganisms were capable of growth. Half-strength Nutrient Agar was used together with various aliquots of concentrated heavy metal solutions (APPENDIX A). The following final concentrations of metal ions were tested:

Cu^{2+} - 100, 150, 200, 250, 500, 1000 mg l^{-1}

Cd^{2+} - 100, 150, 200, 250, 500, 1000 mg l^{-1}

Pb^{2+} - 150, 200, 250, 300, 500, 1000 mg l^{-1}

Cr^{3+} - 150, 200, 250, 300, 500, 1000 mg l^{-1}

Control plates, containing no heavy metals were also inoculated with the same mixed microbial populations, to observe the difference in growth in the presence and absence of metals.

Inoculum (0.1 ml) from the corresponding liquid cultures was spread on each plate with a sterile glass hockey stick. The plates were then inverted and incubated at 30ºC for 48 hours. Plates were observed for growth at 24 and 48 hours.

2.2.4 Methods of Metal Ion Analysis

Two methods were used for metal ion analysis: HPLC (High Performance Liquid Chromatography) and AAS (Atomic Absorption Spectrophotometry). The HPLC method was useful for analysing two or three metal ions simultaneously, when metal combinations were analyzed as in Sections 3.2.2.5, 4.2 and 5.2. However, when using this procedure good sample preparation was vital. The AAS method had the advantage that little clean-up of the samples was required, i.e. organics did not need to be removed; a shortcoming of this method, however, was that only one metal could be analyzed at a time.

Both methods were used to estimate how much metal remained in solution after different periods of incubation, as samples were taken before, during and after uptake of the metals by the microorganisms. The amount of metal taken up by the microbial biomass was determined by the difference between the amount remaining in solution and the initial metal concentration of the solution.

2.2.4.1 HPLC ion analysis

The HPLC (Waters Liquid Chromatography system with Waters 600E System Controller and Autochrome 162 CSI) method employed involved the use of a post column derivatisation procedure. Figure 2.1 is a diagrammatic representation of the HPLC system used.
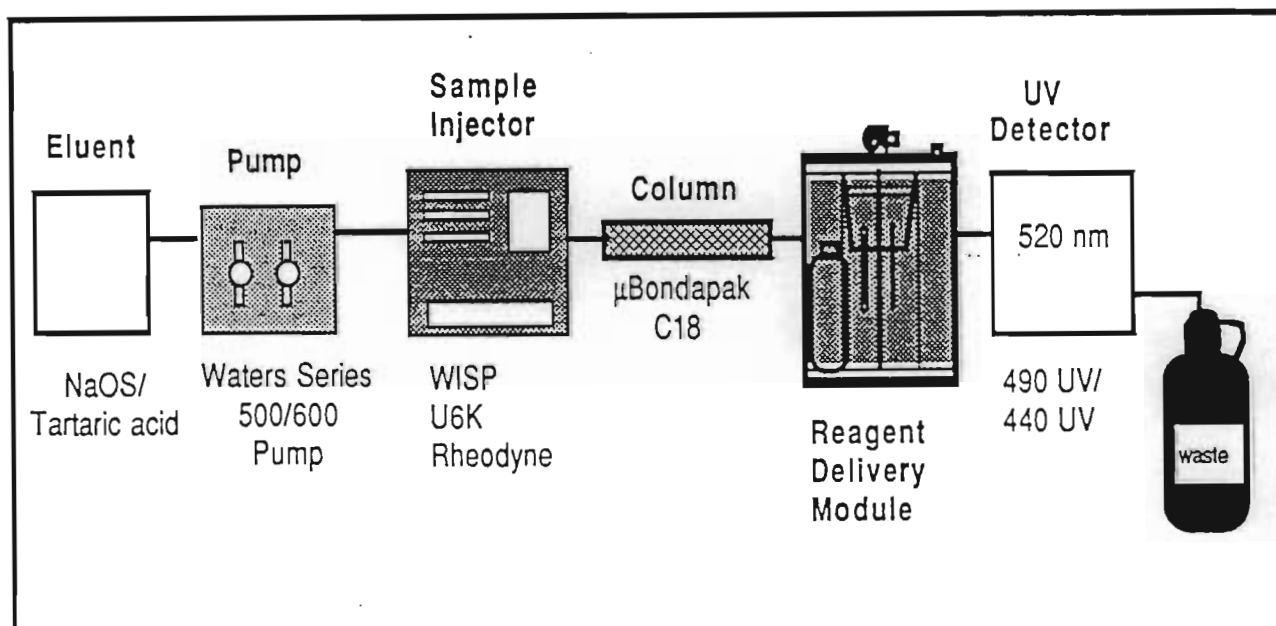
Figure 2.1   Diagrammatic representation of the HPLC system used to detect metal ions in solutions.

The eluent (APPENDIX B) and post-column dye reagent (APPENDIX C) were especially designed to convert a 3.9 mm x 150 mm Nova-Pak C_{18} column (Millipore) into a cation exchange column. The purpose of the octane sulphonate in the eluent was to bind to the metals thereby aiding in their separation over time. Samples (100 μl volume), diluted with eluent to within a range that could be detected, were injected into the system by the

autosampler and the eluent was circulated at a flow rate of 1 ml min^{-1}. The eluent was sparged with Helium gas to prevent any air bubbles from getting into the system. The post column dye binds to the metals after they leave the column and enables them to be detected by the UV detector (Waters 486 Tunable Detector) at 520 nm, as a series of peaks. Figure 2.2 is a spectrum produced when the working metal standards were separated by this HPLC method.
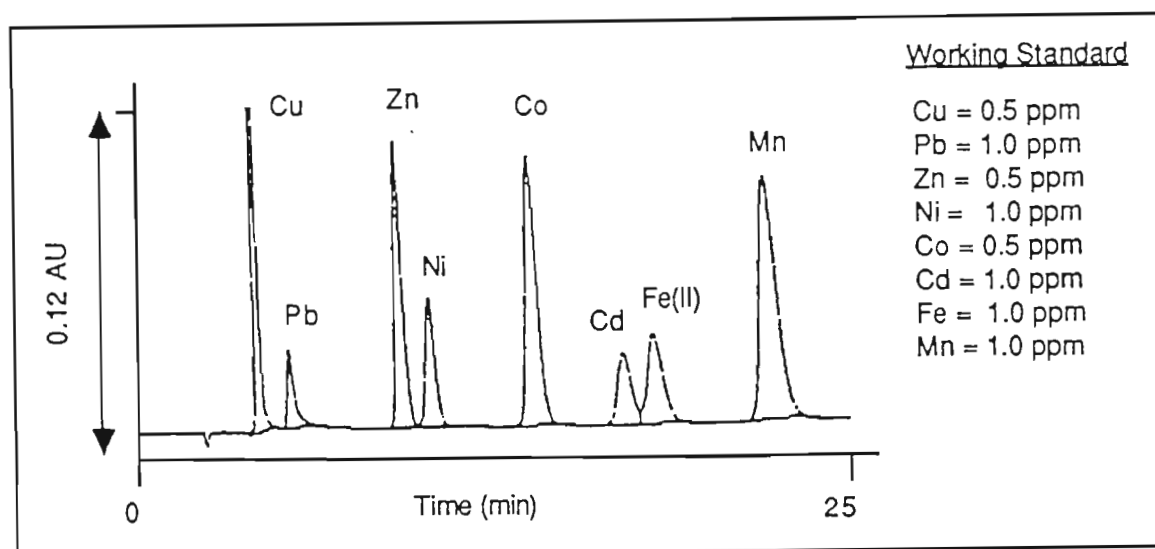
Figure 2.2   Example of spectrum showing separation of metals and working standards used to obtain this spectrum.

Samples for HPLC analysis were initially centrifuged (11000 rpm x g for 3 minutes in an Eppendorf Centrifuge, model 5413) to remove the microorganisms. The supernatant was stored in Eppendorf vials in the freezer (-4°C) until analyzed. Before analysis the samples were filtered through a 0.22 μm cellulose acetate membrane filter to remove any particulates. Samples containing organics were also passed through a Sep-Pak C$_{18}$ cartridge (Millipore) to remove the organics, to prevent interference with the metal peaks and to protect the column. Samples were diluted with the eluent to within a range that could be detected by this HPLC method.

A calibration curve was produced for each metal for each run to ensure that accurate results were obtained. See APPENDIX D for examples of the calibration curves used.

All samples from the experiments were analyzed in duplicate and the average values are presented in tables or graphs in the Results and Discussion sections.

2.2.4.2 Atomic absorption spectrophotometry (AAS)

A Varian AA-275 Series Atomic Absorption Spectrophotometer was used. Samples were aspirated into a flame and atomised. A light beam directed through the flame, into a monochromator and onto a detector measured the amount of light absorbed by the atomised element in the flame. Each metal was analyzed 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, over a limited concentration range. Where possible a nitrous oxide-acetylene flame was used to minimise the chemical interferences often encountered with an air-acetylene flame (Clesceri *et al.* 1989). Standards were made up from ultra pure AAS reagents.

Conditions used for each metal analysis were as follows:

Cu^{2+}: Wavelength, 327.4 nm; Spectral band pass, 0.2 nm; Lamp current, 3 mA; flame, nitrous oxide-acetylene.

Pb^{2+}: Wavelength, 283.3 nm; Spectral band pass, 0.2 nm; Lamp current, 6 mA; flame, air-acetylene.

Cr^{3+}: Wavelength, 357.9 nm; Spectral band pass, 0.2 nm; Lamp current, 5 mA; flame, nitrous oxide-acetylene.

Cd^{2+}: Wavelength, 326.1 nm; Spectral band pass, 1.0 nm; Lamp current, 3 mA; flame, air-acetylene.

Samples were centrifuged (11000 rpm x g for 3 minutes in an Eppendorf Centrifuge, Model 5413), filtered through 0.22 μm cellulose acetate membrane filters and diluted with water to within a range at which the metals could be detected. EDTA was added to the lead samples to obtain a final concentration of 0.1 M EDTA. Organics did not need to be removed as a control showed negligible interference by any of the organic constituents in the half-strength Nutrient Broth used in these experiments.

2.3 RESULTS AND DISCUSSION

2.3.1 Initial Enrichments

2.3.1.1 First stage enrichments

Prahalad and Seenayya (1988) found that the strength of Nutrient Broth used influenced the availability of Zn^{2+} and Cd^{2+} to an *Alcaligenes faecalis* culture and suggested that this effect was due to the metals complexing with compounds in the growth media. Growth was apparent in all the metal-containing cultures, although it differed in amount between the cultures growing in the various metal-containing media. For example, all the cadmium-containing media supported the growth of a bacterium which produced a diffusible fluorescent yellow/green pigment, indicating the possible presence of a green fluorescent *Pseudomonas* species in this microbial association.

Addition of the tannery effluent to the Cr^{3+}-containing medium resulted in a diverse microbial population which was capable of growth in the presence of Cr^{3+}, as many of these microorganisms had previously been exposed to chromium in the tannery effluent.

The addition of glucose to the half-strength Nutrient Broth did not enhance growth of the microorganisms to any extent, and it was, therefore, not added to the media used in subsequent experiments.

2.3.1.2 Second stage enrichments

Samples of the microbial biomass were Gram stained to determine the types of microorganisms present in the various microbial populations which became established in the metal-containing media. The copper-containing medium contained many Gram negative rods together with a few Gram positive cocci (Plate 2.1). Gram negative and Gram positive rods/cocci were present in the cadmium-containing medium (Plate 2.2).
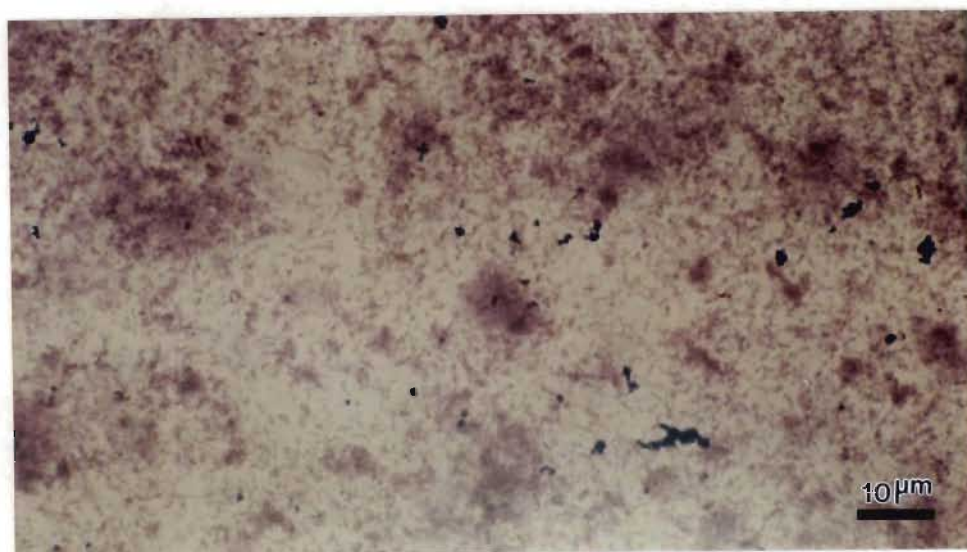
Plate 2.1   Gram stained microorganisms found in a Cu^{2+}-containing medium.
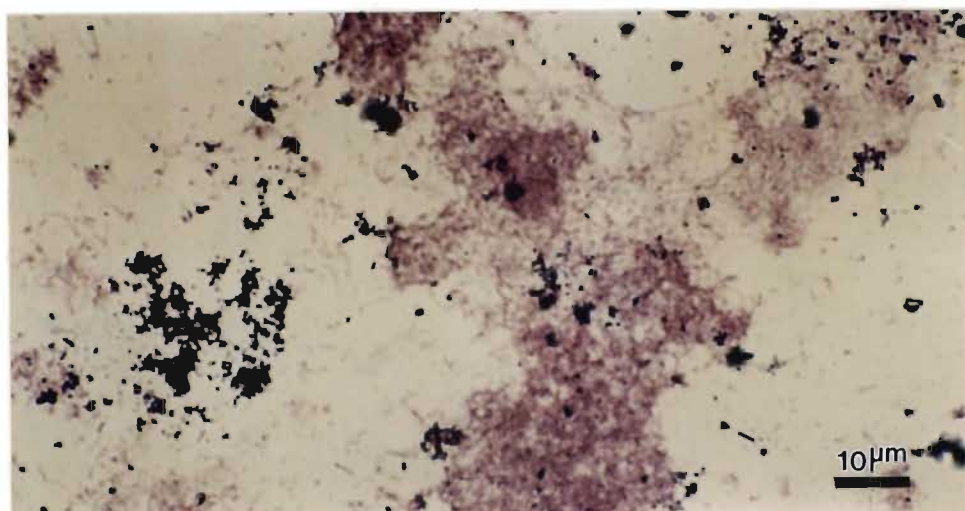
Plate 2.2   Gram stained microorganisms found in a Cd^{2+}-containing medium.

Various Gram positive *Bacillus* species were observed in the lead-containing medium with small Gram negative rods and cocci also present in fairly substantial numbers (Plate 2.3). The chromium-containing medium contained many yeast-like cells, in addition to various Gram positive *Bacillus* species, together with their released endospores (Plate 2.4). Many Gram negative rods and filaments were also visible in the Cr^{3+}-containing medium, as were Gram positive cocci (Plate 2.5). It has been found that, in general, Gram negative bacteria are more resistant to Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+} than Gram positive

bacteria (Doelman 1985). This may explain why there were more Gram negative than Gram positive bacteria present in all the metal-containing solutions tested.
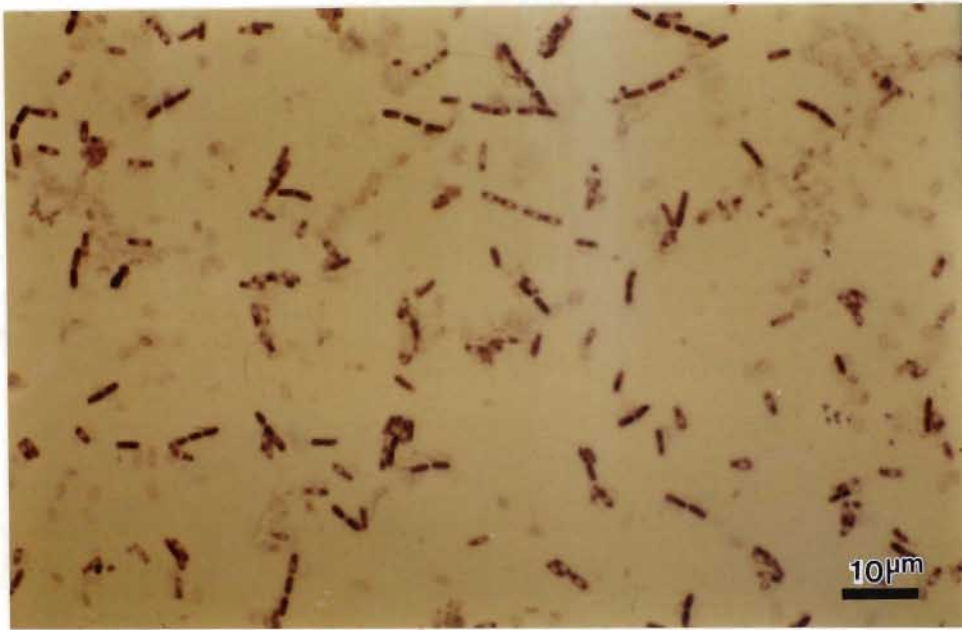
Plate 2.3   Gram stained microorganisms found in a Pb^{2+}-containing medium.
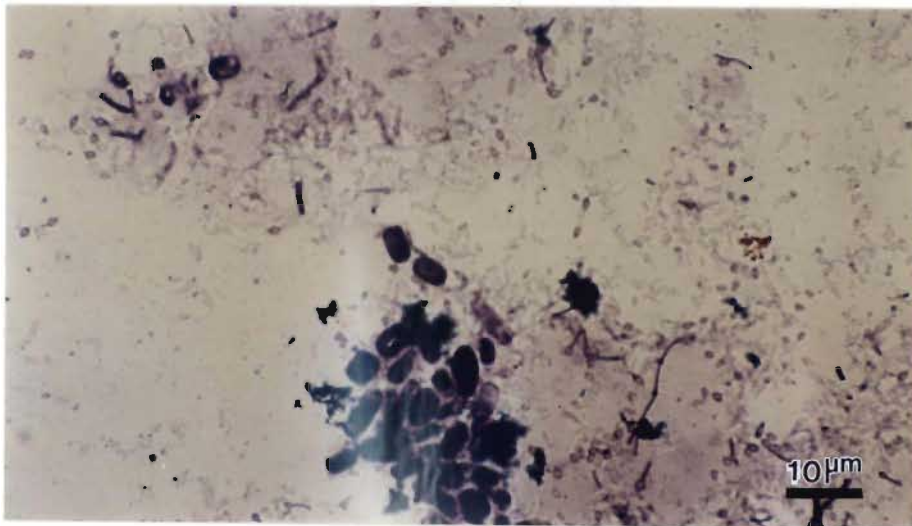
Plate 2.4   Gram stained yeast-like cells and *Bacillus* species found in a Cr^{3+}-containing medium.

Plate 2.5 Gram stained rods and filamentous microorganisms found in a Cr^{3+}-containing medium.

Scanning Electron Microscopy revealed a diverse range of microorganisms in the various metal-containing media. The bacteria found in the copper-containing medium were mainly single rods, a few small cocci, some diphtheroids or coryneforms and a few filamentous microorganisms (Plate 2.6A). Chains or clusters of cocci were observed in the cadmium-containing medium (Plate 2.6B). The chromium-containing medium contained several larger organisms, possibly yeasts (indicated by the Y), together with many bacterial rods, cocci and filaments (Plate 2.6C). The mercury-containing medium contained rods as well as some filaments and cocci (Plate 2.6D). The lead-containing medium contained many large rods, some rounder rods and short filaments, as well as small rods and cocci (Plate 2.6E). A variety of microorganisms have been found to be capable of removing heavy metals from solution (Gadd 1992), and a diverse range of morphotypes was observed in the present enrichments. Different metal ions may be taken up more efficiently by specific microorganisms; for example, uranium was found to be taken up more readily by actinomycetes than by fungi, bacteria and yeasts (Nakajima and Sakaguchi 1986). This metal-induced selectivity was observed in the chromium containing solutions which were the only ones that contained yeast-like cells in addition to the bacteria present.

Plate 2.6 Electron micrographs of microorganisms present in metal-containing media: A. Cu^{2+}-containing medium with single rods, a few cocci and filamentous microorganisms and some diphtheroids or coryneforms; B. Cd^{2+}-containing medium with chains or clusters of cocci; C. Cr^{3+}-containing medium with the larger yeast-like cells (indicated by the Y) and many bacterial rods, cocci and filaments; D. Hg^{2+}-containing medium with rods, cocci and filaments; and E. Pb^{2+}-containing medium with large and small rods, short filaments and small cocci.
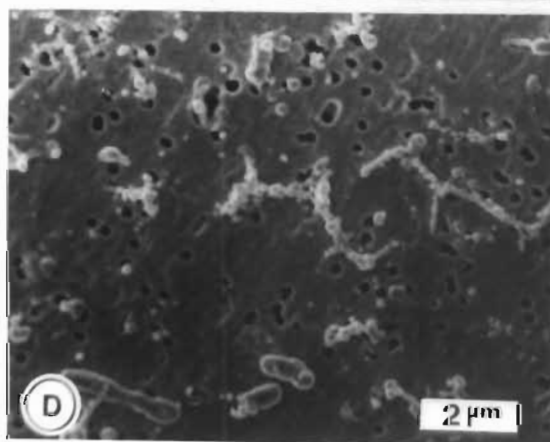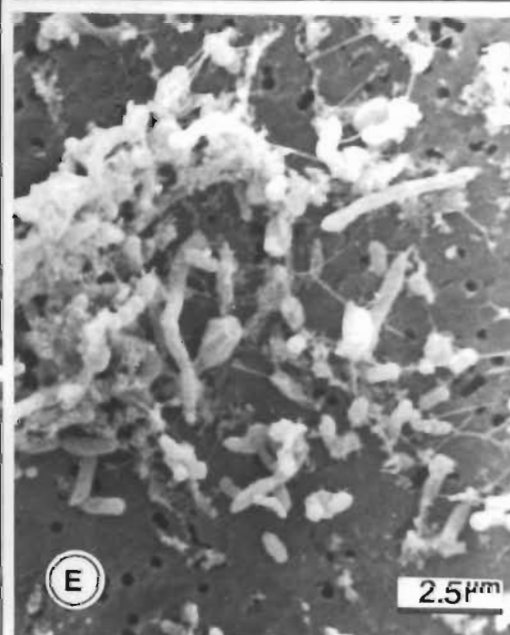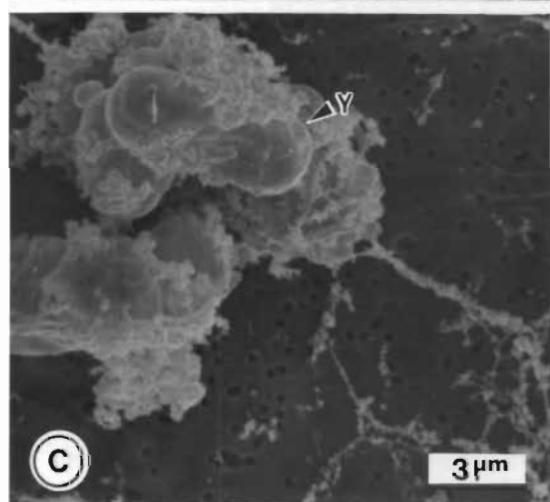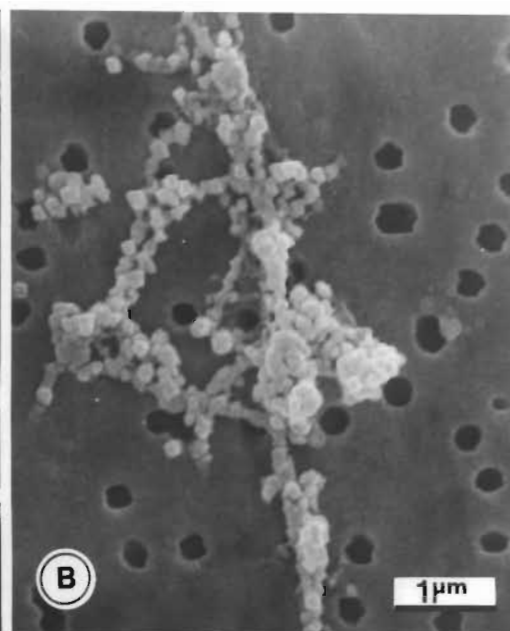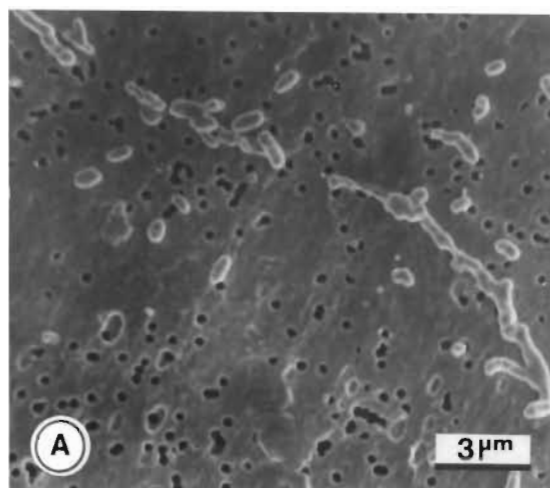
It appears that microorganisms are more stable and better able to withstand high metal ion concentrations when growing in associations (Gadd 1988). Mixed cultures have been successfully used in the treatment of waste waters as a wide range of compounds are found in waste waters and these require a complex microbial population to deal with them (Salmon and Bull 1984). 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). This will allow the entire population to survive, showing that associations are more stable than monocultures, especially under stressed conditions.

2.3.1.3 Growth of mixed cultures under different oxygen regimes

Absorbance readings of the media in the initial aerated flask experiments could not be continued after four days as the combination of 30°C temperature and aeration caused a rapid loss of medium through evaporation which, in turn, resulted in concentration of the medium ingredients, including the metal ions. Therefore, the experiment was repeated using a double volume of each medium to reduce these effects. Absorbance readings showed that, in general, the aerated flasks supported a greater rate of growth than those that were not aerated (results not shown). Due to the limitations of the aeration system used and the evaporation problems encountered, all subsequent cultures were incubated on a rotary shaker which provided some measure of aeration and mixing without excessive evaporation.

The chromium-containing medium developed a slimy blueish growth, suspended in the medium, after three weeks. On microscopic examination this appeared to consist of large, oval cells which could possibly be yeasts (Plate 2.7).
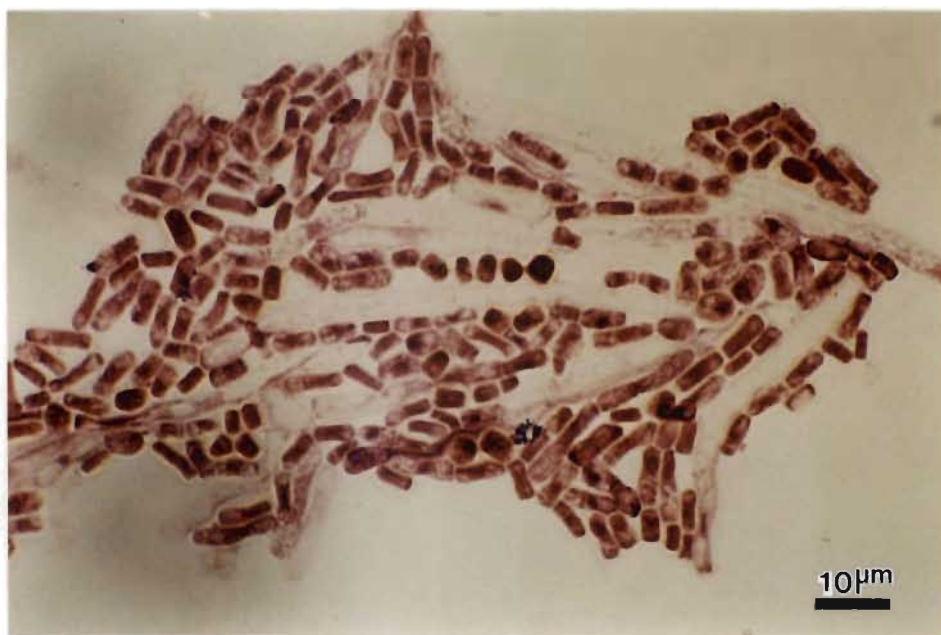
Plate 2.7   Yeast-like cells found in a Cr^{3+}-containing medium.

The aerated and non-aerated cultures were sampled to determine the metal ion concentrations remaining in solution after metal uptake by the microorganisms. Cd^{2+}-containing samples were analyzed by HPLC (see section 2.2.4.1), while Cu^{2+}-, Cr^{3+}- and Pb^{2+}- containing samples were analyzed by AAS (see section 2.2.4.2).

Table 2.1 shows that very little copper was taken up from either the aerated or non-aerated copper-containing medium. The initial concentration of cadmium in the medium was low, i.e. only 32.6 mg l^{-1} rather than the 100 mg l^{-1} which theoretically should have been present. It is thought that precipitation of the cadmium occurred in the half-strength Nutrient Broth, and that this precipitate was removed when the samples were filtered. There did, however, appear to be some uptake of Cd^{2+} ions after 4 days, especially in the aerated culture (65% Cd^{2+} uptake). The uptake of Cr^{3+} was slightly better in the aerated cultures than in those that were not aerated, probably due to the higher growth rate of the aerated culture resulting in the production of more biomass for metal uptake.

Table 2.1   Metal concentrations in medium initially and after 1 and 4 days of microbial growth in aerated and non-aerated cultures and % metal uptake after 4 days.

Culture	Initial metal ion conc * (mg l^{-1})	Metal ion conc on day 1 (mg l^{-1})	Metal ion conc on day 4 (mg l^{-1})	% Metal uptake after 4 days
Cu^{2+}-aerated	104.0	98.9	97.8	6.0
Cu^{2+}-non aerated	104.0	100.9	99.6	4.2
Cd^{2+}-aerated	32.6	30.9	11.4	65.0
Cd^{2+}-non aerated	32.6	67.8	19.5	40.2
Cr^{3+}-aerated	130.2	114.8	89.6	31.2
Cr^{3+}-non aerated	130.2	101.5	93.8	28.0

* = Metal concentrations of corresponding uninoculated controls.

Metal uptake by *Chlorella fusca* cells was delayed under oxygen reduced conditions compared to metal uptake in an oxygen saturated atmosphere; although, the maximum amount of metal ions adsorbed was not influenced by the dissolved oxygen concentration (Wehrheim and Wettern 1994a). A similar effect was observed in the present investigation as metal uptake by the aerated cultures was usually only slightly higher than in the corresponding non-aerated cultures (Table 2.1). However, different microorganisms may have varying requirements for oxygen during metal uptake and this would have to be determined for each system.

No results were obtained for the concentration of Pb^{2+} remaining in solution as precipitation of the metal resulted in it being centrifuged or filtered out of solution prior to analysis by either AAS or HPLC. The lead may have precipitated due to the action of the microorganisms, resulting in the adsorption of some of the Pb^{2+} onto the surface of

the cells by complexation, or the lead may have complexed with organics in the media; however, as no results were obtained for the Pb^{2+} concentration remaining in the medium this could not be confirmed. Macaskie and Dean (1984a;b), however, described a *Citrobacter* species which was capable of secreting inorganic phosphate which complexed with Pb^{2+} to form lead phosphate on the cell surfaces.

2.3.2 Determination of Metal Concentrations at which the Microbial Associations were Capable of Growth in Liquid Cultures

2.3.2.1 Initial increases in metal ion concentrations

The following results were obtained on visual assessment of the cultures after 24 hours:

Cu^{2+} - Very slight growth occurred at 100 mg l^{-1}

Cd^{2+} - Small flocs were observed at 100 mg l^{-1}

Pb^{2+} - Medium was very turbid in all test flasks; concentrations of 100 mg l^{-1} Pb^{2+} were apparently too low to inhibit growth.

Cr^{3+} - Floccular growth was observed at 150 mg l^{-1}

Hg^{2+} - Extremely small flocs were observed at 9 mg l^{-1} metal, but at 4 mg l^{-1} the medium was turbid.

As a result of exposure to increasing concentrations of the different metal ions the microorganisms became more resistant to the metals in question and were thus able to tolerate the elevated metal ion concentrations.

Work on mercury was discontinued due to problems experienced with its analysis, and also because of the very low tolerance of the tested microorganisms to Hg^{2+}. Mercury is also susceptible to transformation and volatilisation as methyl mercury (Wood and Wang 1983), which would complicate the analysis for mercury remaining in solution, thus further distorting the results obtained.

2.3.2.2 Effects of increasing concentration of metal ions on microbial resistance in liquid cultures

Microbial tolerance to heavy metals can be developed by repeatedly subculturing parental strains on media containing progressively increasing toxic levels of metals; this process is known as training (Garcia-Toledo *et al.* 1985). This tolerance to heavy metals has been reported in a wide variety of microorganisms and is thought to be by physiological adaptation rather than by genetic mutation (Garcia-Toledo *et al.* 1985).

Growth after 3 days exposure to increasing metal concentrations varied and the following visual assessments were made:

Cu^{2+} 400 mg l^{-1} - Very little growth

Cu^{2+} 250 mg l^{-1} - Very little growth

Cu^{2+} 200 mg l^{-1} - Growth

Cd^{2+} 500 mg l^{-1} - Growth

Cd^{2+} 250 mg l^{-1} - Growth

Cr^{3+} 400 mg l^{-1} - Little growth

Cr^{3+} 250 mg l^{-1} - Little growth

Cr^{3+} 200 mg l^{-1} - Growth

Pb^{2+} 1000 mg l^{-1} - No growth

Pb^{2+} 500 mg l^{-1} - Growth

The apparent concentration of Pb^{2+} at which growth occurred was probably an overestimate as the Pb^{2+} tended to precipitate out and sink to the bottom of the flask despite rotary shaking. Therefore, the actual concentration available for microbial uptake was probably < 500 mg l^{-1}.

The elevated metal concentration samples were analyzed by HPLC (Cd^{2+}) and by AAS (Cu^{2+} and Cr^{3+}). No results were obtained with the lead samples due to the precipitation problems mentioned earlier.

Table 2.2 shows the percentage uptake of the metals by the microorganisms after 2 days exposure to high metal concentrations. In this case both Cu^{2+} and Cd^{2+} uptake were recorded but little uptake of Cr^{3+} occurred.

Table 2.2 Metal concentrations on day 0 and day 2 in media with high initial metal ion concentrations and % metal uptake after 2 days.

Culture	Initial metal ion conc * (mg l^{-1})	Metal ion conc on day 0 (mg l^{-1})	Metal ion conc on day 2 (mg l^{-1})	% Metal uptake after 2 days
400 mg l^{-1} Cu^{2+}	371.0	348.0	80.5 $\pm$2.1	78.3 $\pm$0.6
500 mg l^{-1} Cd^{2+}	343.0	301.5 $\pm$4.2	44.6 $\pm$31.3	86.9 $\pm$12.9
400 mg l^{-1} Cr^{3+}	428.0	371.0 $\pm$26.9	371.0 $\pm$26.8	13.4 $\pm$6.3

* = Metal concentrations of corresponding uninoculated controls. Duplicate cultures for each metal-containing medium were tested.

With the exception of the Cd^{2+} 500 mg l^{-1} cultures which appeared to grow rapidly after one day, the absorbance readings (results not shown) were very low for most of the cultures, indicating little growth had occurred.

As growth was limited in many cases, it is possible that the metal ion concentration results obtained did not give an accurate assessment of the metal uptake capacity of the microbial associations. Precipitation occurred at these very high metal concentrations and centrifugation and filtration would have removed a large proportion of this precipitate, making it appear that the microorganisms removed a larger proportion of metal ions than was actually the case.

It is important to realise that organic and inorganic components present in the growth medium may affect the concentration of heavy metals that will inhibit microbial growth

or activity (Prahalad and Seenayya 1988), and this should be taken into account when determining the metal concentration at which microbial associations are capable of growth. It is for this reason that half strength Nutrient Broth was used in all these experiments.

2.3.3 Determination of Metal Concentrations at which the Microbial Associations were Capable of Growth on Solid Media

Growth of the different microbial associations on the plates containing elevated metal ion concentrations varied with time and according to the metal concentration.

On solid nutrient media the higher metal concentrations used in this experiment inhibited growth of the microbial associations, i.e. 500 and 1000 mg l^1 Cu^{2+}; 500 and 1000 mg l^1 Cd^{2+}; 1000 mg l^1 Pb^{2+}; 300, 500 and 1000 mg l^1 Cr^{3+} (Table 2.3). This shows that microorganisms can withstand metal ion solutions only up to a certain concentration, unless further exposure is allowed which may result in their adaptation to tolerate higher metal concentrations.

Table 2.3 Extent of growth of microbial associations on plates containing various concentrations of heavy metal ions.

Conc of metal (mg l^{-1})	Amount and type of growth after 24 hours	Amount and type of growth after 48 hours
Cu^{2+} 0 Cu^{2+} 100	Confluent white growth at both concentrations	Same as 24 hour result
Cu^{2+} 150	Single colonies at both concentrations	Same as 24 hour result
Cu^{2+} 200 Cu^{2+} 250	Small single colonies	Same as 24 hour result
Cu^{2+} 500 Cu^{2+} 1000	No growth at either concentration	Same as 24 hour result
Cd^{2+} 0	Cream growth with no pigment production	Same as 24 hour result
Cd^{2+} 100 Cd^{2+} 150 Cd^{2+} 200 Cd^{2+} 250	Cream growth, with green /yellow fluorescent pigment production at all concentrations indicated	Same as 24 hour result
Cd^{2+} 500 Cd^{2+} 1000	No growth evident at either concentration	Slight growth and pigment production at both concentrations
Pb^{2+} 0 Pb^{2+} 150 Pb^{2+} 200 Pb^{2+} 250 Pb^{2+} 300 Pb^{2+} 500	Confluent white growth at all concentrations indicated	Same as 24 hour result
Pb^{2+} 1000	No growth	Slight growth, very small colonies
Cr^{3+} 0 Cr^{3+} 150	White growth at both concentrations	Same as 24 hour result
Cr^{3+} 200 Cr^{3+} 250	Little growth at both concentrations	Same as 24 hour result
Cr^{3+} 300 Cr^{3+} 500 Cr^{3+} 1000	No growth at any of the concentrations indicated	Same as 24 hour result

After 24 hours the microorganisms growing on the cadmium containing plates showed the production of a diffusible yellow/green fluorescent pigment. The control on the other hand

showed only white growth, with no pigment production. This indicates that only in the presence of cadmium did the cells produce the fluorescent pigment. A possibly similar pigment has been found to function as a siderophore in certain *Pseudomonas* species (Stanier *et al.* 1988). Thus the yellow/green pigment might function as a chelator of cadmium ions which might explain its production when cadmium was present in the medium.

The colonies growing on the copper-containing plates were slightly blueish, while the control plates showed whitish growth (Plate 2.8), indicating the copper uptake potential of this microbial population. This coloration of a microbial population was also observed by Dunn and Bull (1983); wall-growth of a ten-membered bacterial community was found to be blue in colour after 13-15 days growth, during which time 92% of the copper was removed from solution.
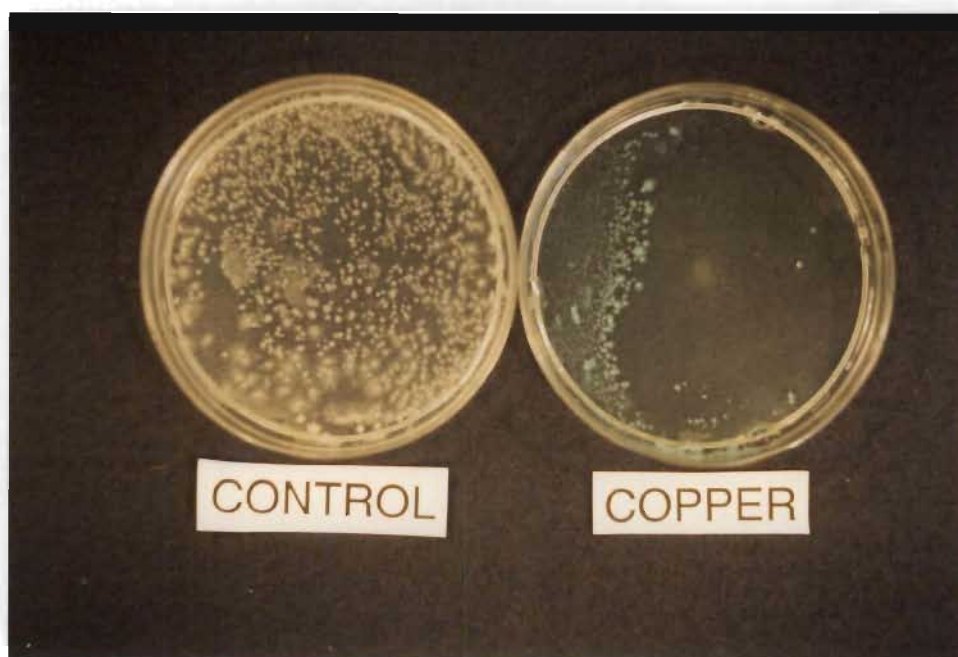
Plate 2.8   Growth and coloration of microorganisms on a plate containing 150 mg l^{-1} copper and a corresponding control plate without copper.

A fungus-like microorganism was also observed on the copper-containing plates. Further work was undertaken to isolate this microorganism and to determine its potential for adsorbing heavy metals (See Chapter 7).

Small white colonies developed on the lead-containing plates while the corresponding control plates showed more abundant growth. Obviously the lead inhibited growth of the microbial population to a certain extent. Whitish/blue colonies developed on the chromium containing plates whereas the corresponding control plates showed only whitish colonies.

2.4 CONCLUSIONS

Since it has been reported that strains of the same species vary considerably in their metal uptake capabilities, it can be concluded that bioaccumulating properties are more strain than species related (Wnorowski 1991). Thus microorganisms for heavy metal removal should be selected with care and tested comprehensively to establish a collection of bioaccumulating strains to treat an effluent of specific chemical composition (Wnorowski 1991). Different mixed microbial associations were established for each of the various metal-containing solutions tested. These were enriched for and subsequently adapted to increasing concentrations of metals. These microbial associations were used in further investigations of their metal-uptake potential.

As growth was not always assured and precipitation became a serious problem as the metal concentrations increased, it was decided that the very high metal concentrations which these experiments showed the microorganisms to be capable of surviving should not be used in subsequent experiments. Therefore, slightly lower metal concentrations, where uptake was measurable, were chosen for use in all subsequent experiments, i.e. Cu^{2+} 100 mg l^{-1}; Cd^{2+} 100-200 mg l^{-1}; Pb^{2+} 150-200 mg l^{-1}; and Cr^{3+} 100-150 mg l^{-1}. Many investigations have shown that there is a relationship between resistance and heavy metal accumulation; in many cases resistant strains take up less metal than their sensitive parental strains (Gadd and Griffiths 1978; White and Gadd 1986; Gadd 1988). For example, Chopra (1971) showed that a resistant strain of *Staphylococcus aureus* took up less Cd^{2+} than did a more sensitive strain; and trained *Saccharomyces cerevisiae* cells that were resistant to elevated concentrations of Cu^{2+}, Co^{2+} and Cd^{2+} also showed much lower rates of uptake than equivalent untrained cells (White and Gadd 1986). It was, therefore, assumed in the present study that the microorganisms that could grow actively at the slightly lower metal concentrations would also have more efficient metal uptake capabilities than their more tolerant counterparts.

CHAPTER 3: ATTACHMENT OF MICROORGANISMS TO VARIOUS SURFACES

3.1 INTRODUCTION

The attachment of bacteria to solid surfaces requires a complex interaction between the bacterium, the solid substratum, and the liquid phase (McEldowney and Fletcher 1986). Each of these components can vary as the environmental conditions change, and so affect attachment (Fletcher 1985; McEldowney and Fletcher 1986). It is well documented that microbial biofilms develop on almost every surface which comes into contact with water (Geesey et al. 1988). Solid surfaces in streams allow an assemblage of non-transient microorganisms to develop (Mills and Mallory 1987).

Biofilms are formed when bacteria and other microorganisms attach to a surface and then replicate, with the produced exopolymers aiding in the attachment (Geesey et al. 1988; Mueller et al. 1992; Gilbert et al. 1993). The thickness of the biofilm can vary from a single cell layer to several hundred millimetres as observed in algal mats (Mueller et al. 1992). Biofilms are found to be responsible for most of the microbial activity in natural and biotechnological situations (Marshall 1994), and have been found to be beneficial for waste water treatment (Mueller et al. 1992); for example, biofilms occur in packed-bed trickling filters, rotating biological contactors, etc (Bryers 1993). Performance of these biofilm systems is dependent on the metabolism of selectively enriched microbial associations in the biofilms (Bryers 1993). Different bacterial species vary in their responses in biofilm formation. No general relationship between growth conditions, cell surface adhesives and attachment ability has been found (McEldowney and Fletcher 1986). Ferris et al. (1989) found that 75-80% of the bacteria growing on experimental filter paper strips in water were Gram negative.

Immobilisation by adsorption onto a surface is the method most closely related to natural attachment phenomena and is also quick and simple to carry out (Monsan et al. 1987). The use of biofilms supported on inert matrices is a way of overcoming the problems associated with entrapment and is thus commonly employed (Macaskie and Dean 1989).

Ideal supports have a large surface area, such as planar surfaces (glass, plastics), uneven surfaces (wood shavings, clays, crushed rock) and porous materials (foams and sponges) (Macaskie and Dean 1989). A *Citrobacter* species was observed by Macaskie and Dean (1987) to form a cohesive biofilm that accumulated uranium and lead via cell surface phosphatase while growing on glass helices.

Immobilised biofilm systems provide more stable operating performance, inherent biomass/fluid separation, good mechanical stability, excellent adsorbing characteristics, the ability to use sequential reactors for different bioconversion processes, and to enable repeated use of biomass and mass loading of the pollutant as the biomass is independent of the fluid phase (Nakajima and Sakaguchi 1986; Bryers 1993). Physical, chemical and biological heterogeneity is inherent to biofilm systems (Bryers 1993).

Most associations between microorganisms and interfaces in aquatic systems are of the non-specific type, in that associations do not involve any specific bonding between complementary molecular structures on the microorganism and the interface (Marshall 1976). Non-specific interactions are thought to originate from the entire microbial cell, due to Van der Waals and electrostatic forces, and H-bonding, while specific interactions are a combination of the same forces but originate from highly localised chemical groups which form a specific stereochemical combination (Busscher *et al.* 1992). Attachment may be reduced or increased depending on the type and the number of physico-chemical interactions between the solid surface and the bacterium (McEldowney and Fletcher 1986).

Various surfaces were investigated to determine which surface material offered the best support for the development of microbial biofilms capable of removing heavy metals from solution.

3.2 MATERIALS AND METHODS

3.2.1 Initial Flask-based Biofilm Development Investigations

Initially four different surfaces were investigated in flask cultures, namely smooth glass, ground glass, polystyrene and perspex. Individual cultures comprising half-strength Nutrient Broth (89 ml), 10 ml inoculum and various aliquots of concentrated heavy metal solutions (APPENDIX A) to obtain initial concentrations of 100 mg l^1 Cd^{2+}; 150 mg l^1 Cr^{3+}; and 150 mg l^1 Pb^{2+}, were set up. Cu^{2+} was not used in this experiment. Each of the four surfaces was tested in separate metal-containing media. These flasks were incubated stationary at 30°C for two weeks to allow for biofilm formation, after which the extent of biofilm development was assessed.

After four weeks the surfaces with biofilms were transferred into fresh metal-containing media to promote further growth as there seemed to be nutrient limitation. Liquid samples (1.5 ml) for metal analysis were taken immediately after transfer and again after a further four weeks, to determine the metal uptake potential of these biofilms. These samples were prepared for analysis by High Performance Liquid Chromatography (HPLC) or Atomic Absorption Spectrophotometry (AAS) (see section 2.2.4).

3.2.2 Model Stream Experiments

A glass model stream was developed to investigate various factors, such as surface texture for attachment, which might affect microbial biofilms exposed to heavy metals and the effect of metal combinations (see Chapter 5). Plate 3.1 shows the stream model initially used. Problems, including non constant flow rates, and patchy development of the biofilm, were encountered with this model and it was subsequently modified. The original single channel system was converted to a five channel model, each channel having its own weir system (Plate 3.2). This provided even flow of liquid over each individual weir and hence down each channel, ensuring the entire surface of each channel-bed was completely submerged at all times.
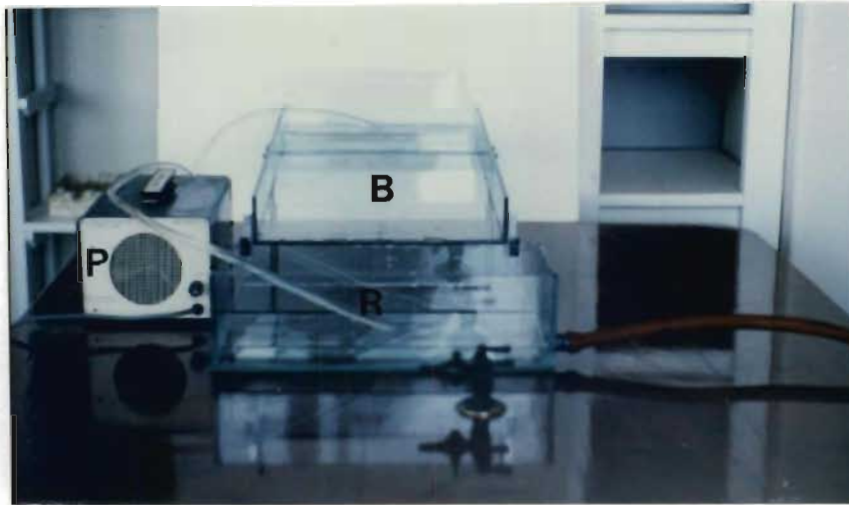
Plate 3.1 Initial model stream with single stream channel bed (B), reservoir (R) and peristaltic pump (P).
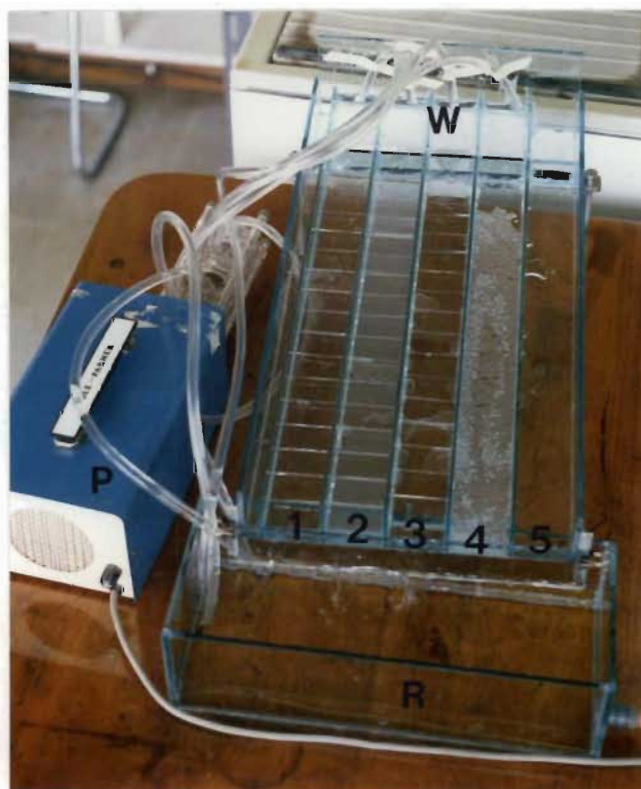
Plate 3.2 Modified model stream with additional weirs (W) and channels (indicated by the numbers), reservoir (R) and peristaltic pump (P).

Four types of glass, each with a different surface characteristic, were investigated for their ability to support microbial adhesion. Each material was placed in a separate channel of the model stream. Thus, from left to right in Plate 3.2: channel 1, rippled glass; channel 2, ground glass; channel 3, smooth glass; and channel 4, glass beads (2 mm diameter). Channel 5 was not used in this experiment. See Plate 3.3 for the detailed topography of the stream-bed materials. The first three materials were cut into sectional plates (3x5x0.5 cm) to facilitate subsequent Au/Pd- or carbon-coating and SEM viewing. These plates were placed side by side and covered the stream-bed of each channel. Each microbial association capable of growing in the presence of the individual heavy metals was tested in the model stream to determine the best surface for biofilm formation and metal uptake.
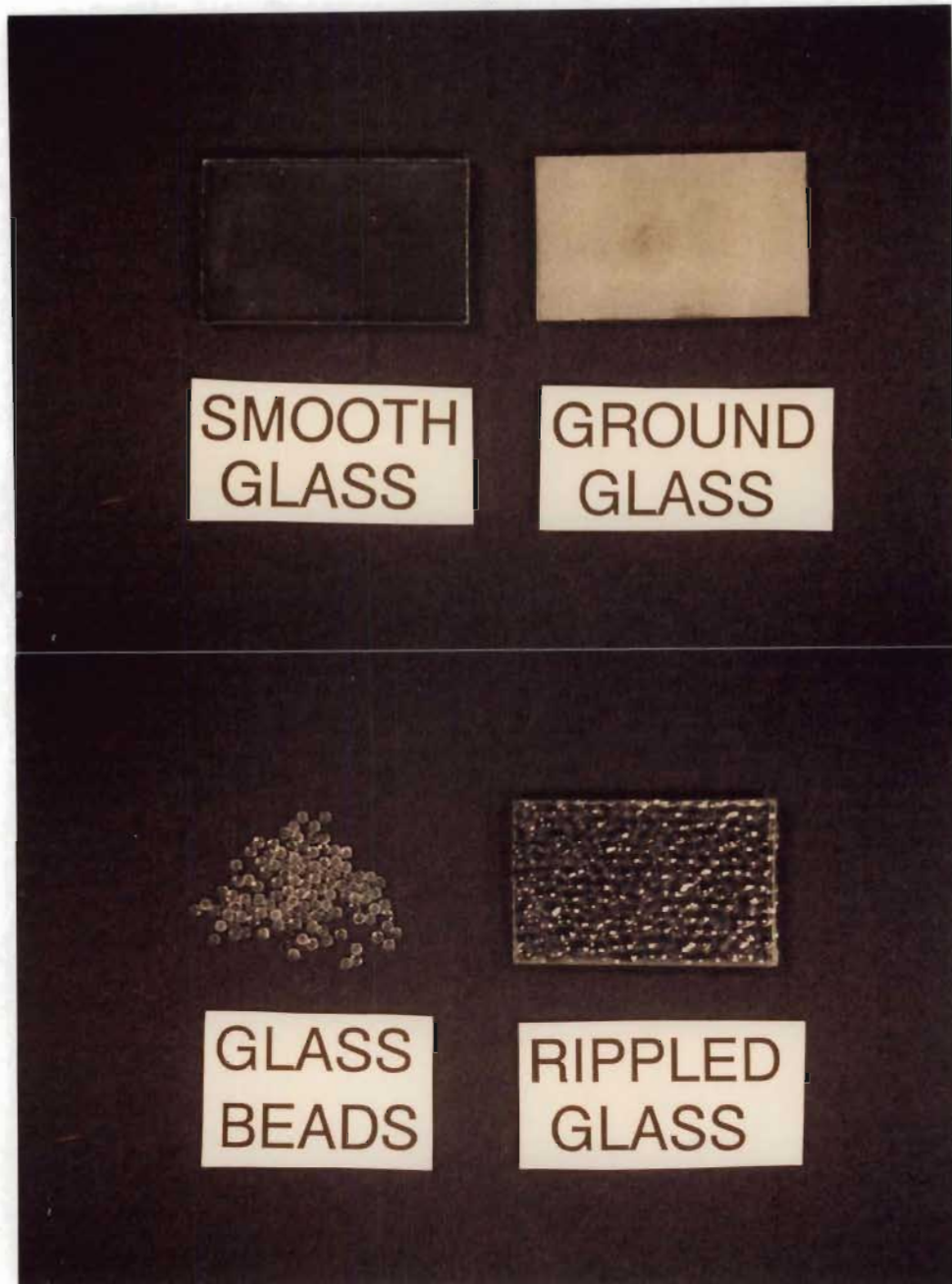
Plate 3.3   Stream bed materials, smooth and ground glass, glass beads and rippled glass used for biofilm development.

3.2.2.1   Investigations of lead uptake by microbial biofilms developed in the Model Stream

Inoculum for the biofilm was cultured in 250 ml Erlenmeyer flasks containing 196 ml half-strength Nutrient Broth with 4 ml of a concentrated Pb^{2+} solution to obtain a concentration of 200 mg l^{-1}.   After three days these cultures were centrifuged at 5000 rpm x g in a

Beckman J2 centrifuge for 10 minutes, and the pellet washed several times in quarter-strength Ringers solution to remove all traces of Pb^{2+} ions. Finally, the pellet was resuspended and centrifuged at 5000 rpm x g for a further 10 minutes. Half-strength Nutrient Broth (400 ml) was used to resuspend the pellet, and this inoculum was added to sufficient fresh half-strength Nutrient Broth to fill the model stream system (approximately 4 l). This medium was circulated at an average flow rate of 222 ml min^{-1}. Cling wrap was used to cover the entire model stream and reservoir to prevent excess evaporation and to minimise contamination from the environment (Plate 3.4). The biofilms were allowed to develop for one week at room temperature.
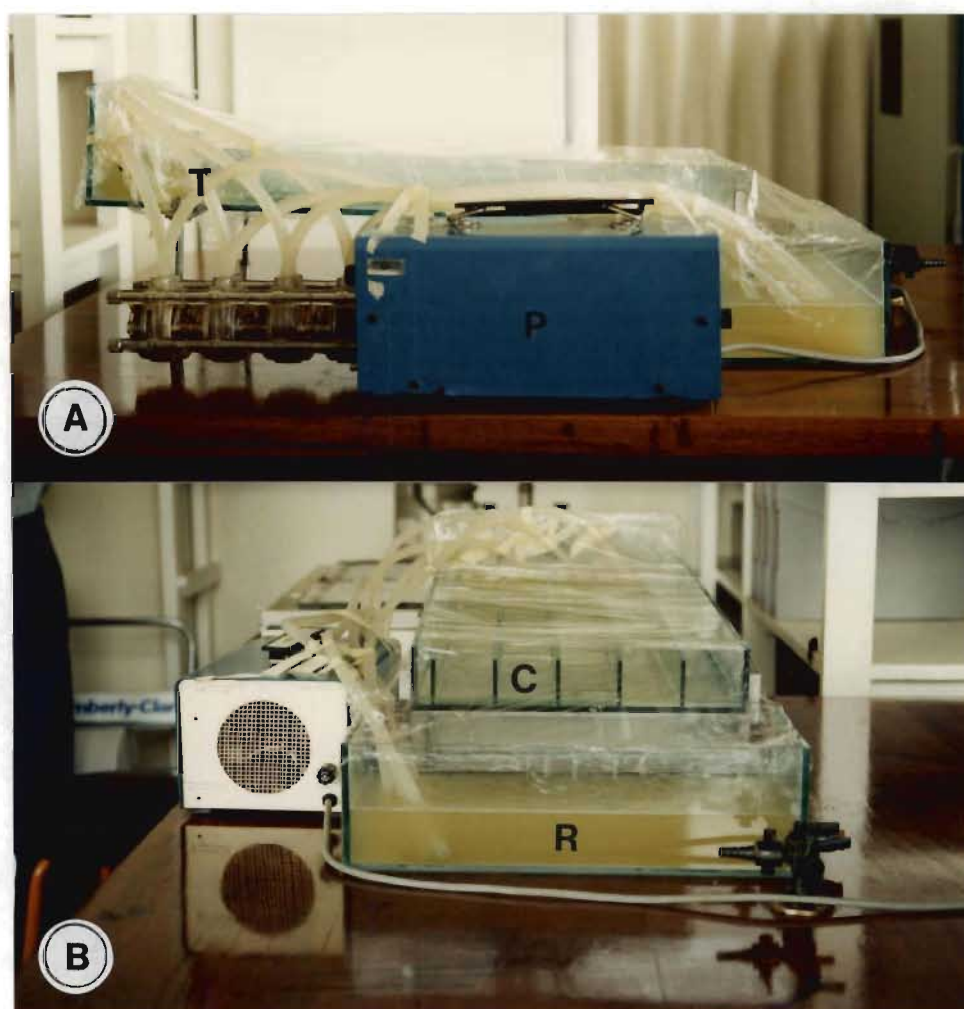
Plate 3.4   Model stream system with circulating half-strength Nutrient Broth and Pb^{2+}-adapted microorganisms; A. side view showing peristaltic pump (P) with tubing (T); B. front view showing channels (C) and reservoir (R).

After biofilm formation aliquots from a stock solution of Pb^{2+} (10000 mg l^{-1}, APPENDIX A) were added to each weir to obtain an overall initial concentration of 200 mg l^{-1} in each channel system. Liquid samples (1.5 ml) were taken immediately after the addition of the metal solution, and then after 30, 60, 90, 120 minutes, and 18, 20, 22, 24, 44 and 48 hours. All samples were taken from the downstream end of each channel. The samples were centrifuged (11000 rpm x g for 3 minutes) to remove the microorganisms and stored in Eppendorf vials in the freezer (-4°C) until analyzed. Leaks from the tubing in the peristaltic pump led to decreased flow rates in some of the channels and, therefore, the experiment was terminated after 48 hours. Sample glass plates from each channel and some of the glass beads were put into 3% (v/v) glutaraldehyde and refrigerated (4°C) prior to preparation for Scanning Electron Microscopy (SEM).

3.2.2.2 Investigations of copper uptake by microbial biofilms developed in the Model Stream

The model stream was again set up to investigate the biofilm developing potential of the microorganisms previously adapted to growing in copper-containing nutrient solutions. Inoculum was cultured for three days in 198 ml half-strength Nutrient Broth containing 2 ml of a concentrated Cu^{2+} (APPENDIX A) solution to obtain a concentration of 100 mg l^{-1}. The inoculum was washed and resuspended in half-strength Nutrient Broth as stated above (see section 3.2.2.1). The model stream was set up as described above to promote biofilm development. Aliquots of a stock copper solution (10000 mg l^{-1}, APPENDIX A) were then added to each of the weirs after 1 week, to obtain an overall concentration of 100 mg l^{-1} Cu^{2+} in each channel. Liquid samples (1.5 ml) for metal analysis were taken immediately after the addition of the metal and thereafter at timed intervals (15, 30, 60, 120 min, 24 and 72 hours), centrifuged (11000 rpm x g for 3 minutes) and stored in the freezer (-4°C) until analyzed by HPLC. Half-strength Nutrient Broth was mixed with a concentrated Cu^{2+} solution (APPENDIX A) to obtain a medium with a Cu^{2+} concentration of 100 mg l^{-1}, to be used as a time 0 sample to determine the metal concentration in the model stream prior to microbial uptake. Sample glass plates supporting biofilms were transferred to 3% (v/v) glutaraldehyde and refrigerated (4°C) prior to preparation for scanning electron microscopy to investigate biofilm development on the various surfaces.

3.2.2.3 Investigations of cadmium uptake by microbial biofilms developed in the Model Stream

Due to various problems, including the presence of free-living cells in the model stream, the following alterations were made to the system:

a) The liquid in the reservoir was stirred continuously with six magnetic stirrer bars after the addition of the metal solution, to ensure thorough mixing of the metal and nutrients.

b) The glass plate at the downstream end of the channels, was modified. Silicone was used to make ridges that separated the outflows of the channels from one another, so that samples could be taken, without interference from neighbouring channels.

c) The metal salt solutions were added to the bottom reservoir not to each separate weir as before, to prevent or decrease precipitation problems, and to ensure thorough mixing in the overall volume.

d) The actual stream bed was levelled so that there was no gradient. This ensured a better flow over all the glass pieces and beads, i.e. the surfaces were uniformally covered by the metal-containing nutrient medium at all times.

e) The biofilms were developed without circulation of the medium, i.e. half-strength Nutrient Broth was added to each channel together with inoculum and left for one week to allow biofilm development.

Microorganisms capable of growing in the presence of Cd^{2+} were cultured in half-strength Nutrient Broth as described above (see section 3.2.2.1). After centrifugation and washing in quarter-strength Ringers solution, the microorganisms were resuspended in half-strength Nutrient Broth. This inoculum (50 ml) and 100 ml half-strength Nutrient Broth were added to each channel of the model stream. The model stream was covered in cling wrap to prevent excess contamination, and to ensure that the biofilms did not dry out due to evaporation. Additional half-strength Nutrient Broth was added if the biofilms started to dry out. The room temperature was kept at about 20-25°C using the air-conditioner, as experience had shown that the higher temperatures initially used led to extensive evaporation of the liquid resulting in drying out of the biofilm.

After one week the biofilm was washed with sterile distilled water to remove any free-living or weakly attached microorganisms. Half-strength Nutrient Broth (3.5 l) containing Cd^{2+} ions at a concentration of 200 mg l^{-1} was then added to the system and circulated at an average flow rate of 228 ml min^{-1}. Liquid samples (1.5 ml) for metal analysis were taken to determine the rate of Cd^{2+} removal from the medium at various time intervals (*viz.* 0, 2, 15, 30, 60, 120 minutes, and 24 and 48 hours).

3.2.2.4   Investigations of chromium uptake by microbial biofilms developed in the Model Stream

The model stream was set up as before to investigate the uptake of chromium ions (Cr^{3+}), i.e. an inoculum was cultured in half-strength Nutrient Broth in the presence of chromium ions at 200 mg l^{-1}. After centrifugation, washing and resuspension, 50 ml of this inoculum in half-strength Nutrient Broth were added to each channel with approximately 100 ml of half-strength Nutrient Broth. Note that a fifth surface, polystyrene, was added to the experiment at this stage. The model stream was again levelled to allow for development of the biofilms. In this experiment the development of the biofilms during the period preceding addition of the metal ions and after metal addition, was assessed using scanning electron microscopy. Sample plates with each type of surface were removed from the channels after 2 days, 7 days (at this stage the biofilm was washed with water and half-strength Nutrient Broth containing Cr^{3+} at 200 mg l^{-1} was added to the model system and circulated at an average flow rate of 230 ml min^{-1}) and 10 days (3 days after addition of metal ions). These glass plates were washed with distilled water to remove loosely attached microorganisms and then fixed in 3% (v/v) glutaraldehyde overnight, washed twice in cacodylate buffer, dehydrated in an alcohol series (10 minutes each in 30, 50, 70, 80, 90% and 3 times in 100%) and critical point dried in an Hitachi HCP-2 CPD. After coating with gold/palladium the sample plates were viewed with an Hitachi S-570 SEM.

Liquid samples (1.5 ml) were taken before the metal-containing medium was exposed to the microorganisms and thereafter from the downstream end of each channel at various times after metal addition (2, 15, 30, 60, 120 minutes, 24 and 48 hours) for metal ion

analysis using AAS (2.2.4.2). The spot analysis EDX technique was used to determine the Cr^{3+} uptake potential of the biofilm grown on the polystyrene (see section 3.2.3.1).

3.2.2.5 Investigations of metal uptake from an artificial effluent by microbial biofilms developed in the Model Stream

The downstream reservoir was subdivided into five smaller reservoirs to enable different metal uptake systems to be investigated concurrently.

Polystyrene was used in every channel as the surface for biofilm formation, as it is cheap and readily available. Mixed microbial associations adapted to grow in the presence of the various metal ions were added to fresh half-strength Nutrient Broth in each channel as before, and the biofilms allowed to develop for two weeks. After washing the biofilms with running water, the metal solutions were added, each spiked with 5% (v/v) Nutrient Broth to make up an artificial effluent. The channels contained the following metal solutions:

Channel 1: Cu^{2+} 100 mg l^{-1} (pH = 3.94)

Channel 2: Cd^{2+} 200 mg l^{-1} (pH = 5.28)

Channel 3: Pb^{2+} 200 mg l^{-1} (pH = 3.37)

Channel 4: Cr^{3+} 150 mg l^{-1} (pH = 2.13)

Channel 5: Cu^{2+} 100 mg l^{-1} + Cd^{2+} 200 mg l^{-1} + Pb^{2+} 200 mg l^{-1} (pH = 3.12)

The metal-containing solutions were circulated at an average flow rate of 213 ml min^{-1} and liquid samples (1.5 ml) were taken immediately and at 2, 30, 60, 120 minutes, and 24, 48 and 96 hours from the downstream end of each channel for metal analysis by HPLC (see section 2.2.4.1). After four days, sample polystyrene plates from each channel were removed and prepared for scanning electron microscopy and EDX mapping (3.2.3.2).

3.2.3 Energy Dispersive X-ray Microanalysis (EDX)

The electron beam/specimen interaction in the SEM produces, amongst its electron beam interactions, an X-ray signal. An X-ray spectrometer, attached to the microscope, collects the signal based on the energy of the X-ray, to provide information on the elemental composition of the specimen (Morgan 1985).

Each element produces a discrete and fairly easily characterised spectrum. It is this characteristic X-ray signal which is collected and used to present information on the nature and position of the elements within the specimen. The elemental composition of the organic matrix itself cannot be determined since the major constituents are elements of low Atomic Number (carbon, hydrogen, oxygen and nitrogen) and are thus not detected by this system (Morgan 1985).

Important features of this technique are :
1) X-ray spectrometry detects elements but is not capable of distinguishing between ionic and non-ionic species.
2) Since electrons and X-rays are strongly absorbed by air molecules, all samples must be analyzed under relatively high vacuum.
3) Elements are detected and measured *in situ* with a spatial resolution ranging from about 10 nanometres to a few micrometres.
4) The technique is considered to be non-destructive but specimens are non-functional.
5) The method can be quantitative (Morgan 1985).

An Hitachi S-570 Scanning Electron Microscope fitted with a Link eXL II EDX system was used. Specimens in the SEM were tilted to an angle of 15° towards the X-ray gun, and the working distance in the microscope was set at 15 mm to obtain the most accurate results.

3.2.3.1 Spot analysis technique

In this technique a specific microorganism or cluster of microorganisms was selected and the EDX then used to detect the presence of any elements in or on that specific microorganism or cluster. Samples were prepared for SEM viewing by fixing in 3% (v/v) glutaraldehyde overnight, washing twice in cacodylate buffer, dehydrating in an alcohol series (10 minutes each in 30, 50, 70, 80, 90% and 3 times in 100%), critical point drying in a Hitachi HCP-2 CPD and carbon coating as a clear picture was needed to enable selection of specific points. The samples were carbon coated rather than gold/palladium coated to prevent interference by the coating material.

3.2.3.2 Digital mapping technique

The mapping technique involved specifying the metal or metals of interest and mapping an area on the specimen for the presence of that metal or metals. The location(s) in which the metal is concentrated is depicted as a white area on the map. Depending on the nature of the sample, longer exposure times, i.e. extended dwell times (the time the X-ray covers a specific point on the specimen), or more frames (the number of times the X-ray covers a specific point on the specimen), were needed to obtain a clearer map of where the metals were located on the biomass. This mapping technique is useful when simultaneously determining the location of a number of metals in a specific area.

3.2.3.3 Dot mapping technique

As in digital mapping a specific metal is chosen and a specified area is mapped to determine the location of that metal in the specimen. Dots on the screen and micrograph indicate the presence and location of the particular metal. This mapping technique is simple to carry out, results can be obtained rapidly, and it is useful when investigating the location of one specific metal/element in a specimen.

3.3 RESULTS AND DISCUSSION

3.3.1 Initial Flask-based Biofilm Development Investigations

After two weeks contact with the nutrient medium the various surfaces under investigation were observed for biofilm development.

Table 3.1   Amount of macroscopically visible microbial biofilm established on the various surfaces in the presence of metals after two weeks.

Metal	Ground Glass	Smooth Glass	Polystyrene	Perspex
Cadmium	+	+ +	+	+ +
Chromium	+ + +	+	+ + +	+ + +
Lead	+ + +	+	+ + +	+ +

+ = poor biofilm formation; + + = average biofilm formation; + + + = extensive biofilm formation

Overall ground glass and polystyrene offered the best surfaces for biofilm development (Table 3.1). The microorganisms growing in the cadmium containing solutions did not appear to attach well to any of these surfaces.

Plate 3.5 shows the extensive microbial biofilm that developed on polystyrene in the chromium-containing medium. Note the presence of many yeast-like cells (indicated by the Y).
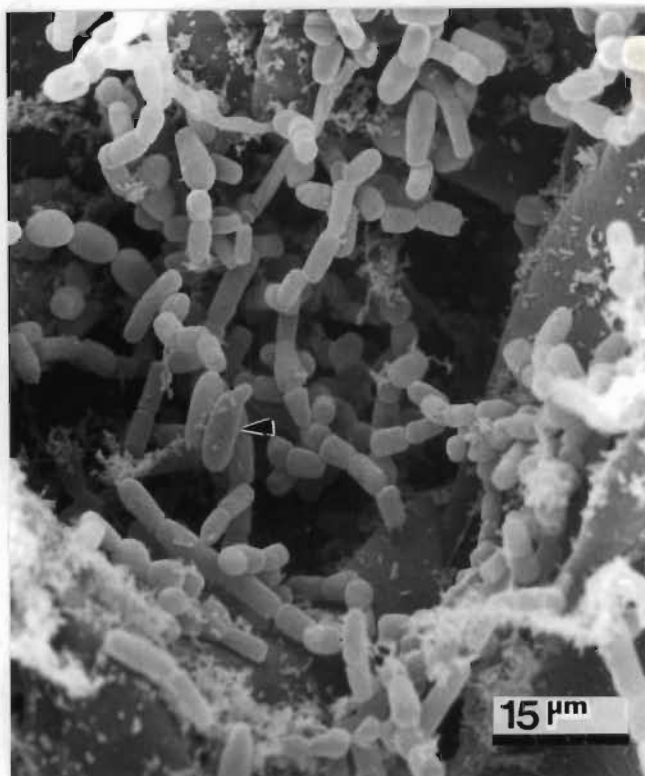
Plate 3.5   Electron micrograph of biofilm development on polystyrene after 4 weeks
exposure to a Cr^{3+}-containing medium; Y = yeast-like cell.

After transfer of the materials supporting biofilms into fresh metal-containing half-strength
Nutrient Broth, liquid samples were taken for metal ion analysis by HPLC (Cd^{2+} and Pb^{2+})
and AAS (Cr^{3+}) (see section 2.2.4), and the results are shown in Table 3.2.

Table 3.2   Initial and final metal concentrations in supernatant of cultures grown on different attachment matrices.

Culture	Metal ion concentration in fresh medium (mg l^{-1})	Metal ion concentration after 4 weeks contact with biofilm (mg l^{-1})	% metal uptake after 4 weeks
Cr (P)	102.2	104.3	0
Cr (Pl)	93.8	98.0	0
Cr (S)	96.6	48.3	50.0
Cr (G)	97.3	98.0	0
Cd (P)	37.2	12.7	66.0
Cd (Pl)	34.9	11.9	66.0
Cd (S)	39.1	13.0	66.0
Cd (G)	64.5	3.2	95.0

(P) = perspex; (Pl) = polystyrene; (S) = smooth glass; and (G) = ground glass.

The only microbial biofilm by which Cr^{3+} was taken up was the one that formed on the smooth glass. However, as biofilm development on this surface was poor, the 50% Cr^{3+}-uptake indicated could be erroneous; it is possible that metal ions might have been lost during sample preparation. There appeared to be some uptake of cadmium which may have been due to free-living microorganisms as biofilm development was poor on all the surfaces. Biofilm development did, however, improve after addition of fresh medium. Lead could not be analyzed due to precipitation problems. Subsequent experiments were sampled more regularly and close to the time of initial exposure of the metal to the microbial association, to obtain a better idea of the microbial metal uptake capacity.

3.3.2 Model Stream Experiments

3.3.2.1 Lead

Extensive microbial biofilms developed on the glass beads, ground glass and rippled glass surfaces over 40 hours. Thick biofilms consisting mainly of bacterial cells and extracellular polysaccharide were clearly visible over much of the ground glass (Plate 3.6A), rippled glass (Plate 3.6B) and glass bead (Plate 3.6D, E) surfaces examined. The smooth glass (Plate 3.6C) showed very little cell attachment, indicating that the roughness of the surface plays an important role in biofilm development.

Using glass (high surface charge) and polystyrene (low surface charge) in a colonisation experiment, it was found that the colonisation of the two materials was not significantly different (Baker 1984). However, colonisation of the rough-surfaced glass and polystyrene was found to be significantly faster than that of smooth-surfaced glass and polystyrene respectively (Baker 1984). It was suggested that surface roughness not only increases the surface area, but may enable the glycocalyx to attach more easily, i.e. surface irregularities serve as anchoring points, and microorganisms are protected from fluid dynamic shear forces (Verran *et al.* 1991), thereby aiding colonisation (Baker 1984). Mueller *et al.* (1992) investigated the adsorption of *Pseudomonas aeruginosa* to various surfaces and determined that, in general, the rate of adsorption increased with increasing surface free energy and surface roughness. Roughness 100X smaller than a microbial cell influences adsorption, and thus adhesion may be influenced by changing the surface topography with coatings of organic substrates or polymers (Mueller *et al.* 1992).

Baier (1981) also suggested that more hydrophilic surfaces produce firmer bacterial adhesion than do more hydrophobic surfaces, as the former surfaces have high energy polarity and absorb macromolecules more strongly than do the latter surfaces. The phenomenon of surface roughness aiding in the colonisation of that surface and the production of a biofilm was clearly illustrated in all the model stream experiments.

Plate 3.6   Scanning electron micrographs of microbial biofilms on various surfaces exposed for 48 hours to a medium containing 200 mg l^{-1} Pb^{2+}; ground glass (A) and rippled glass (B) with thick bacterial biofilms; C. smooth glass with little cell attachment; and D. glass bead with thick biofilm and detail of biofilm on glass bead (E) corresponding to boxed area in D.

A 4 μm
B 4 μm
C 4 μm
D 0.2mm
E 9 μm
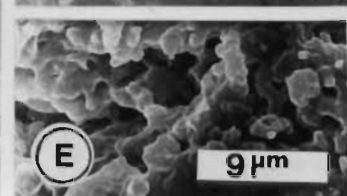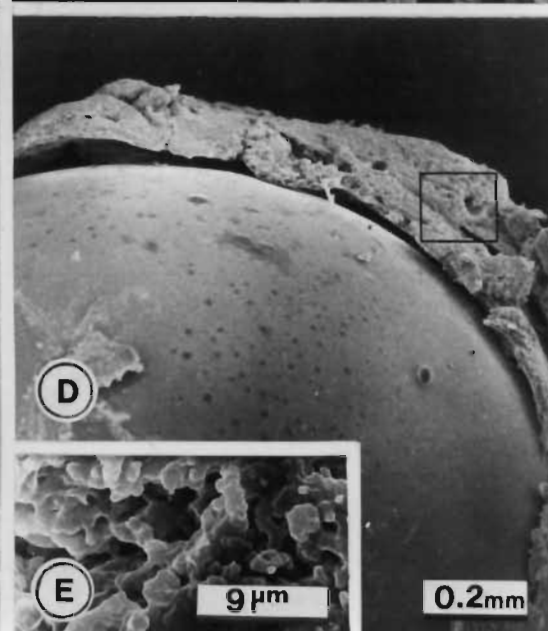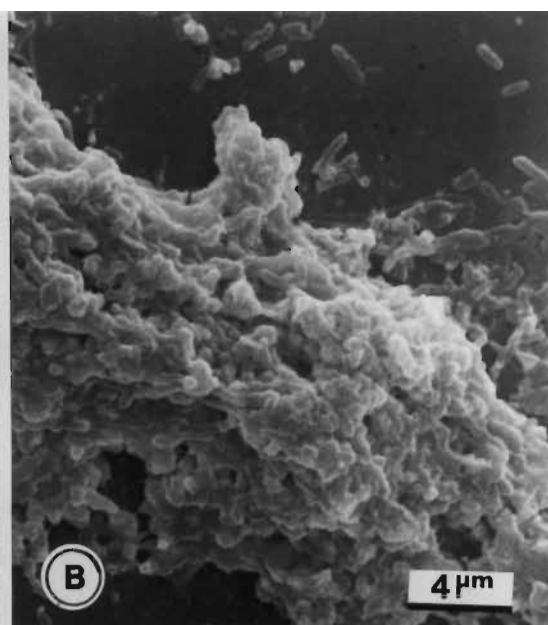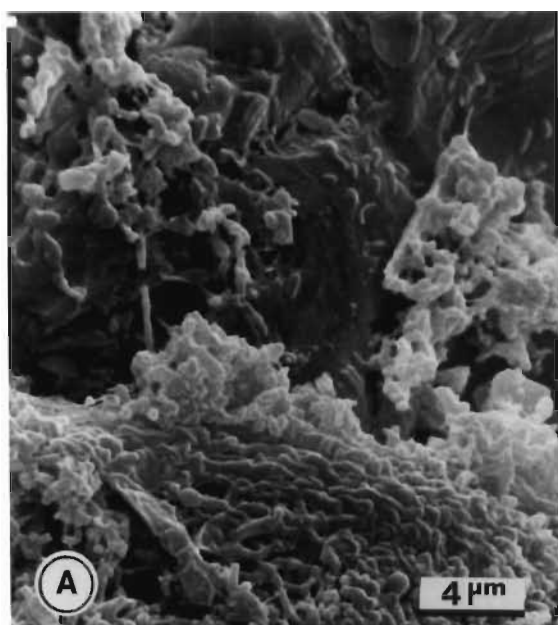
EDX analysis of the microbial biofilm on the rippled glass indicated the presence of lead (Fig. 3.1).
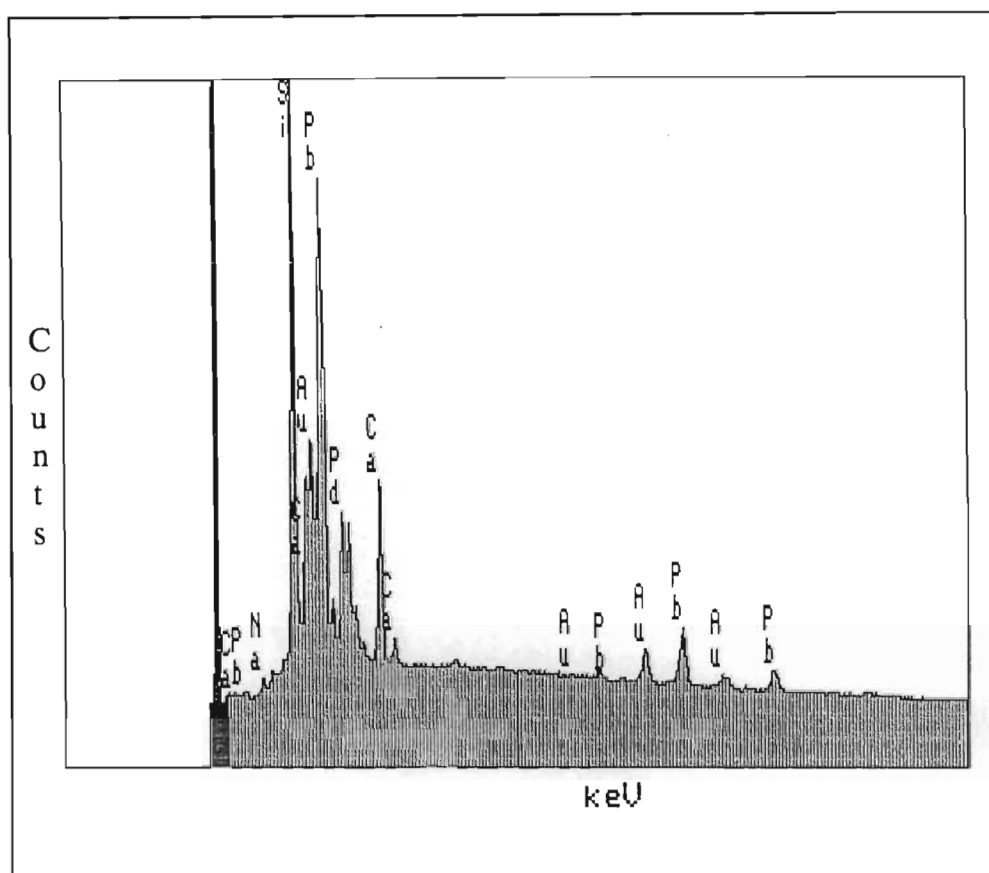
Figure 3.1   EDX spectrum of microbial biofilm attached to a rippled glass plate exposed for 48 hours to a medium containing 200 mg l^{-1} Pb^{2+}.

The X-ray signal intensity is quantified by counts (y-axis label) and is the relative concentration of the elements subject to a number of factors; the orientation of the elements in the specimen and the topography of the specimen in relation to the detector (Chandler 1978). The gold (Au) and palladium (Pd) peaks shown on the EDX spectrum resulted from the coating used on these samples. The silicon (Si) and calcium (Ca) detected were from the glass plate. Unfortunately, the concentration of Pb^{2+} remaining in the medium could not be determined since precipitation of the metal made quantitative analyses impossible. The precipitated lead was centrifuged and/or filtered out of the sample before analysis by HPLC or AAS.

3.3.2.2 Copper

A problem encountered when the copper ion solution was added to an already growing culture was that it precipitated out of the medium in the reservoir and thus the amount of copper available for uptake by the biofilm was considerably lower than that which was intended. On macroscopic observation, the glass beads, ground glass and rippled glass again supported more extensive biofilm development than the smooth glass.

Scanning Electron Microscopy (SEM) was used to investigate the different biofilm support materials removed from the channels 3 days after the addition of the copper solution. Both the ground glass (Plate 3.7A) and rippled glass (Plate 3.7B) surfaces provided for good biofilm development, and extracellular polysaccharide was associated with the biofilm on the rippled glass plate (Plate 3.7B). The stability of biofilms was shown by Vess *et al.* (1993) who demonstrated that the surface is colonised with bacteria that excrete extracellular material and that this results in embedded cells and an increased physical thickness of the biofilm, due to the continuous layering of this material. This biofilm can persist and remain viable as individual cells are protected and thus more likely to survive to continue biofilm development. Allison and Sutherland (1987) also observed polymeric material attaching Gram negative cells to a surface and to each other, and indicated that extracellular polysaccharide was not directly involved in initial attachment, but with the establishment of the biofilm. In the present study remnants of extracellular polysaccharide were observed associated with biofilms on the roughened surfaces, indicating that extracellular polysaccharides are associated with biofilm formation.

Only isolated, small clusters of cells were observed on the smooth glass, with some extracellular polysaccharide present (Plate 3.7C), once again indicating the need for surface roughness for biofilm development. A problem was encountered with the glass beads in that the dehydration and critical point drying procedures removed most of the microbial growth from the bead surfaces and thus little biofilm was observed under the SEM (Plate 3.7D). In the model stream, however, the glass beads provided an irregular surface which was quickly colonised by the microorganisms.

Plate 3.7 Scanning electron micrographs of microbial biofilms on various surfaces exposed for 3 days to a medium containing 100 mg l^{-1} Cu^{2+}; ground glass (A) and rippled glass (B) with good biofilm development (arrows represent the remnants of extracellular polysaccharide); C. smooth glass with isolated small clusters of cells (arrow represents the remnants of extracellular polysaccharide); and D. glass bead with little apparent biofilm development.
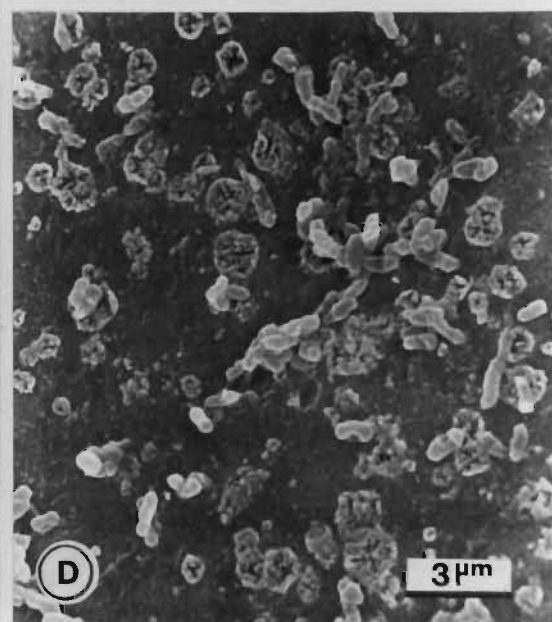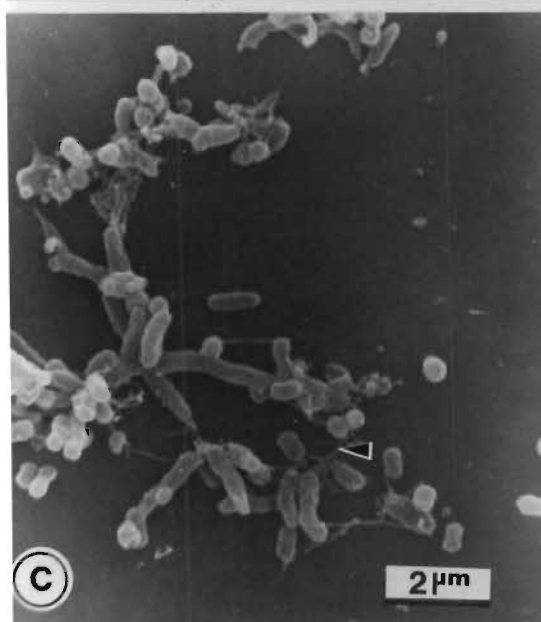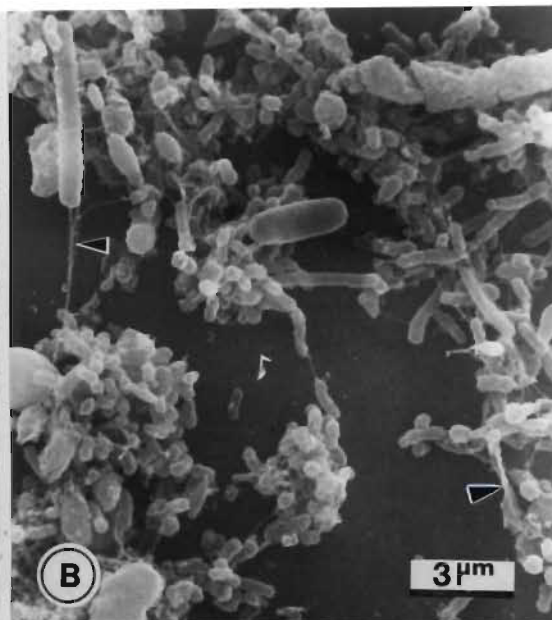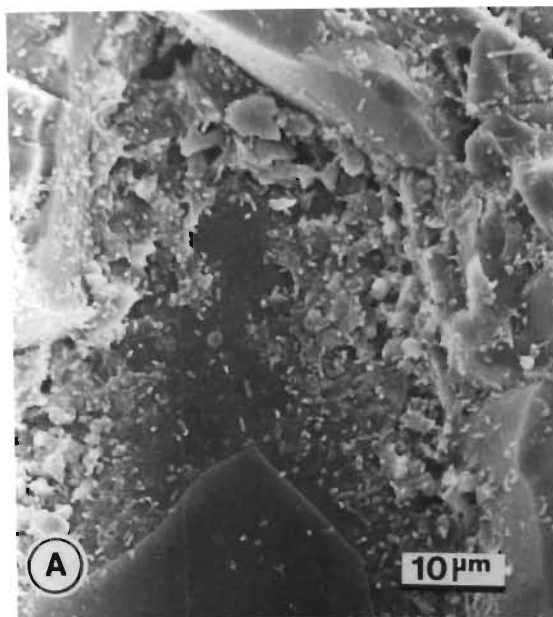
Samples of the liquid medium were analyzed by HPLC (see section 2.2.4.1) to determine the amount of metal remaining in solution after uptake by the microbial biomass. Figure 3.2 shows the percentage copper remaining in solution after microbial metal uptake. It appears that all the microbial biofilms removed the copper to a large extent. It must be noted, however, that there was precipitation of the copper in the model stream reservoir, possibly due to complexation between organics in the half-strength Nutrient Broth and the Cu^{2+} ions, as well as inadequate mixing, which may have contributed to the high percentage copper removal ascribed to the microbial biofilms.
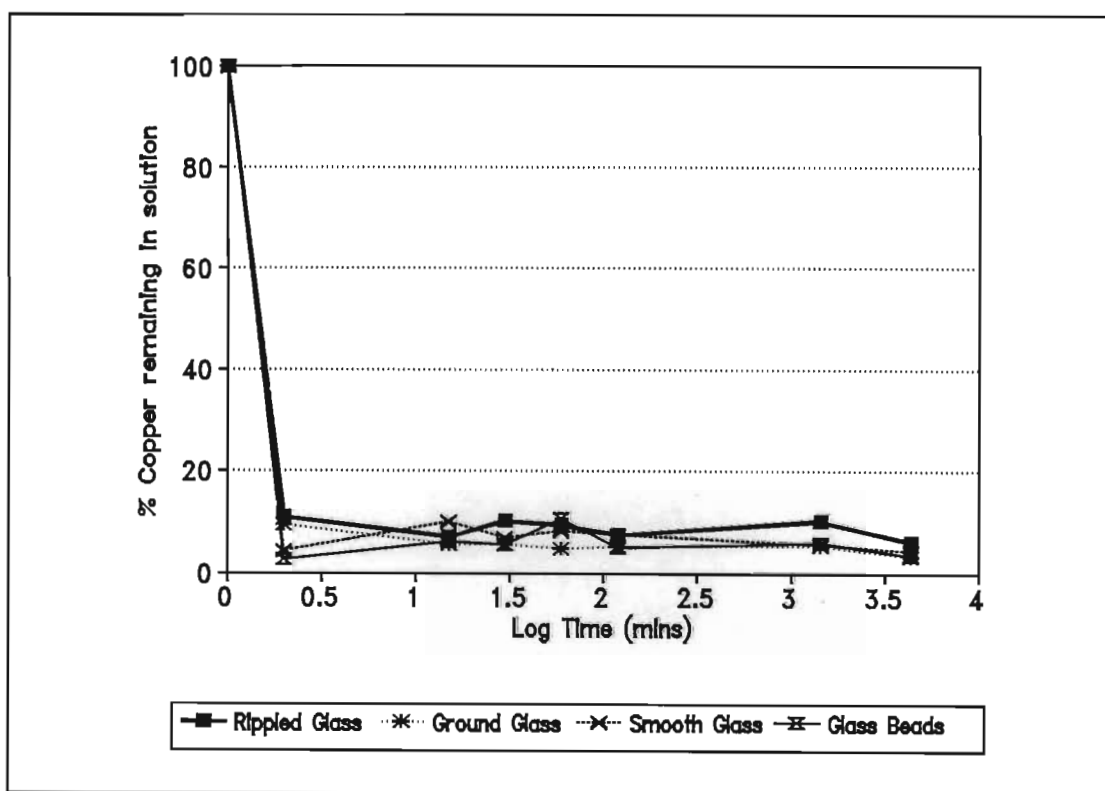
Figure 3.2   Percentage Cu^{2+} remaining in solution after various periods of metal uptake by the microbial biofilms developed on the different surfaces.

3.3.2.3 Cadmium

In this model stream experiment it was observed that after cadmium was added and circulation of the medium commenced, there were both attached and free-living microorganisms present. The biofilm also seemed to improve, probably due to the

addition of fresh half-strength Nutrient Broth. Good biofilm development was observed, using Scanning Electron Microscopy, on both the ground and rippled glass plates. Plate 3.8A and B show the depth of the biofilm developed on the ground glass. A similar situation was also observed on the rippled glass plates (Plates 3.8C and D), where extracellular material seemed to play a role in biofilm formation, as discussed above.

The smooth glass (Plate 3.8E) supported only weak biofilm development as observed before. No micrographs of the glass beads are shown due to loss of the biofilm during the dehydration and CPD steps in the preparation of the samples for SEM.

Plate 3.8 Scanning electron micrographs of microbial biofilms on various surfaces exposed for 48 hours to a medium containing 200 mg l^{-1} Cd^{2+}; A and B. ground glass surfaces showing the depth of biofilm development; C and D. rippled glass surfaces with dense biofilm development and extracellular material; and E. smooth glass surface with poor cell attachment.
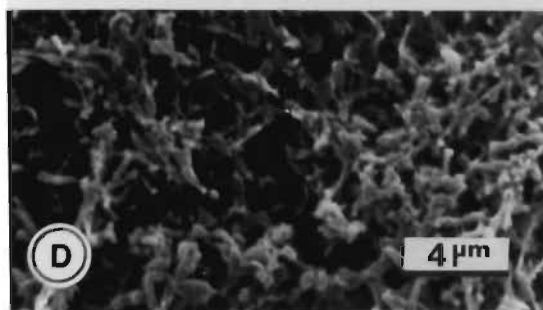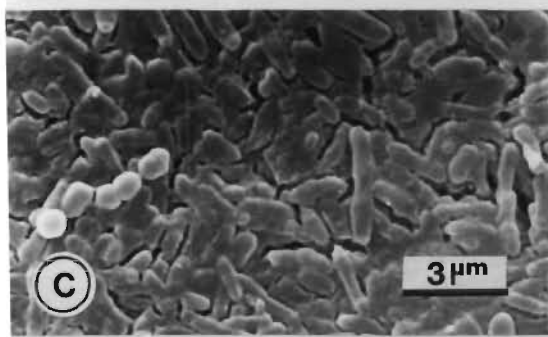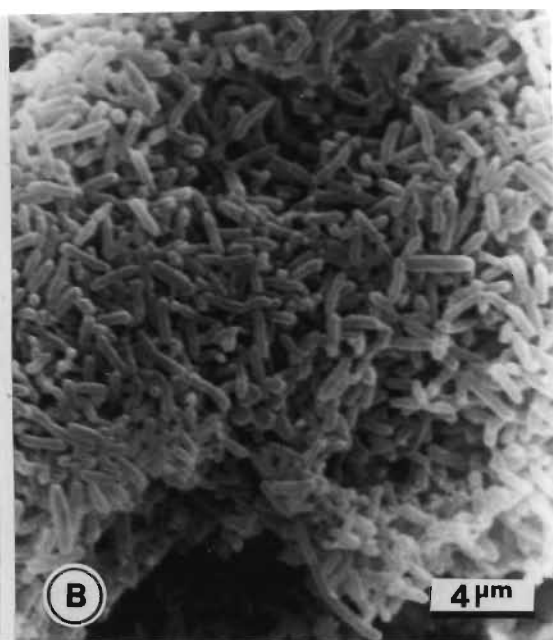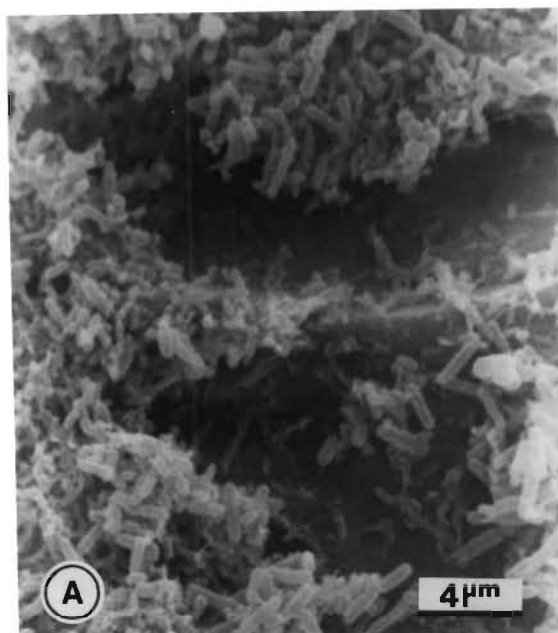
HPLC was used to analyze these liquid medium samples to determine the amount of Cd^{2+} removed by the microorganisms. In Fig. 3.3 it can be seen that the biofilms on both the rippled glass and the glass beads showed the highest concentrations of Cd^{2+} removal after 48 hours. Initially, however, the biofilm on the ground glass showed the best Cd^{2+} removal rate. The shapes of the graphs suggest that there were periods of desorption of the metal ions, possibly associated with sloughing of the biofilm from the support materials or, possibly, due to changes in the pH of the medium. However, the pH did not fluctuate but increased over time from an initial pH of 6.8 to a final pH of 8.2. Thus, part of the cadmium which was removed may have been lost due to precipitation as the pH of the solution increased (Fig. 3.3) and these precipitates may have been filtered out of solution before the samples were analyzed. At pH ≤ 8 cadmium exists predominantly as the divalent ion, Cd^{2+}, with $CdOH^+$ beginning to form at pH 7 to 7.5 (Hahne and Kroontje 1973). Cadmium ions are known to complex with specific chelating groups in organic matter and also precipitate as phosphate salts (Babich and Stotzky 1978). The organics present in the medium and metabolic products released by the microorganisms may also have resulted in precipitation of the Cd^{2+} ions. Cadmium uptake by *Zoogloea ramigera* 115 was shown to be unaffected at pH 6 to 8 (Kuhn and Pfister 1990). Thus, it is unlikely that the adsorption/desorption periods were due to changes in pH. The better established biofilms were capable of removing more metal from solution. Thus surfaces which provide for good biofilm development would also facilitate a more efficient metal uptake system.
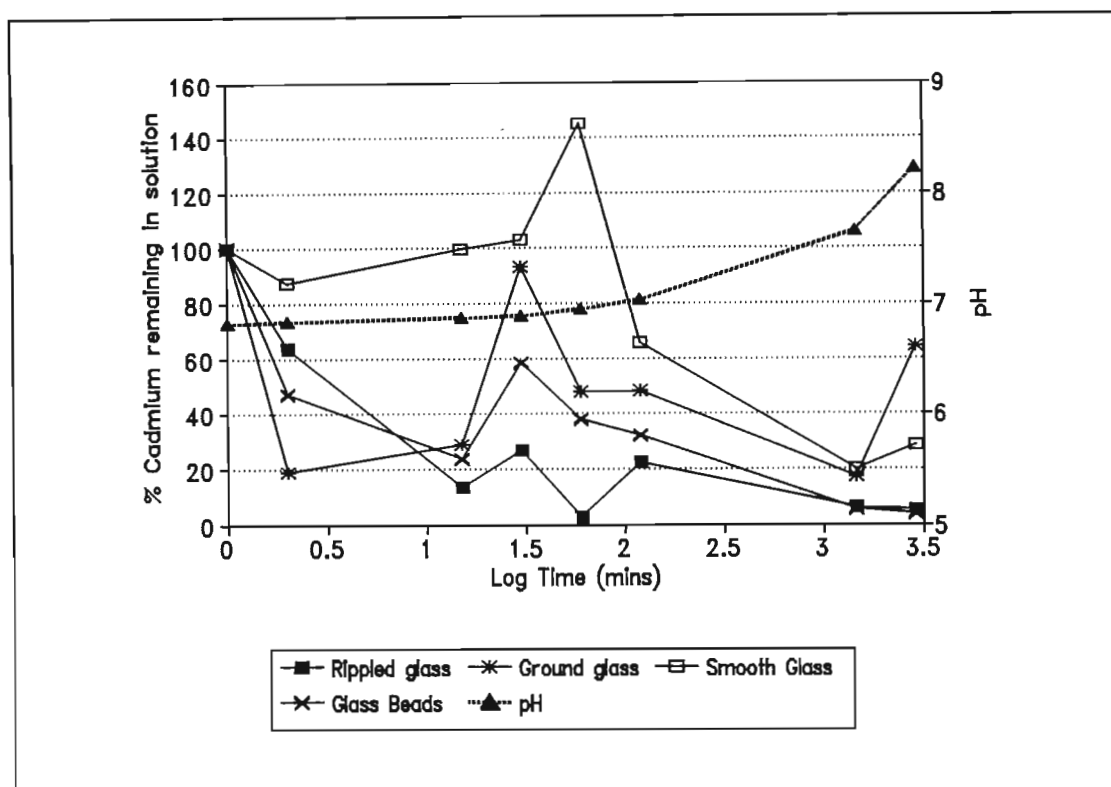
Figure 3.3   Percentage Cd^{2+} remaining in solution after various periods of metal uptake by the microbial biofilms developed on the different surfaces, and changes in solution pH.

3.3.2.4 Chromium

Plates 3.9 - 3.12 depict the extent to which the biofilms had developed on the various surfaces on day 2, day 7 (when the metal was added), and day 10. The ground glass (Plate 3.9) supported the most extensive biofilm development. There appeared to be a progression of microorganisms colonising the surface during biofilm development, with only bacteria present on day 2 (Plate 3.9A), while yeast-like cells and bacteria were present on day 7 (Plate 3.9B) and day 10 (Plate 3.9D). Filamentous microorganisms were also visible in parts of the biofilm on day 7 (Plate 3.9C). Polystyrene also provided a surface suitable for good biofilm formation (Plate 3.10). In this case yeast-like cells were present from day 2 (Plate 3.10A), through day 7 (Plate 3.10B) to day 10 (Plate 3.10C), with filamentous microorganisms making their appearance only on day 10 (Plate 3.10D). The yeast-like cells seemed to prefer to attach to the polystyrene, a phenomenon also

observed in earlier experiments (Plate 3.5). The rippled glass showed a slower rate of colonisation (Plate 3.11A-C), with the smooth glass showing poor biofilm development (Plate 3.12A-C). No micrographs were obtained for the glass beads due to the loss of biofilm during preparation for SEM.

Plate 3.9   Scanning electron micrographs showing the development of microbial biofilms on ground glass exposed to a Cr^{3+}-containing solution (200 mg l^{-1}) for different periods of time; A. day 2 (no metal present); B and C. day 7, bacteria and yeast-like cells (indicated by the Y) were present together with various filamentous microorganisms; D. day 10, bacteria and both yeast-like cells and filamentous microorganisms were present.

A 7.5 μm

B 6 μm C 20 μm

4 μm
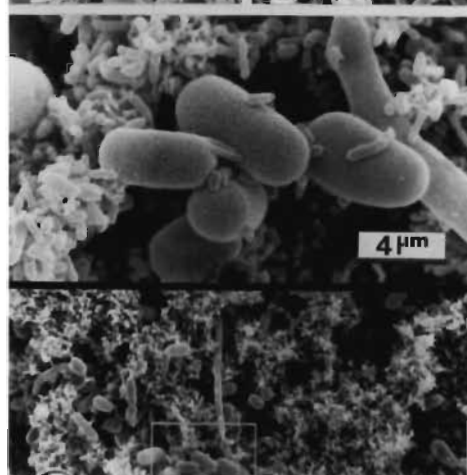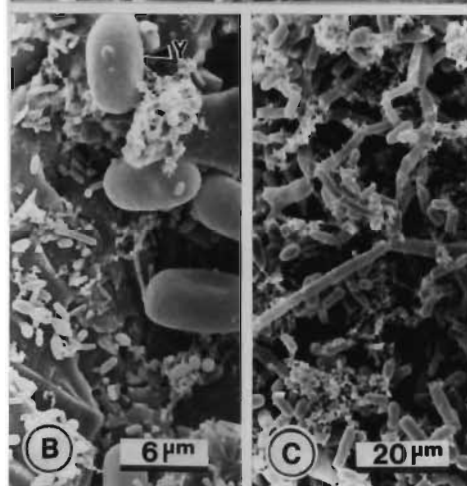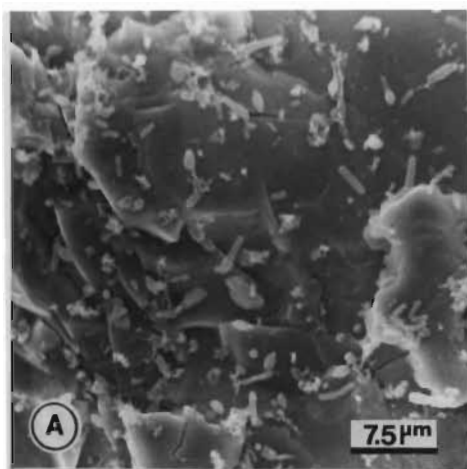
Plate 3.10   Scanning electron micrographs showing the development of microbial biofilms on polystyrene exposed to a Cr^{3+}-containing solution (200 mg l^{-1}) for different periods of time; A. day 2 (no metal present; Y = yeast-like cell); B. day 7, showing the presence of bacteria and the yeast-like cells indicated in A; C and D. day 10 (arrow indicates filamentous microorganism) and the yeast-like cells indicated in A were also observed together with bacteria.

A   6 μm

B   4 μm

C   4 μm   D   15 μm
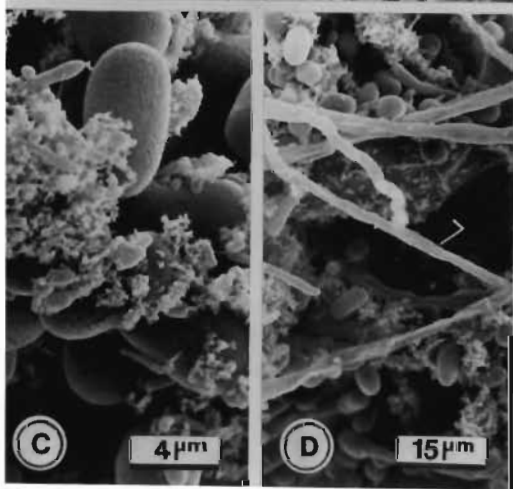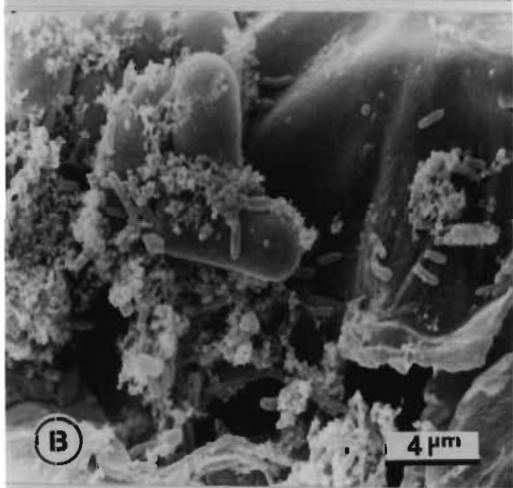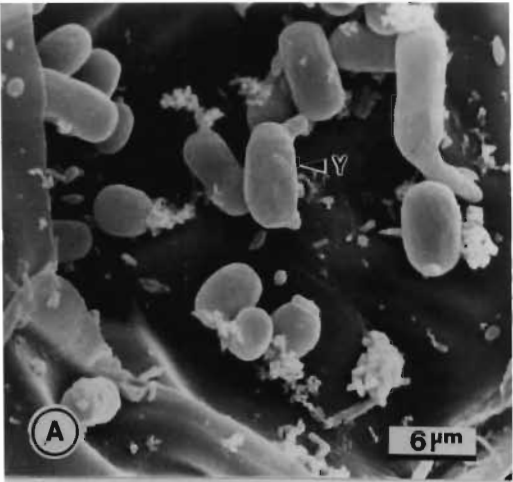
Plate 3.11   Scanning electron micrographs showing the development of microbial biofilms on rippled glass exposed to a Cr^{3+}-containing solution (200 mg l^{-1}) for different periods of time; A. day 2 (no metal present); B. day 7; C. day 10.
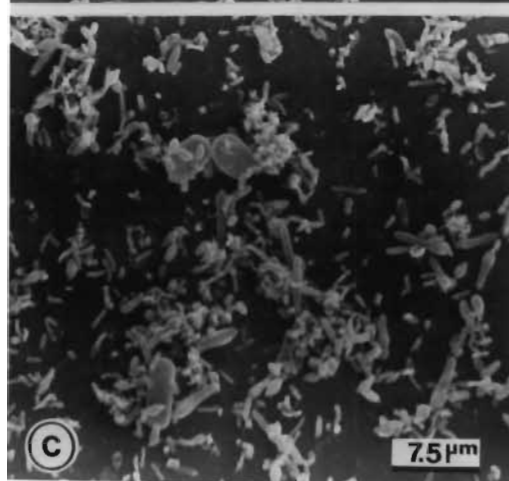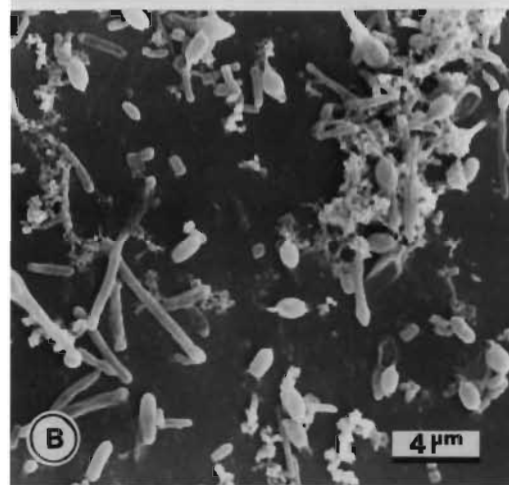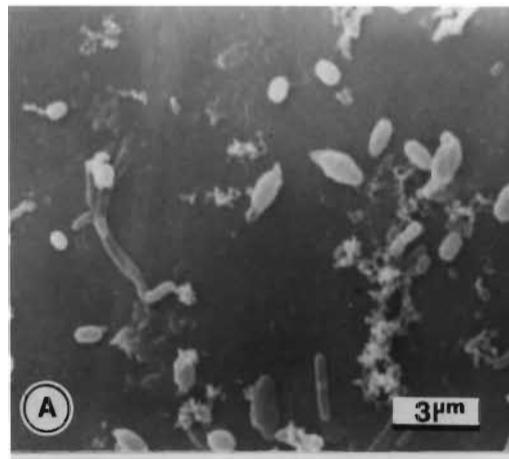
Plate 3.12   Scanning electron micrographs showing the development of microbial biofilms on smooth glass exposed to a Cr^{3+}-containing solution (200 mg l^{-1}) for different periods of time; A. day 2 (no metal present); B. day 7, few yeast-like cells were present; C. day 10 (note the unusual surface of the yeast-like cells indicated by the arrow).
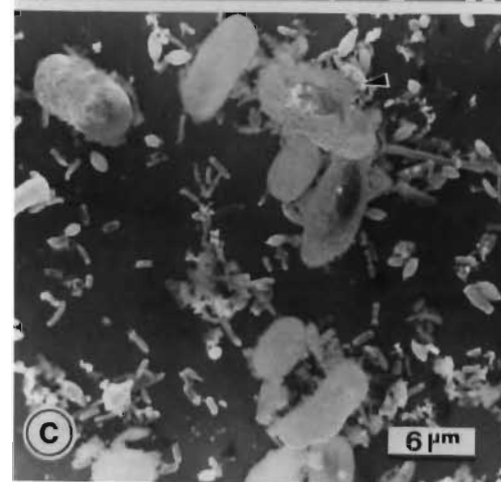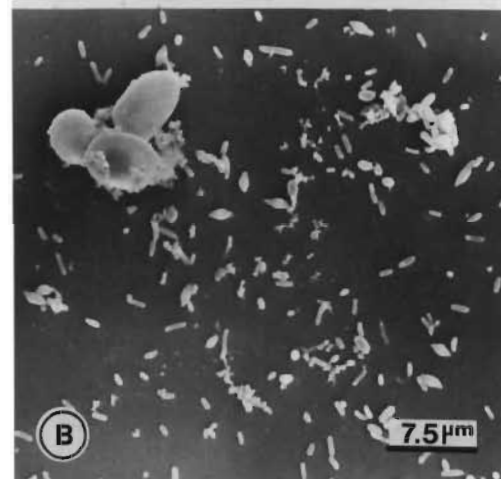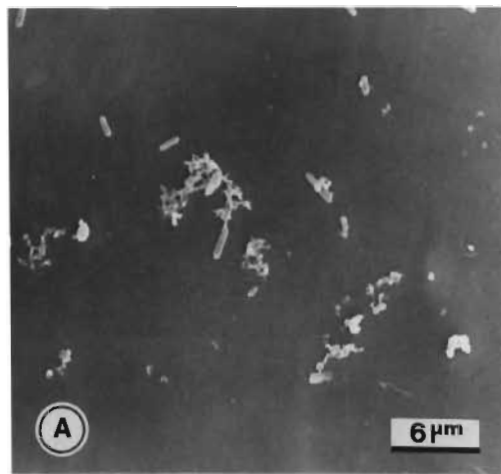
EDX analysis showed that chromium was present on or in the microbial biofilm which formed on the surface of the rippled glass (Fig. 3.4).
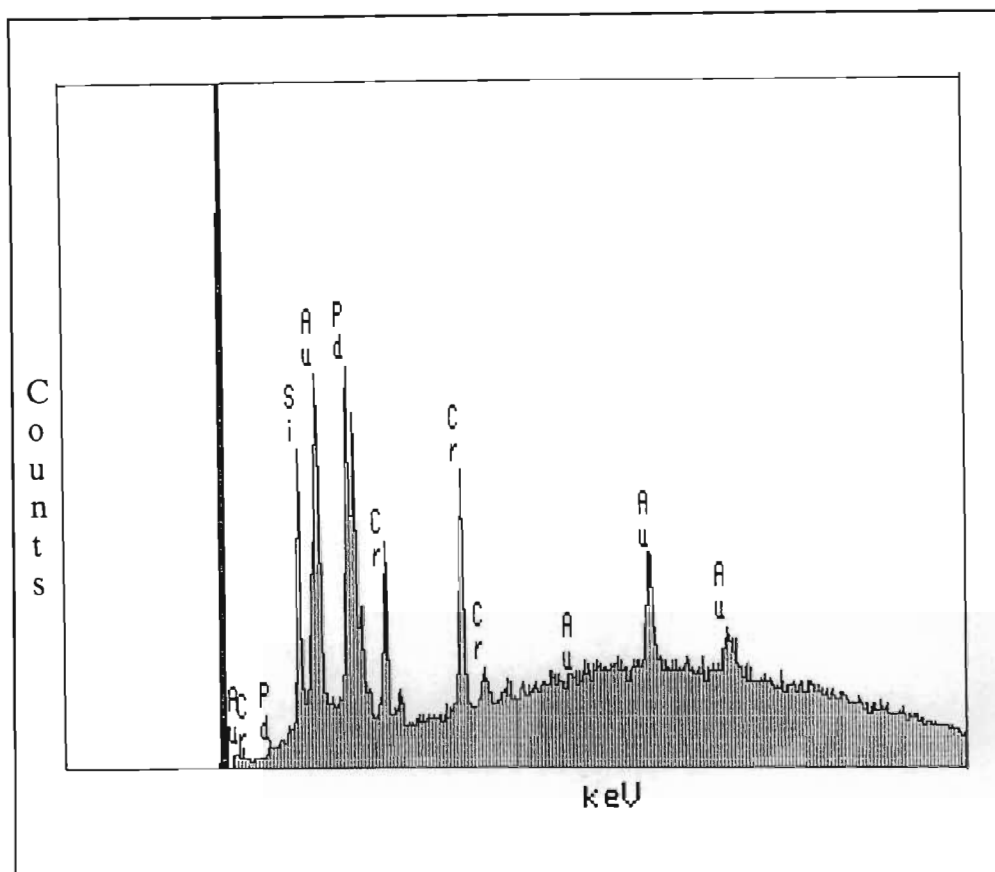
Figure 3.4   EDX spectrum of microbial biofilm which became established on a rippled glass plate during exposure for 48 hours to a medium containing 200 mg l^{-1} Cr^{3+}.

Other elements were detected by the EDX, i.e. Si from the glass plate, and Au and Pd from the gold/palladium coating.   Atomic Absorption Spectrophotometry (see section 2.2.4.2) was used to determine the concentration of chromium remaining in the liquid medium and the results are shown in Figure 3.5. The most active chromium uptake was by the biofilm formed on the polystyrene. Maximum chromium removal occurred after two hours but this subsequently decreased, possibly due to desorption of the metal ions or perhaps due to evaporative loss of liquid from the system thereby causing an increase in the metal concentration in the medium. The biofilm on the glass beads showed an initial

rapid uptake of Cr^{3+} which subsequently decreased, perhaps due to desorption of the metal ions from the biofilm. There seemed to be little uptake of Cr^{3+} by the microorganisms formed on the rippled, ground and smooth glass surfaces, even though biofilm development was good on the former two surfaces.
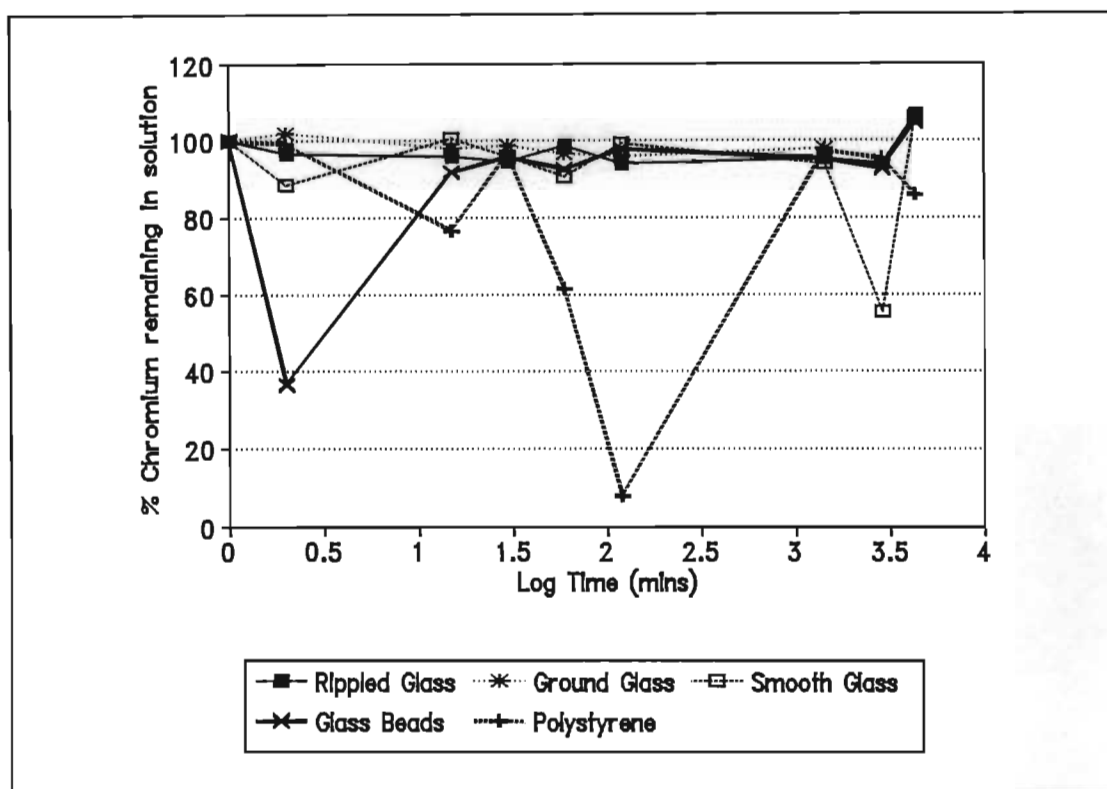
Figure 3.5   Percentage Cr^{3+} remaining in solution after various periods of metal uptake by the microbial biofilms developed on the different surfaces.

Spot analysis of a yeast-like cell in the biofilm on polystyrene showed the presence of chromium (Fig. 3.6), indicating that Cr^{3+} had been taken up by the cells. Whether this uptake was due to surface adsorption, intracellular accumulation or both, could not be ascertained.
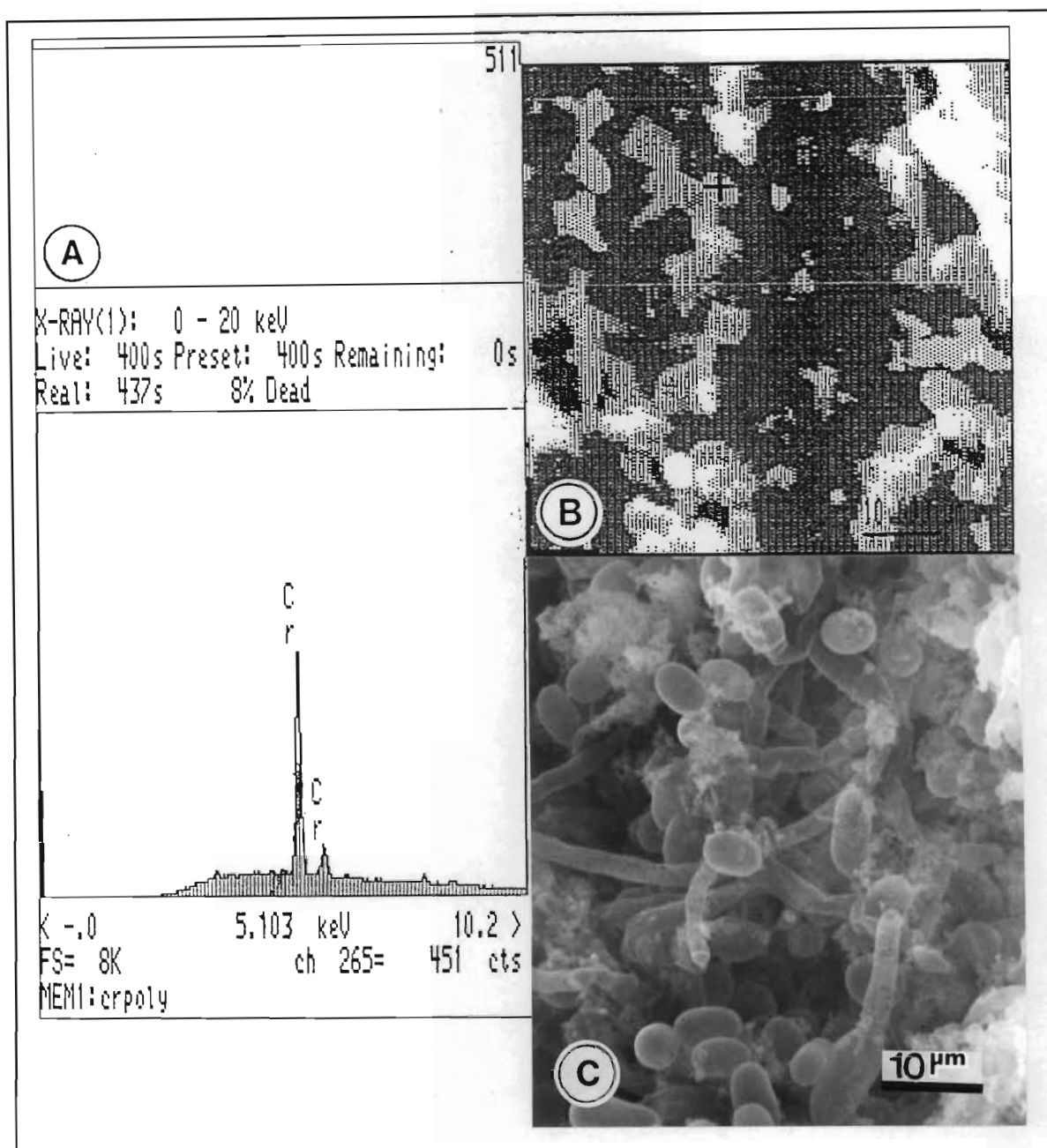
Figure 3.6 Spot analysis of microbial biofilm on polystyrene surface exposed for 48 hours to 200 mg l^{-1} Cr^{3+}; A. EDX spectrum of yeast-like cell indicated by the $+$ symbol on the secondary image (B); C. electron micrograph of portion of the biofilm corresponding to the secondary image (arrow indicates the yeast-like cell subjected to spot analysis).

3.3.2.5 Model Stream fed with artificial effluent

Figure 3.7 shows the amounts of individual metals remaining in solution after microbial metal uptake. In all cases the biofilm was immobilised on polystyrene and the metals were present initially at the following concentrations: Cu^{2+} - 100 mg l^{-1}, Cd^{2+} - 200 mg l^{-1}, Pb^{2+} - 200 mg l^{-1}, and Cr^{3+} - 200 mg l^{-1}. The channel fed with the copper-containing solution (100 mg l^{-1}) showed a high percentage removal of Cu^{2+} over the four days duration of the experiment, with the highest rate of uptake occurring after one day (Fig. 3.7A). There was a slight initial uptake of Cd^{2+} followed by renewed uptake after two hours, with maximum uptake occurring after 72 hours (Fig. 3.7B). The initial stages of Pb^{2+} uptake were slower but then increased compared to the rate of Cd^{2+} uptake with an unexplained drop after 24 hours (Fig. 3.7C). Chromium uptake increased steadily with time reaching a maximum after 60 minutes, and then decreased again (Fig. 3.7D).

In the channel containing a combination of copper, cadmium and lead at the concentrations indicated above, the removal of Cu^{2+} and Cd^{2+} through adsorption to the biomass reached a maximum at 120 minutes and then uptake decreased (Fig. 3.8). The metal ions were possibly released back into the solution, or evaporation caused the concentration of the remaining metal ions in the medium. The Pb^{2+} on the other hand was rapidly taken up within the first few minutes and at no time was it released back into the solution. It must be noted that the initial concentration of lead was much lower than the intended 200 mg l^{-1}, i.e. only approximately 8 mg l^{-1} were found in the time 0 sample. However, this was completely removed during the first two minutes of exposure to the biofilm.
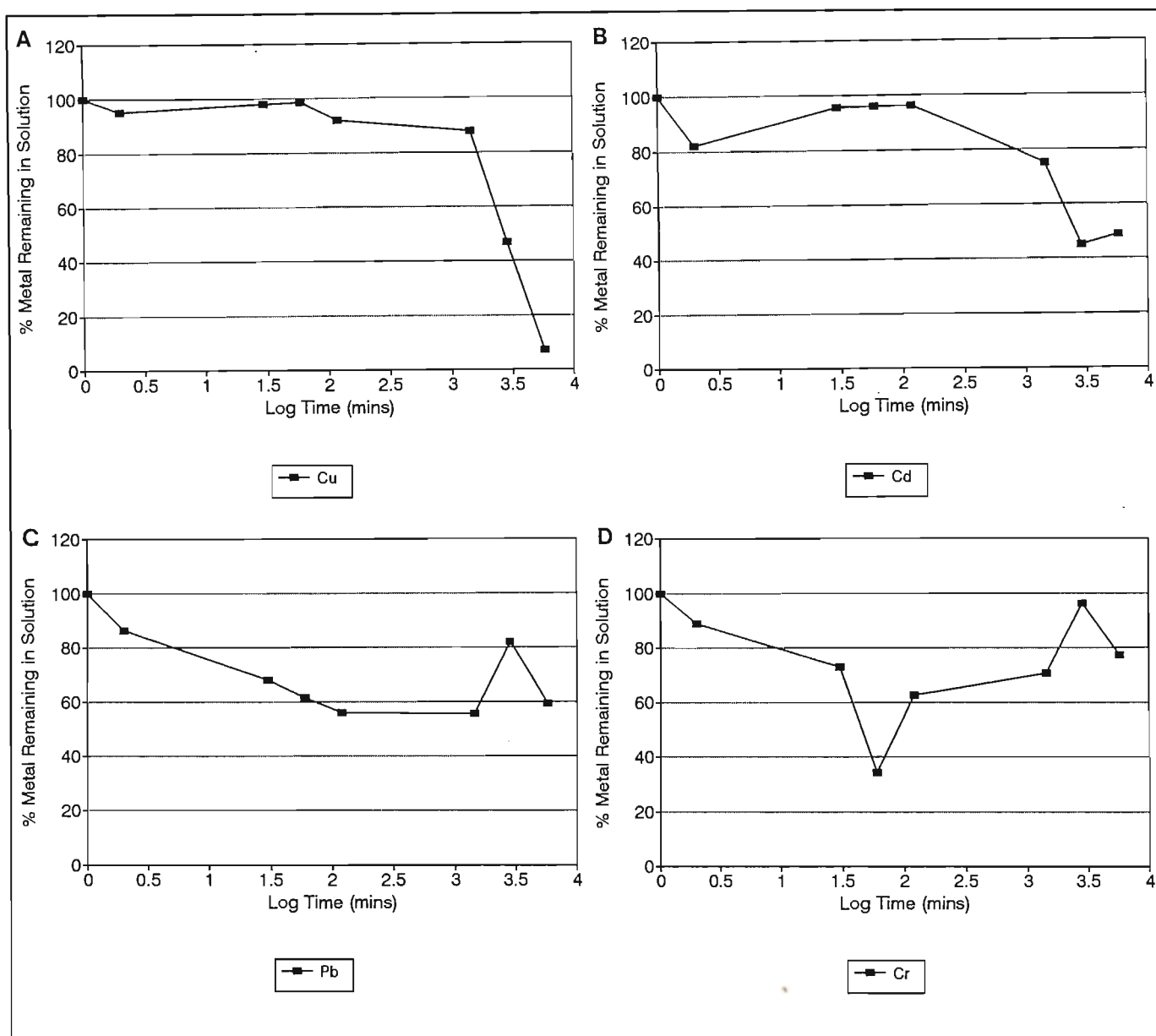
Figure 3.7   Percentage metal remaining in solution after various periods of metal uptake by the microbial biofilms developed on polystyrene; A. Cu^{2+}; B. Cd^{2+}; C. Pb^{2+}; D. Cr^{3+}.
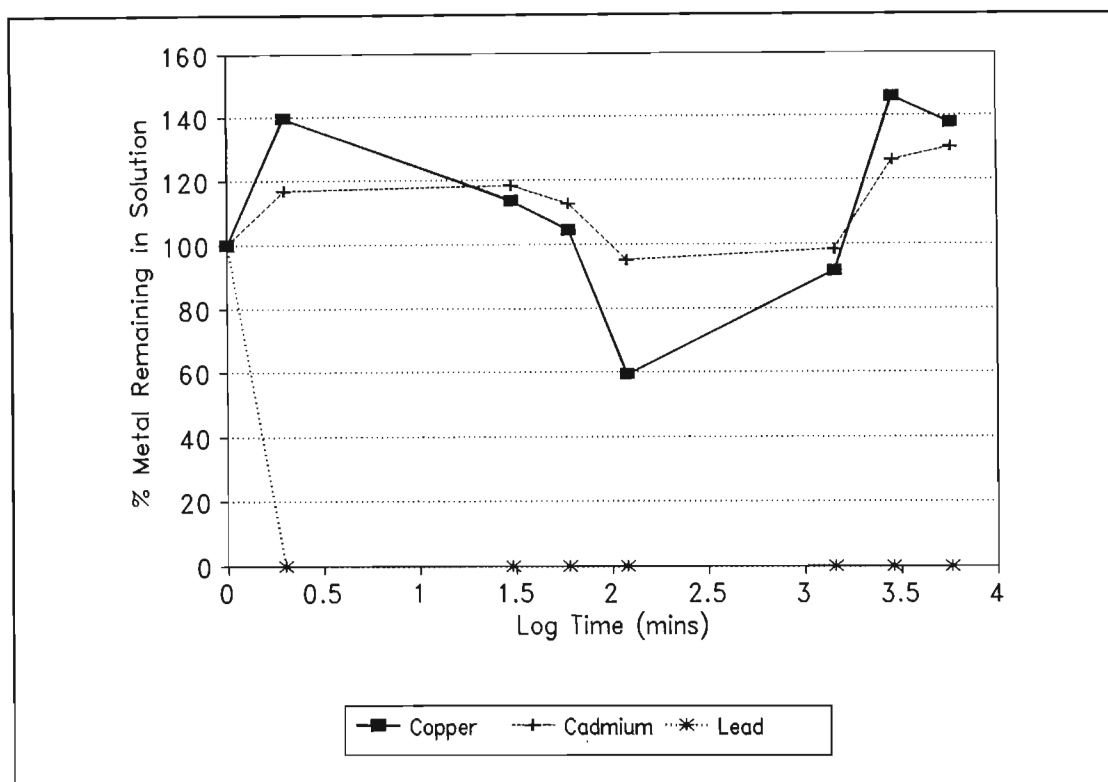
Figure 3.8   Percentage metal remaining in solution after various periods of metal uptake from a mixed metal solution by a microbial biofilm developed on polystyrene.

The EDX digital mapping technique (see section 3.2.3.2) was used to determine if the metal was actually adsorbing onto the microbial biofilm or onto the polystyrene.

The polystyrene-supported microbial biofilm to which the Pb^{2+} -containing solution was added showed good uptake of the metal (Fig. 3.7C). The electron micrograph (Fig. 3.9C) and secondary image (Fig. 3.9B) show the extensive biofilm that developed on the polystyrene and the EDX map (Fig. 3.9A) shows that the Pb^{2+} accumulated on the bacterial biofilm, rather than on the polystyrene. The spectrum also shows the presence of lead in association with the biomass (Fig. 3.10).
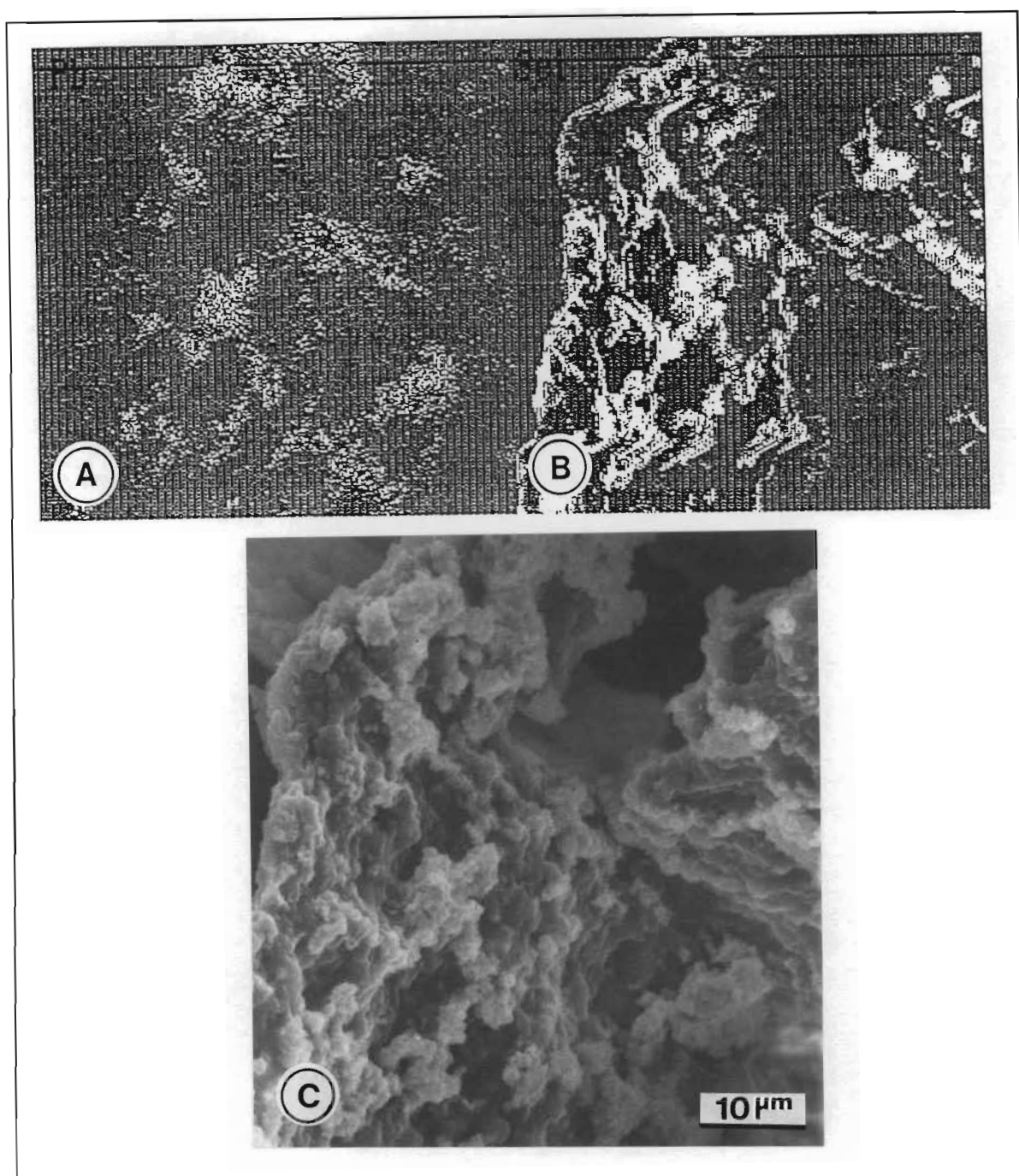
Figure 3.9 EDX digital map of microbial biofilm developed on polystyrene exposed for 4 days to a 200 mg l^{-1} Pb^{2+}-containing medium; A. lead map; B. secondary image; C. electron micrograph corresponding to secondary image.
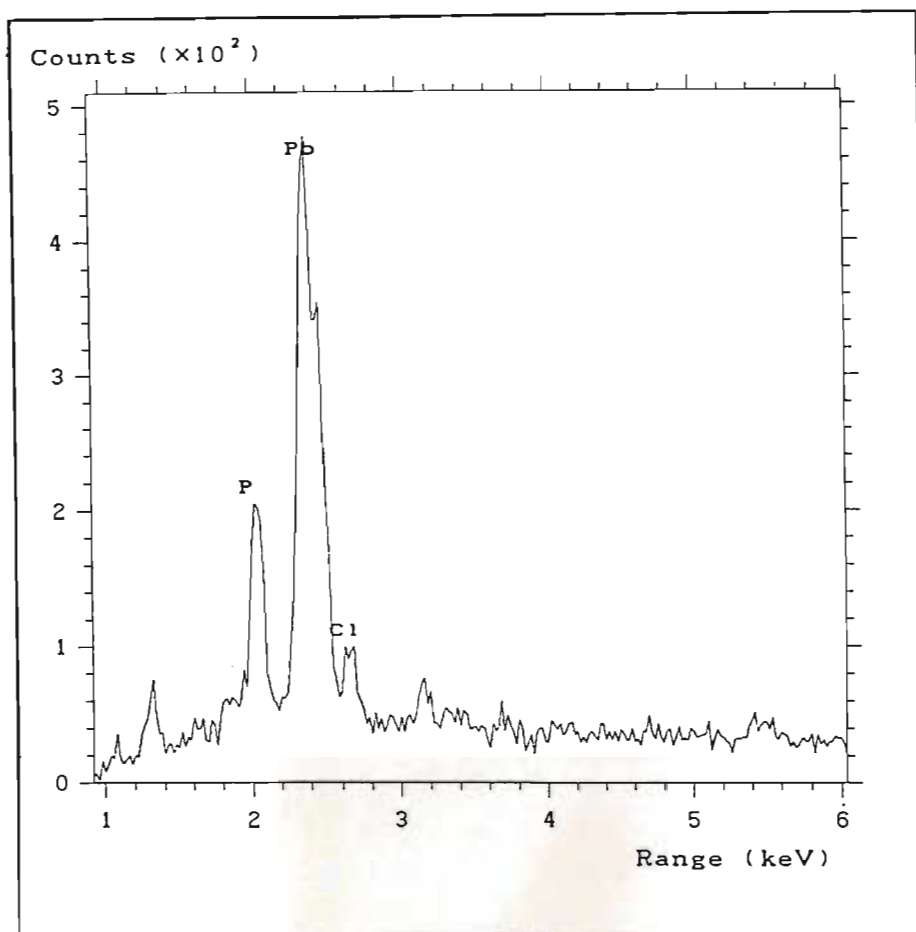
Figure 3.10   EDX spectrum of microbial biofilm which became established on polystyrene exposed for 4 days to a medium containing 200 mg l^{-1} Pb^{2+}.

Despite the poor quality of the secondary image, due to charging effects as the specimen was carbon coated, Figure 3.11, clearly verifies that the Pb^{2+} had accumulated on the actual microbial cells comprising the biofilm. Long dwell times (20-30 milliseconds) are required to obtain clear maps of the metals present and this also may lead to a very unclear secondary image.
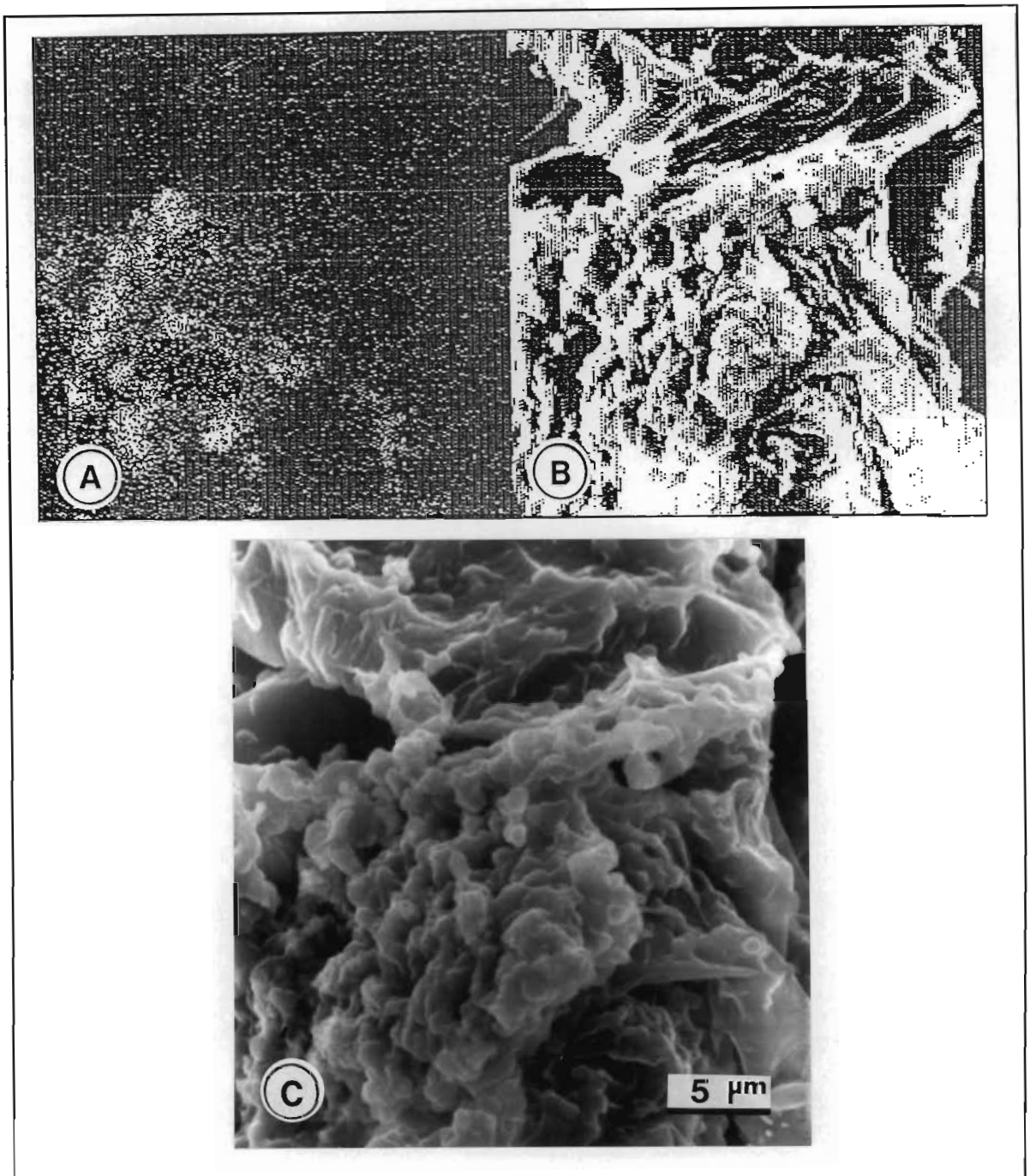
Figure 3.11 EDX digital map of microbial biofilm established on polystyrene and exposed for 4 days to a medium containing 200 mg l^{-1} Pb^{2+}; A. lead map; B. secondary image; C. electron micrograph corresponding to secondary image.

The biofilm exposed to a mixed metal solution was also mapped using the EDX digital mapping technique. Despite the poor quality of the secondary image it is apparent from

Plate 3.13 that the lead which can be clearly seen in the EDX map was located on the microbial biofilm attached to the polystyrene. For EDX mapping it was found that the best results were obtained with one frame with a longer exposure time, or dwell time (20-30 milliseconds). Various other examples (not shown) revealed the same pattern of mapping with lead predominating, followed by cadmium and then copper. This could be because the cadmium and copper were not as highly concentrated on the surface of the biofilm or because they were not detected as easily by this mapping technique. HPLC metal analysis results support the former possibility.

Plate 3.13 EDX digital map of microbial biofilm established on polystyrene exposed for 4 days to a medium containing 100 mg l^{-1} Cu^{2+} + 200 mg l^{-1} Cd^{2+} + 200 mg l^{-1} Pb^{2+}; A. lead map; B. cadmium map; C. copper map; D. secondary image; E. electron micrograph corresponding to secondary image.
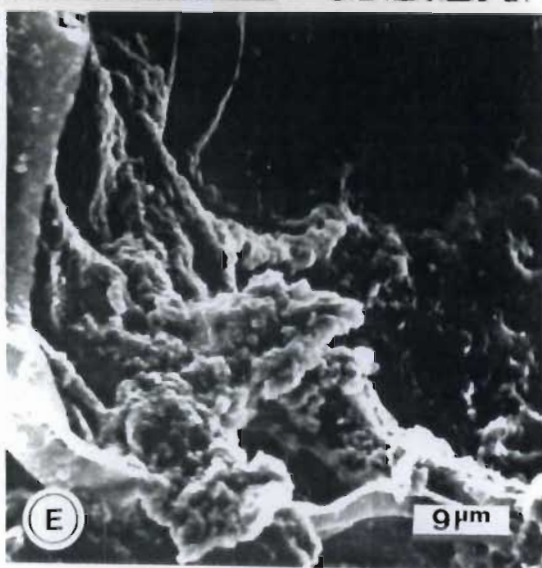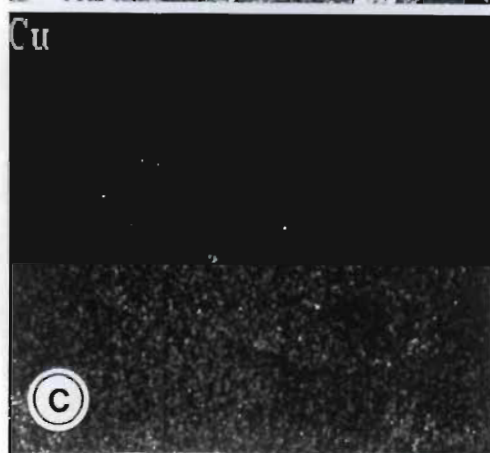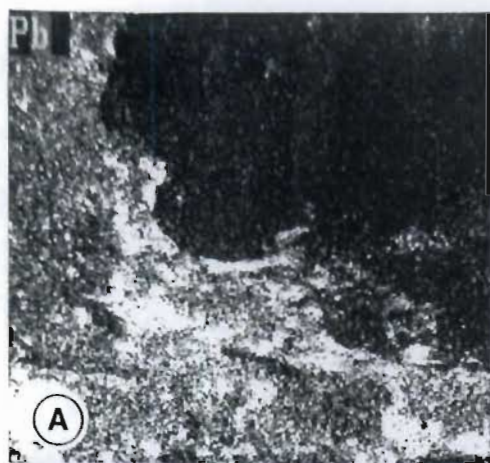
The biofilm exposed to a mixed metal solution was also dot mapped using EDX. The spectrum (Fig. 3.12) shows the presence of lead (48.9%), cadmium (13.8%) and a small amount of copper (5.1%) on or within the biofilm. The maps show the distribution of these metals in relation to the microbial cells comprising the biofilm (Plate 3.14A, B and C). It is clear from these maps that large amounts of lead were associated with the microbial biofilm (Plate 3.14A), but only small amounts of cadmium (Plate 3.14C) and very little, if any copper (Plate 3.14B). Lead seems to be preferentially taken up over cadmium and copper. This selective accumulation of heavy metals was reported to be due to interionic competition, as certain actinomycete species were observed to accumulate uranyl, mercury, lead and copper ions more readily than zinc, manganese, cobalt, nickel and cadmium (Nakajima and Sakaguchi 1986).
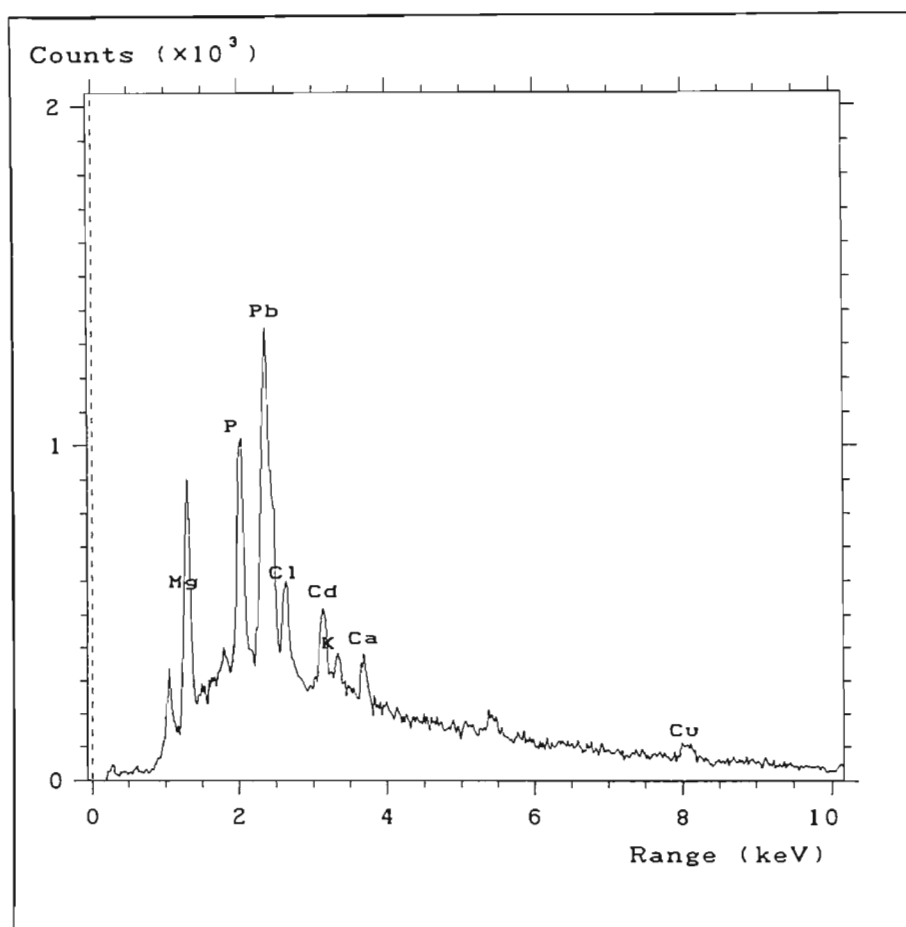
Figure 3.12   EDX spectrum of microbial biofilm established on polystyrene exposed for 4 days to a medium containing 100 mg l^{-1} Cu^{2+} + 200 mg l^{-1} Cd^{2+} + 200 mg l^{-1} Pb^{2+}.

Plate 3.14 EDX dot maps of bacterial biofilm developed on polystyrene and exposed for 4 days to a mixed metal-containing medium; A. lead map and corresponding electron micrograph; B. copper map and corresponding electron micrograph; C. cadmium map and corresponding electron micrograph.
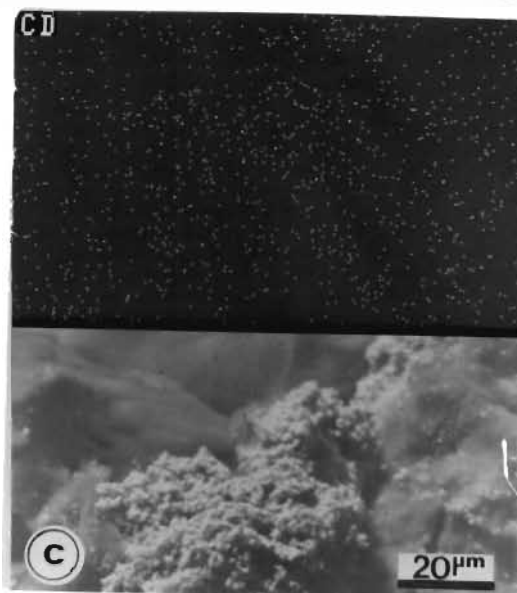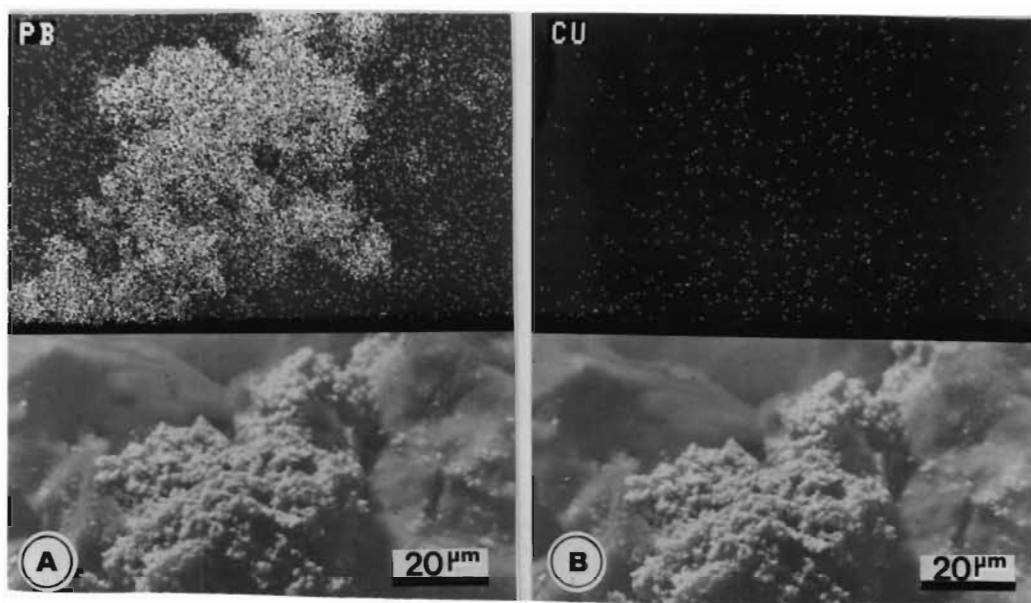
3.4 CONCLUSIONS

Immobilisation through attachment to solid support materials leads to the stabilisation of the microbial association and also to an efficient uptake of the metals examined. Biofilm development was found to be better on the roughened surfaces, i.e. ground and rippled glass (high surface charge) and on the polystyrene (low surface charge), than on the smooth glass. This result correlated with the results obtained by Baker (1984), who concluded that the microtopography of a surface, or surface roughness, was more important in determining colonisation rates than the electrostatic surface charge. The production of extracellular material was observed to aid in biofilm development and protection of the individual cells in the biofilm (Plates 3.6, 3.7 and 3.8). Vess *et al.* (1993) also reported that the production of extracellular material caused an increase in physical biofilm thickness, with more embedded cells, and enabled the biofilm to persist and remain viable in high heavy metal concentrations.

Polystyrene provides an ideal support as it has a large roughened surface area, is quickly colonised for biofilm formation and is also cheap and readily available, which would make the process more economical. Ground glass was used in subsequent experiments as it provided for extensive biofilm formation in all cases, and is efficient to use on a small scale.

There appeared to be selective accumulation of the various metal ions when the metals were present in combinations. This phenomenon was investigated further and is discussed in Chapters 4 and 5.

Thus, a good biological metal uptake system should consist of an attached microbial population which has previously been exposed to various metals. Also, it should be stable, and have the ability to remove these metals from solution.

CHAPTER 4: QUANTITATIVE COMPARISON OF METAL UPTAKE BY PLANKTONIC AND ATTACHED MICROORGANISMS

4.1 INTRODUCTION

The form of biomass to be used in metal accumulation processes is important (Gadd 1988). For rigorous industrial applications unsupported microbial biomass has many disadvantages: it is generally of small particle size, low mechanical strength, low density (Gadd 1988) and it may cause problems in the operation of reactors by blocking flow lines and clogging filters (De Rome and Gadd 1991). This limits the choice of reactor system and makes biomass/effluent separation difficult (Gadd 1988; De Rome and Gadd 1991). Planktonic or free-living cells offer limited scope for commercial applications. Conversely immobilised cells or microorganisms adhering to surfaces may offer great potential and ease of use (Gadd 1992). Immobilised systems are more tolerant of environmental changes and less sensitive to toxic substances such as heavy metals and are, therefore, more advantageous (Costerton *et al.* 1981).

It has been found that the adhesiveness of microorganisms is altered by heavy metals (Bhattacherjee and Saxena 1983). At low bacterial densities microorganisms grown in the presence of 100 μM zinc showed impaired adherence compared with a control containing no zinc (Bhattacherjee and Saxena 1983). With increasing bacterial density this inhibitory effect was overcome, and at high cell densities zinc-grown organisms exhibited enhanced adherence compared to the control population (Bhattacherjee 1986). Metals can influence the hydrophobic and hydrophilic nature of microbial cell walls, but it should be noted that only animal cells were used in these studies (Bhattacherjee and Saxena 1983; Bhattacherjee 1986) and further research is required to determine the effect of heavy metals on the adherence of microbial cells to plants, rocks and sediments.

The physiology and viability of microorganisms may be strongly affected by adhesion to surfaces, which also offers several advantages (Bar-Or 1990). These include a continuous supply of nutrients flowing over the biofilm, continuous removal of toxic metabolic waste products and higher nutrient levels (Bar-Or 1990). In dilute or hostile environments growth

of microbial cells at surfaces confers advantages on the component cells: localised concentration of nutrients and, in mixed cultures, cooperative mobilisation of nutrients by different organisms within consortia (Gilbert *et al.* 1993). Biofilms promote interactions between cells of the same and different species that would not occur in environments where hydrodynamic forces dominate movement of individual cells (Geesey *et al.* 1992). Immobilised microorganisms perform activities that are in some instances more diverse, efficient and economically important than their free living counterparts (Geesey and White 1990).

Microbial associations were grown as both free-living cultures and as biofilms attached to ground glass surfaces, to determine which system was the more efficient with regard to metal uptake from both single and multi ion solutions.

4.2 MATERIALS AND METHODS

4.2.1 Copper/Cadmium Combinations

Microbial associations adapted to growing in the presence of copper or cadmium were cultured separately at 30°C in 200 ml half-strength Nutrient Broth cultures without any metal(s) added to obtain pregrown inoculum for the model stream and flask cultures.

The beds of the model stream channels were overlaid with ground glass plates (3x5x0.5 cm). Pregrown inoculum was added together with half-strength Nutrient Broth to each of the separate channels as follows:

Channel 1: 100 ml inoculum of Cu^{2+}-adapted microorganisms + 40 ml half-strength Nutrient Broth.

Channel 2: 100 ml inoculum of Cd^{2+}-adapted microorganisms + 40 ml half-strength Nutrient Broth.

Channels 3, 4 and 5: 50 ml inoculum of each of Cu^{2+}- and Cd^{2+}-adapted microorganisms + 40 ml half-strength Nutrient Broth.

The entire model stream was covered with plastic to prevent contamination from the environment, and the biofilms allowed to develop for two weeks at room temperature. During this time extra half-strength Nutrient Broth was added to the channels to prevent drying out of the biofilms.

After two weeks the biofilms were washed with sterile distilled water to remove excess half-strength Nutrient Broth and non-attached microorganisms. Metal ion solutions containing no nutrients were prepared and then added to the channels as follows:

Channel 1: 100 mg l^{-1} Cu^{2+} (pH = 5.34)

Channel 2: 100 mg l^{-1} Cd^{2+} (pH = 6.34)

Channel 3: 100 mg l^{-1} Cu^{2+} + 100 mg l^{-1} Cd^{2+} (pH = 5.49)

Channel 4: 200 mg l^{-1} Cu^{2+} + 50 mg l^{-1} Cd^{2+} (pH = 5.15)

Channel 5: 50 mg l^{-1} Cu^{2+} + 50 mg l^{-1} Cd^{2+} (pH = 5.66)

These metal ion solutions were circulated at a flow rate of 205 ml min^{-1} with a peristaltic pump, and liquid samples (1.5 ml) were taken from the downstream end of each channel at various time intervals after contact with the biofilms (2, 15, 30, 60, 120 minutes and 24 hours) for metal analysis by HPLC (see section 2.2.4.1). Samples of the prepared metal ion solutions were taken before the metal solutions were exposed to the biofilms to determine the initial concentrations of the metals in the solutions.

Before adding the metal ion solution(s), a ground glass plate from each channel was removed and stored in 3% (v/v) glutaraldehyde prior to preparation for SEM to observe biofilm formation. Sample plates were also removed after 24 hours exposure to the metal solutions for SEM and EDX investigations. The EDX spot analysis technique (see section 3.2.3.1) was used to investigate some of the plates removed from the model stream after 24 hours.

Free-living or planktonic cultures were set up concurrently in 100 ml Erlenmeyer flasks as follows:

Flask 1: 20 ml pregrown inoculum of Cu^{2+}-adapted microorganisms + 30 ml half-strength Nutrient Broth.

Flask 2: 20 ml pregrown inoculum of Cd^{2+}-adapted microorganisms + 30 ml half-strength Nutrient Broth.

Flasks 3, 4 and 5: 10 ml pregrown inoculum of each of Cu^{2+}- and Cd^{2+}-adapted microorganisms + 30 ml half-strength Nutrient Broth.

These flask cultures were incubated for two weeks at 30°C on a rotary shaker (Thermolyne Big Bill Rotary Shaker) operating at 150 rpm. The cultures (50 ml) were then centrifuged for 10 minutes at 9000 rpm x g in a BHG Hermle Z-380 centrifuge. The cells were washed by resuspending the pellet in 30 ml water, and then recentrifuging and the resulting pellets were resuspended in 50 ml of the same metal ion solutions as were used in the model stream. Liquid samples (1.5 ml) were taken for HPLC metal analysis (see section 2.2.4.1) at various time intervals (2, 30, 60, 120 minutes and 24 hours) after the final resuspension. Samples of the metal ion solutions were also taken prior to exposure to the free-living cells to determine their initial metal concentrations.

The dry weights of the microorganisms cultured in these free-living flask cultures were determined as follows:

After the initial centrifugation and resuspension of the pellet in 30 ml water, 5 ml were removed from each sample. This 5 ml aliquot was centrifuged (9000 rpm x g for 10 minutes) and the pellet dried on pre-weighed filter paper at 100°C overnight, reweighed and the dry mass of the biomass in the 50 ml culture calculated.

Due to leakage between the channels in the model stream, the experiment was aborted after 24 hours. A repeat experiment was set up to determine the uptake of Cu^{2+} and Cd^{2+} over a longer time period (5 days). The experimental conditions were identical to those described for the first experiment except the pH values of the metal ion solutions differed slightly:

Channel 1: 100 mg l^1 Cu^{2+} (pH = 5.17)

Channel 2: 100 mg l^1 Cd^{2+} (pH = 6.69)

Channel 3: 100 mg l^1 Cu^{2+} + 100 mg l^1 Cd^{2+} (pH = 5.07)

Channel 4: 200 mg l^1 Cu^{2+} + 50 mg l^1 Cd^{2+} (pH = 4.81)

Channel 5: 50 mg l^1 Cu^{2+} + 50 mg l^1 Cd^{2+} (pH = 5.20)

4.2.2 Copper/Lead Combinations

The above experiment was repeated using Cu^{2+}/Pb^{2+} combinations, with mixed microbial populations adapted to grow in Cu^{2+}- and Pb^{2+}-containing media. The flow rates of the metal ion solutions in each channel were on average 210 ml min^{-1}. Liquid samples (1.5 ml) were taken for HPLC metal ion analysis from the metal ion solutions before exposure to the microorganisms and at various times thereafter (2, 15, 30, 60, 120 minutes and 24, 48, 72 and 96 hours and on day 5. Some EDX investigations were also carried out.

The metal ion solutions used in each channel, and in the corresponding free-living flask cultures were as follows:

Channel/Flask 1: 100 mg l^1 Cu^{2+} (pH = 5.30)

Channel/Flask 2: 100 mg l^1 Pb^{2+} (pH = 5.71)

Channel/Flask 3: 100 mg l^1 Cu^{2+} + 100 mg l^1 Pb^{2+} (pH = 5.28)

Channel/Flask 4: 200 mg l^1 Cu^{2+} + 50 mg l^1 Pb^{2+} (pH = 5.06)

Channel/Flask 5: 50 mg l^1 Cu^{2+} + 200 mg l^1 Pb^{2+} (pH =5.13)

In this experiment three of the glass plates were removed from each channel after three days to determine the dry weight of the biofilm in each entire channel. The microbial biofilm was scraped off the glass plates into 5 ml of water and centrifuged. The resulting pellets were dried overnight at 100°C on pre-weighed filter paper, reweighed and the total biofilm biomass calculated. The dry weights of the free-living cultures were determined as described in section 4.2.1.

4.2.3 Cadmium/Lead Combinations

The experiment was repeated using Cd^{2+}/Pb^{2+} combinations with microbial associations capable of growing in the presence of Cd^{2+} and Pb^{2+}. The flow rates of the metal ion solutions in the channels were on average 175 ml min^{-1}.

The metal ion solutions used in the various channels and in the corresponding free-living flask cultures were as follows:

Channel/Flask 1: 100 mg l^{-1} Cd^{2+} (pH = 6.34)

Channel/Flask 2: 100 mg l^{-1} Pb^{2+} (pH = 5.73)

Channel/Flask 3: 100 mg l^{-1} Cd^{2+} + 100 mg l^{-1} Pb^{2+} (pH = 5.76)

Channel/Flask 4: 200 mg l^{-1} Cd^{2+} + 50 mg l^{-1} Pb^{2+} (pH = 5.89)

Channel/Flask 5: 50 mg l^{-1} Cd^{2+} + 200 mg l^{-1} Pb^{2+} (pH = 5.68)

In this experiment four plates were removed from each channel after two days of metal-microorganism contact to determine the dry weight of the biofilm, using the same procedure as described in section 4.2.2. Liquid samples (1.5 ml) were taken for metal ion analysis by HPLC as described above.

The EDX digital mapping technique (see section 3.2.3.2) was used to investigate sample glass plates from this experiment, to determine whether or not the metals were being adsorbed by the microbial biofilm.

4.3 RESULTS AND DISCUSSION

4.3.1 Copper/Cadmium Combinations

Before adding the metal ion solutions, good biofilm development had occurred in the various channels of the model stream (Plate 4.1).

Plate 4.1 Biofilm comprising various metal-adapted microbial associations attached to ground glass; A. clusters of Cu^{2+}-adapted microorganisms; B. large clusters of Cd^{2+}-adapted microorganisms covering most of the surface; clumped patches of microbial growth (C) and a continuous biofilm layer (D) formed by mixtures of Cu^{2+}- and Cd^{2+}-adapted microorganisms.
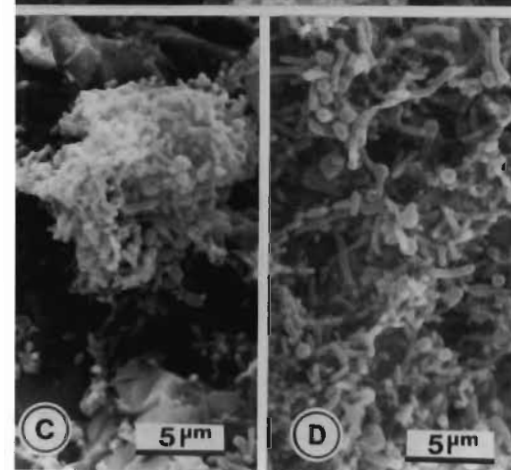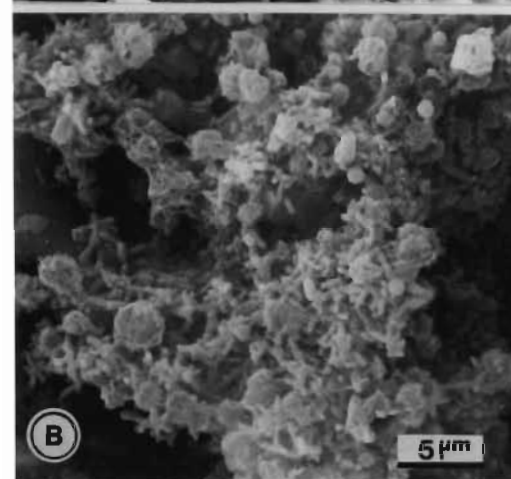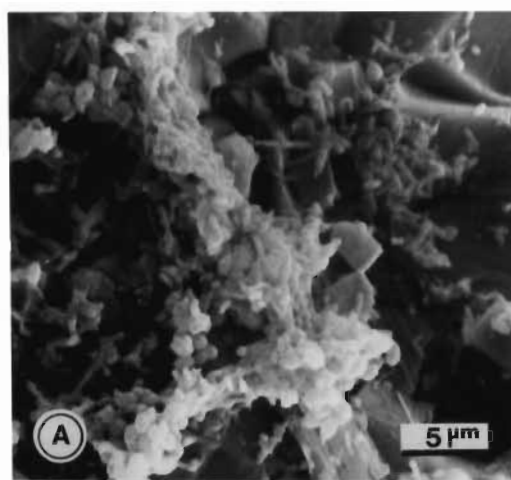
Plate 4.1A shows the clusters of Cu^{2+}-adapted microorganisms present on the ground glass surface taken from channel 1. The microorganisms previously exposed to Cd^{2+} developed in large clusters covering most of the ground glass surface (Plate 4.1B). When a combination of Cu^{2+}- and Cd^{2+}-adapted microorganisms were cultured together in a 1:1 ratio on the ground glass, clumped patches of microbial growth were seen in some parts (Plate 4.1C) while a continuous biofilm layer developed in others (Plate 4.1D).

Figure 4.1 shows the results obtained for the free-living flask cultures and for the first model stream experiment which was terminated after 24 hours due to leakage. Initial uptake of Cu^{2+} by the microbial biofilm exposed to 100 mg l^{-1} of the metal ion was good but decreased over time, probably due to inter-channel leakage, while the free-living cultures showed an initial Cu^{2+} uptake of 20% which appeared to be their equilibrium point, possibly due to saturation of the biomass (Fig. 4.1A). The biofilm in the channel exposed to 100 mg l^{-1} Cd^{2+} showed a good initial uptake of Cd^{2+} of 35%, with a subsequent increase in the Cd^{2+} concentration in the medium, due to leakage from adjacent channels. The free-living flask cultures once again showed an initial uptake and subsequent establishment of an equilibrium (Fig. 4.1B). The cells were either saturated or did not take up the Cd^{2+} ions to any great degree.

The biofilm in channel three and the free-living cells in flask three were exposed to a mixture of Cu^{2+} and Cd^{2+} ions, both at 100 mg l^{-1}. The free-living culture did not appear to take up Cd^{2+} or Cu^{2+} to any great extent, while the attached microbial biofilm took up Cd^{2+} and Cu^{2+} initially, with a greater amount of Cu^{2+} than Cd^{2+} being removed with time. Interchannel leakage problems could, however, have been responsible (Fig. 4.1C). Figure 4.1D shows the results of the microbial population exposed to 200 mgl^{-1} Cu^{2+} and 50 mg l^{-1} Cd^{2+}. The free-living cells showed little uptake of Cu^{2+} ions and no uptake of Cd^{2+} ions. The concentration of Cd^{2+} in solution actually increased, possibly due to errors made during sample preparation or in the HPLC analysis. Whatever the reason, the results show that Cd^{2+} ions were not adsorbed onto these cells. Attached cells showed an initial uptake of both metals with the concentration of Cu^{2+} remaining in solution decreasing while that of Cd^{2+} increased during the later stages of the experiment. Again the increase in Cd^{2+} was probably caused by leakage from adjacent channels. The biofilm

exposed to 50 mg l^{-1} Cu^{2+} and 200 mg l^{-1} Cd^{2+} showed greater uptake of Cu^{2+} initially; the subsequent increase in Cu^{2+} concentration in the medium being due to the leakage problems. The free-living microorganisms showed an initial uptake, particularly of Cu^{2+} ions, before again reaching equilibrium (Fig. 4.1E).

Figure 4.1   Graphs of % metal remaining in solution in Cu^{2+}/Cd^{2+} combinations after uptake by attached (model stream, experiment 1) and free-living microorganisms; A. Channel/flask 1, biomass exposed to 100 mg l^1 Cu^{2+}; B. Channel/flask 2, biomass exposed to 100 mg l^1 Cd^{2+}; C. Channel/flask 3, biomass exposed to 100 mg l^1 of both Cu^{2+} and Cd^{2+}; D. Channel/flask 4, biomass exposed to 200 mg l^1 Cu^{2+} and 50 mg l^1 Cd^{2+}; E. Channel/flask 5, biomass exposed to 50 mg l^1 Cu^{2+} and 200 mg l^1 Cd^{2+}.
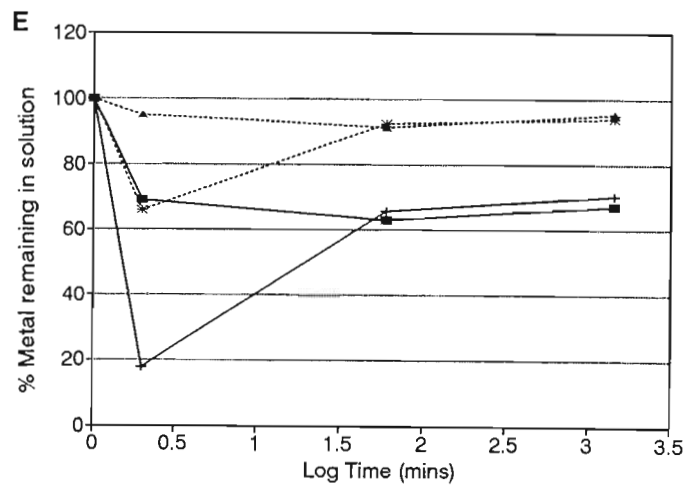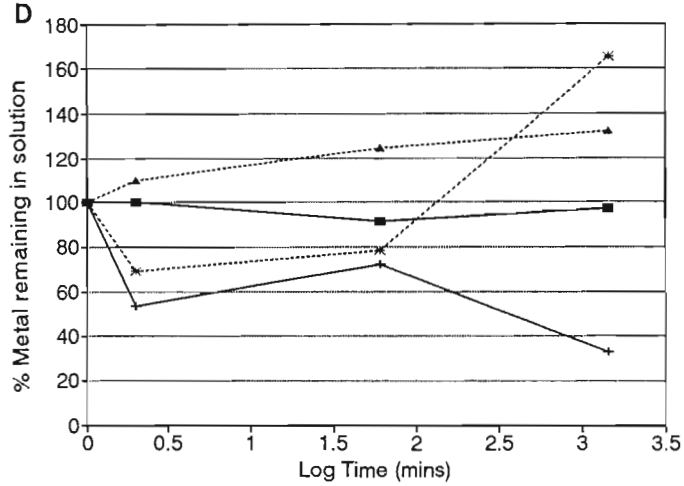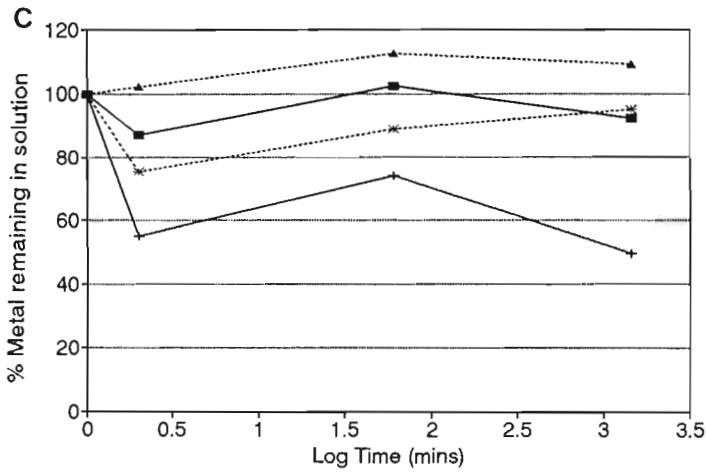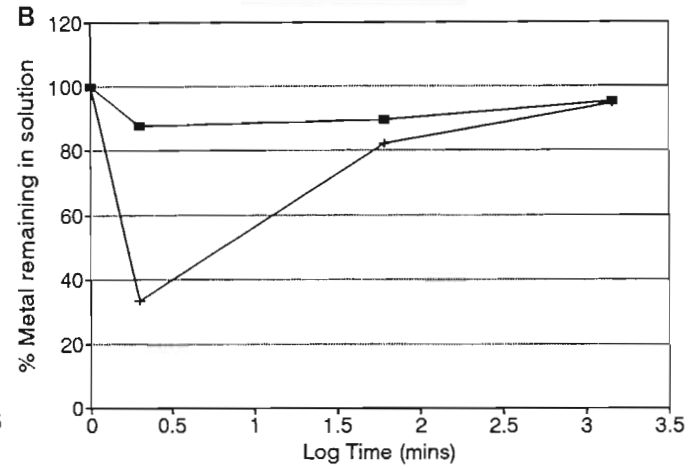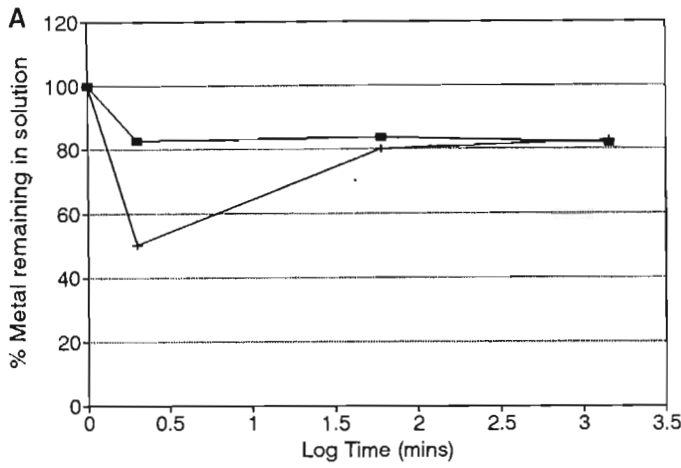
In general, metal uptake by the attached microbial biofilm was better than that by the free-living cells. This could have been due to the greater biomass found in the microbial biofilm as well as the increased stability of the attached biofilm compared to the free-living, suspended cells in the flask cultures. It has been reported that an attached or epilithic bacterial community displayed a greater degree of adaptation to metal stress than did the corresponding free-living cultures (Dean-Ross and Mills 1989). These microorganisms remain in contact with the pollutant and respond to its presence by increasing the proportion of resistant members in the population (Dean-Ross 1990). Planktonic microorganisms showed the lowest adaptation to heavy metals as there was little contact between the free-living bacteria and dissolved heavy metals (Dean-Ross and Mills 1989).

Kirchman and Mitchell (1982) observed that in certain aquatic ecosystems, surface-associated bacteria were metabolically more active per cell than unattached bacteria, as solid-liquid interfaces provide an enriched nutrient status for attached microorganisms (Fletcher 1985). It has also been shown that biofilm-associated microorganisms have increased resistance to various industrial biocides compared to planktonic bacteria of the same species (Vess et $al.$ 1993). It should also be noted that Cu^{2+} ions were taken up more readily than the Cd^{2+} ions from the multi-ion solution.

The results of the second model stream experiment are shown in Fig. 4.2. There appeared to be little uptake of either Cu^{2+} or Cd^{2+} in most of the channels, except in channel 5, where the concentration of Cu^{2+} decreased by 35% over the time period. Biofilm development was not good on this occasion, perhaps due to a lower ambient temperature than in the first Cu^{2+}/Cd^{2+} model stream experiment, and evaporation problems which led to an increase in the concentration of the metal ions in the medium, particularly as the exposure time increased. It has been reported that microorganisms show specific trends in their adsorption/desorption rate and that if microorganisms are kept in contact with metal solutions beyond an optimum time, the rate of desorption may exceed the rate of adsorption (Pradhan and Levine 1992b). This may have occurred in this experiment and been responsible for the apparent increase in the Cu^{2+} and Cd^{2+} concentrations observed on day 5 (Fig. 4.2).

Figure 4.2 Graphs of % metal remaining in solution in Cu^{2+}/Cd^{2+} combinations (model stream, experiment 2) after uptake by attached microorganisms; A. Channel 1, biomass exposed to 100 mg l^{-1} Cu^{2+}; B. Channel 2, biomass exposed to 100 mg l^{-1} Cd^{2+}; C. Channel 3, biomass exposed to 100 mg l^{-1} of both Cu^{2+} and Cd^{2+}; D. Channel 4, biomass exposed to 200 mg l^{-1} Cu^{2+} and 50 mg l^{-1} Cd^{2+}; E. Channel 5, biomass exposed to 50 mg l^{-1} Cu^{2+} and 200 mg l^{-1} Cd^{2+}.
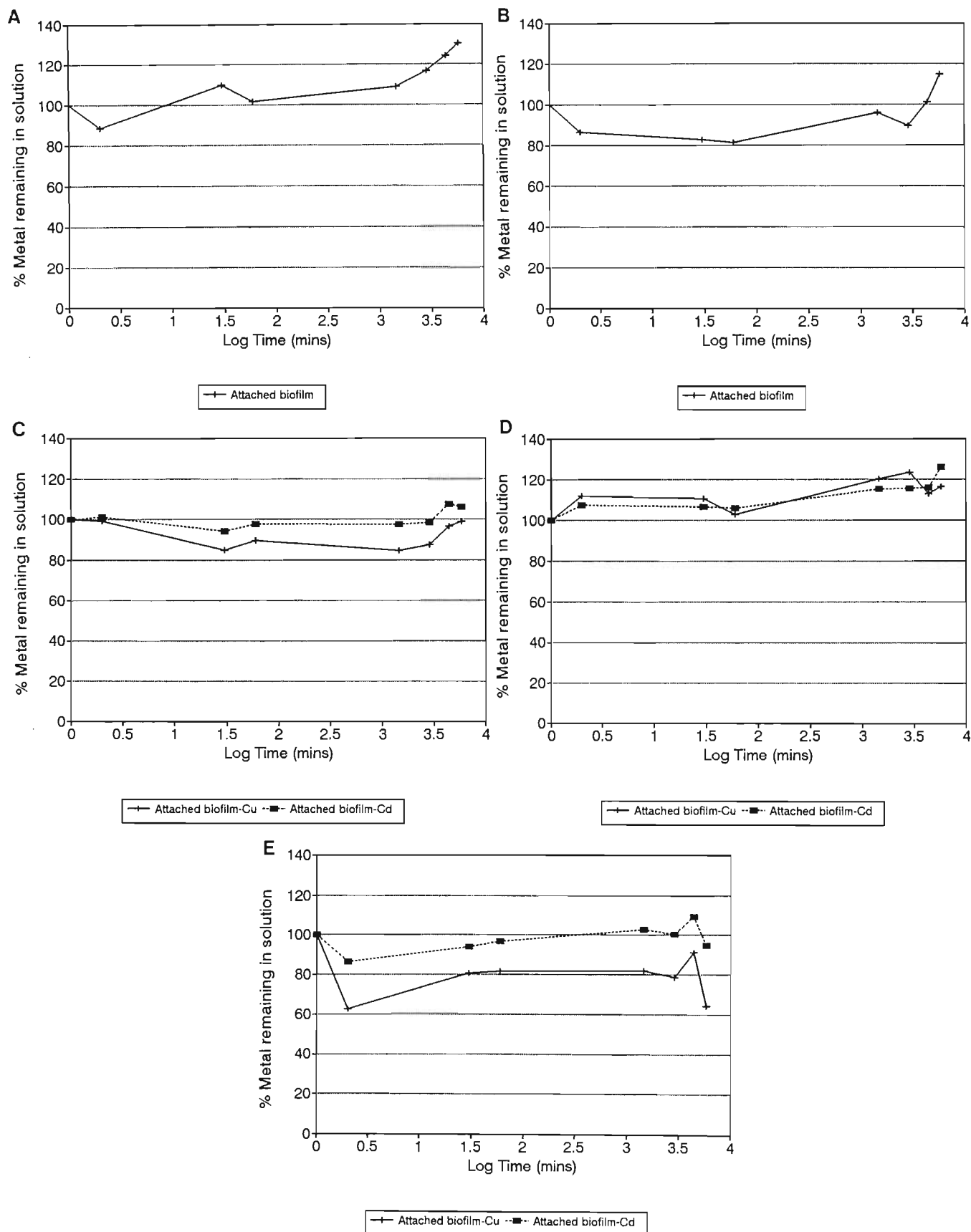
Table 4.1   Dry weight determinations of free-living microorganisms in flask cultures.

Culture	Dry weight (mg) of cells in 50 ml free-living flask cultures
Cu^{2+} 100 (1)	11.3
Cd^{2+} 100 (2)	48.8
Cu^{2+} 100 Cd^{2+} 100 (3)	21.4
Cu^{2+} 200 Cd^{2+} 50 (4)	10.1
Cu^{2+} 50 Cd^{2+} 200 (5)	12.3

Numbers in parentheses indicate the different flask cultures.

The dry weights of the free-living microbial populations in the cultures were quite low, possibly due to nutrient limitation as only a small volume of half-strength Nutrient Broth was used (Table 4.1).

The spot analysis technique (see section 3.2.3.1) was used to investigate the uptake of Cu^{2+} and Cd^{2+} by the attached microorganisms. In channel 1 where only Cu^{2+} was added, at an initial concentration of 100 mg l^{-1}, the bacterium selected for spot analysis showed a very small uptake of copper (Fig. 4.3A) after 24 hours, possibly due to the small size of this bacterium (Fig. 4.3C). If spot analysis of other cells had been done the results might have been different as HPLC analysis of the supernatant from this channel showed a decrease of approximately 50% in the copper concentration after 24 hours (Fig. 4.1A).
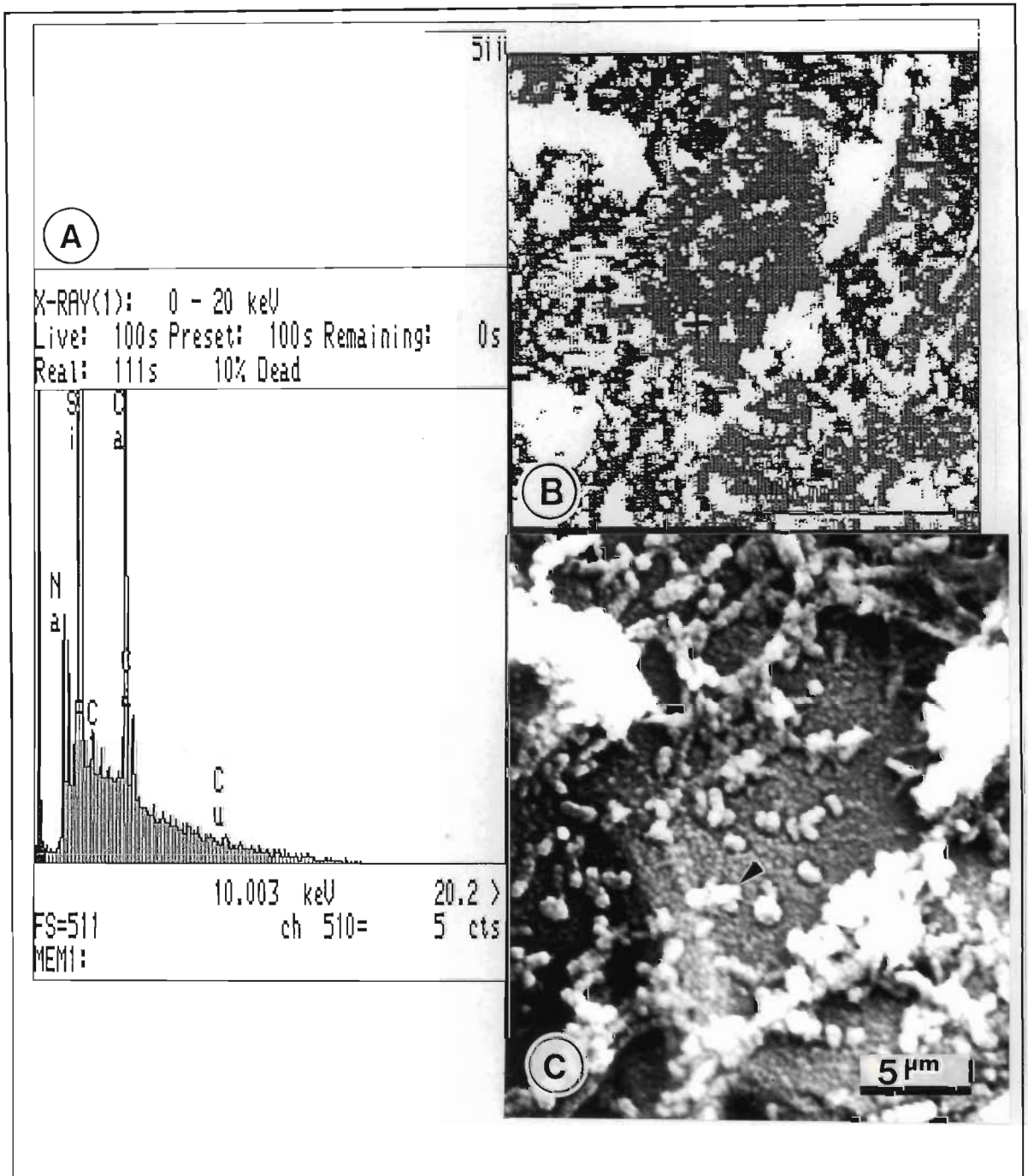
Figure 4.3   Spot analysis (A) of an individual bacterium (indicated by the + symbol in B) in the biofilm (C, bacterium indicated by the arrow) exposed for 24 hours to 100 mg l^1 Cu^{2+} in channel 1.

In channel 2 only 100 mg l^1 Cd^{2+} were initially added. However, from the EDX result (Fig. 4.4A) both cadmium and copper were detected on the large cell (indicated by the arrow in Fig. 4.4C), possibly a yeast, which was also associated with smaller bacterial

cells (Fig. 4.4C). This was due to interchannel leakage. Analysis of the supernatant by HPLC showed that the Cd^{2+} concentration did decrease initially but subsequently increased (Fig. 4.1B) due to the leakage. Copper was also present in the supernatant at this stage at a concentration of 41 mg l^{-1} (result not shown).
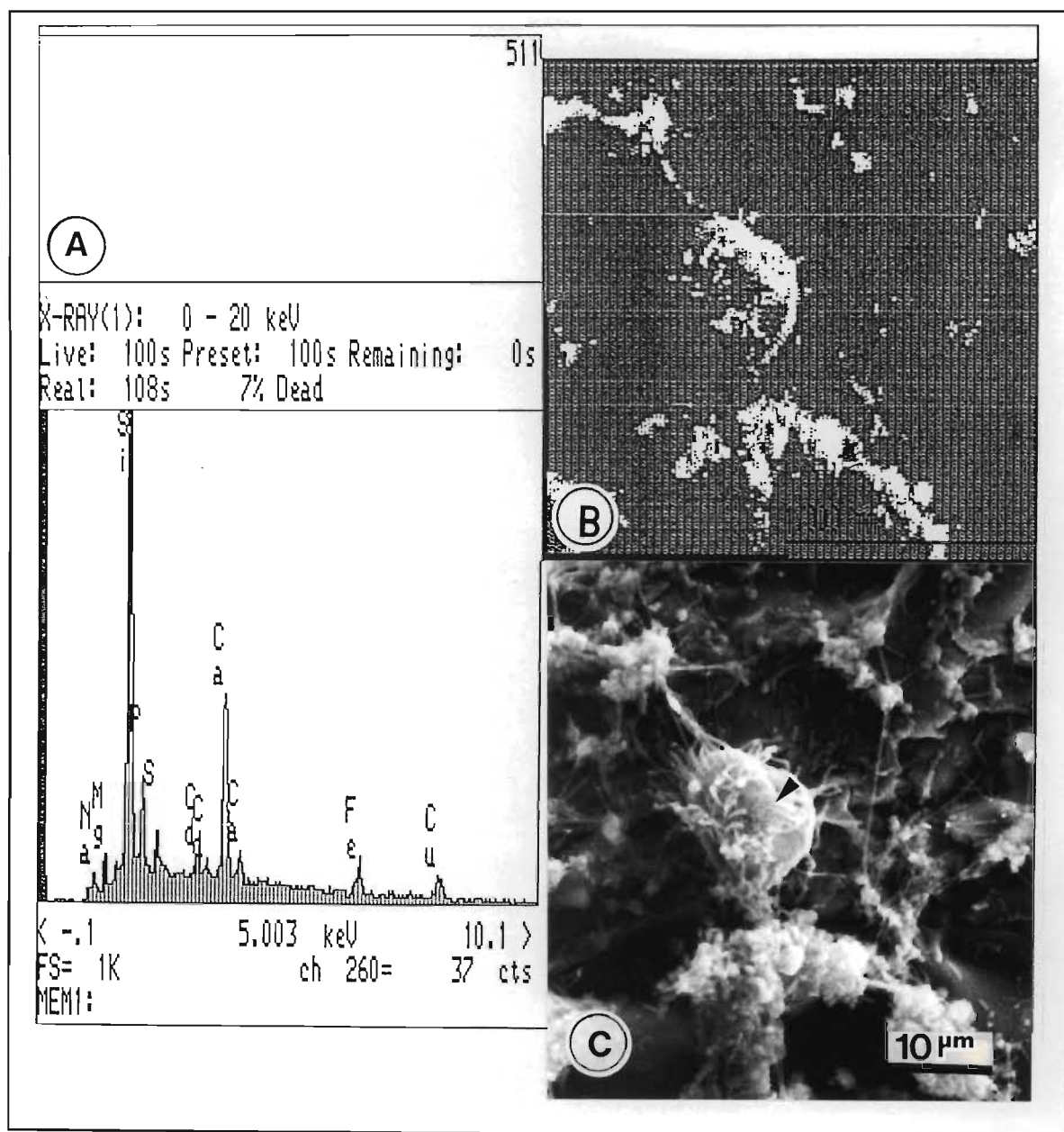
Figure 4.4   Spot analysis (A) of an individual cell (indicated by the + symbol in B) in the biofilm (C, cell indicated by the arrow) exposed to 100 mg l^{-1} Cd^{2+} in channel 2.

Cu^{2+} and Cd^{2+} were added to channel 3 at an initial concentration of 100 mg l^{-1} each. A site on a cluster of bacterial cells (indicated by the + symbol in Fig. 4.5B and the arrow

in Fig. 4.5C) was chosen for spot analysis and copper but no cadmium was detected (Fig. 4.5A). This result correlates with the HPLC analysis of the associated supernatant which showed that there was a decrease of 50% in the concentration of Cu^{2+} in the supernatant, while the Cd^{2+} concentration in the supernatant showed only a small initial decrease followed by a subsequent increase, due to the leakage problem (Fig. 4.1C).
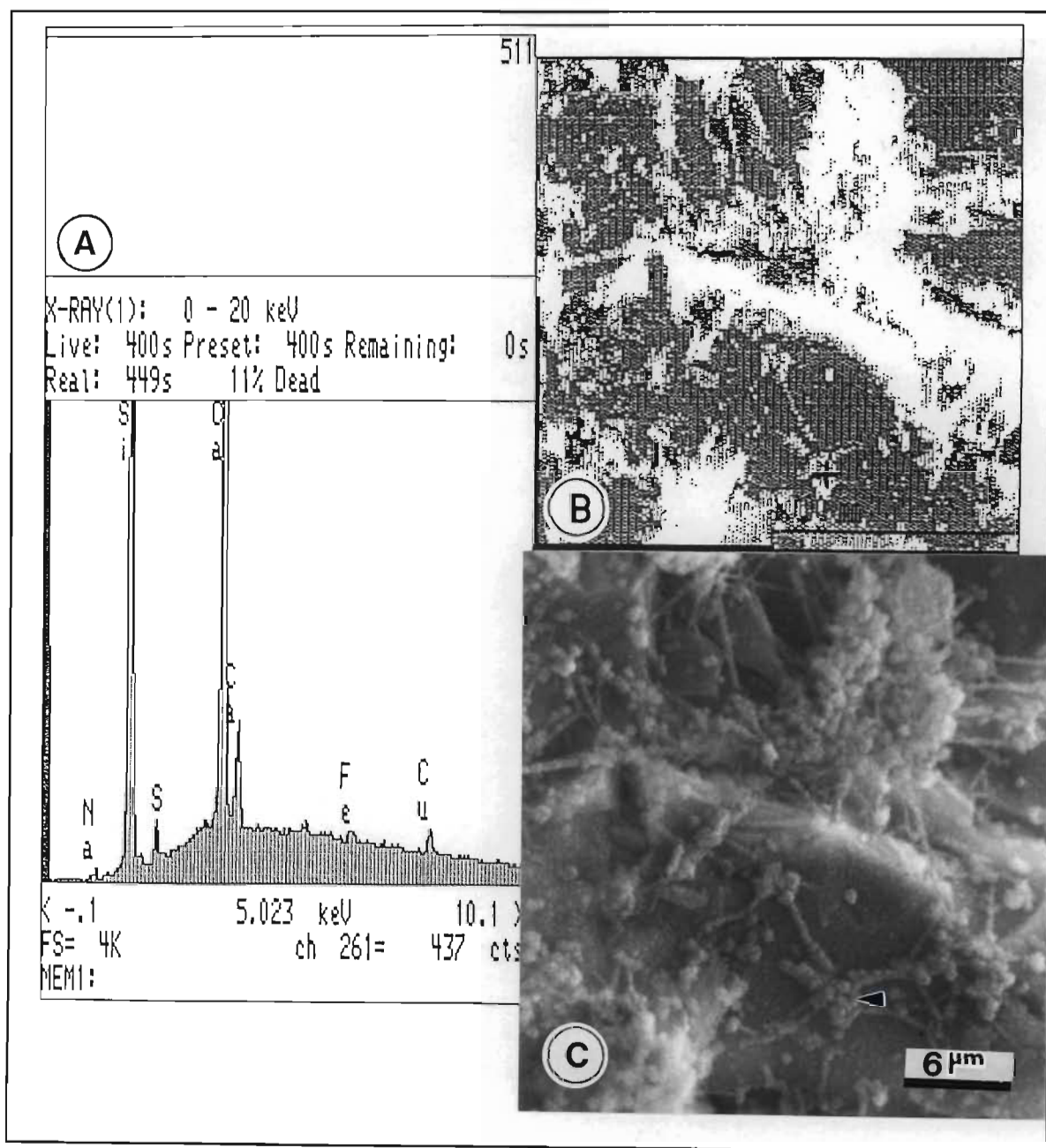
Figure 4.5   Spot analysis (A) of a cluster of bacterial cells (indicated by the + symbol in B) in the biofilm (C, cluster of cells indicated by the arrow) exposed for 24 hours to 100 mg l^{-1} of both Cd^{2+} and Cu^{2+} in channel 3.

To channel four 200 mg l^{-1} Cu^{2+} and 50 mg l^{-1} Cd^{2+} were added initially. A small cluster of bacterial cells (indicated by the symbol + in Fig. 4.6B and the arrow in Fig 4.6C) was selected for EDX spot analysis after 48 hours exposure to the metal ions, and only copper was detected on/in these cells (Fig. 4.6A). The associated supernatant samples were analyzed using HPLC and the results correlated with this spot analysis result in that the Cu^{2+} concentration of the supernatant had decreased after 24 hours while that of Cd^{2+} had increased indicating that little, if any, Cd^{2+} had been taken up by the biofilm (Fig. 4.1D).
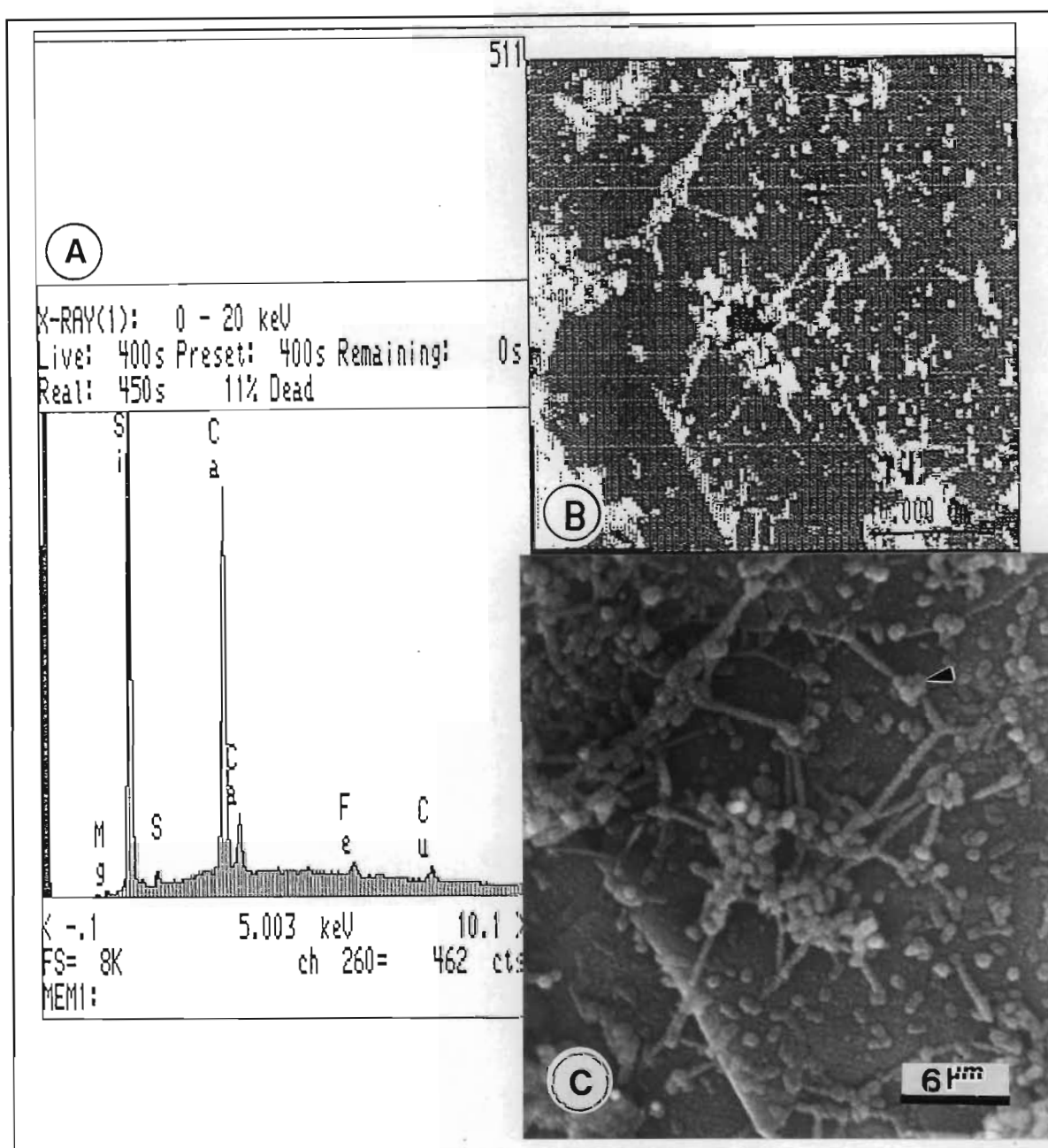
Figure 4.6   Spot analysis (A) of a cluster of bacteria (indicated by the + symbol in B) in the biofilm (C, cluster of bacteria indicated by the arrow) exposed for 24 hours to 200 mg l^{-1} Cu^{2+} and 50 mg l^{-1} Cd^{2+} in channel 4.

Cu^{2+} (50 mg l^{-1}) and Cd^{2+} (200 mg l^{-1}) were added initially to channel 5. Spot analysis of a cluster of cells (indicated by the + symbol in Fig. 4.7B and the arrow in Fig. 4.7C) showed the presence of both copper and cadmium in small amounts (Fig. 4.7A). A second spot analysis conducted on a strand of filamentous growth showed only the presence of copper (result not shown); perhaps the area selected for examination was too small. The

associated supernatant was analyzed using HPLC and the results showed an initial decrease in both the Cu^{2+} and Cd^{2+} concentrations followed by gradual increases possibly due to the leakage problems. Higher concentrations of Cu^{2+} than Cd^{2+} were detected (Fig. 4.1E).
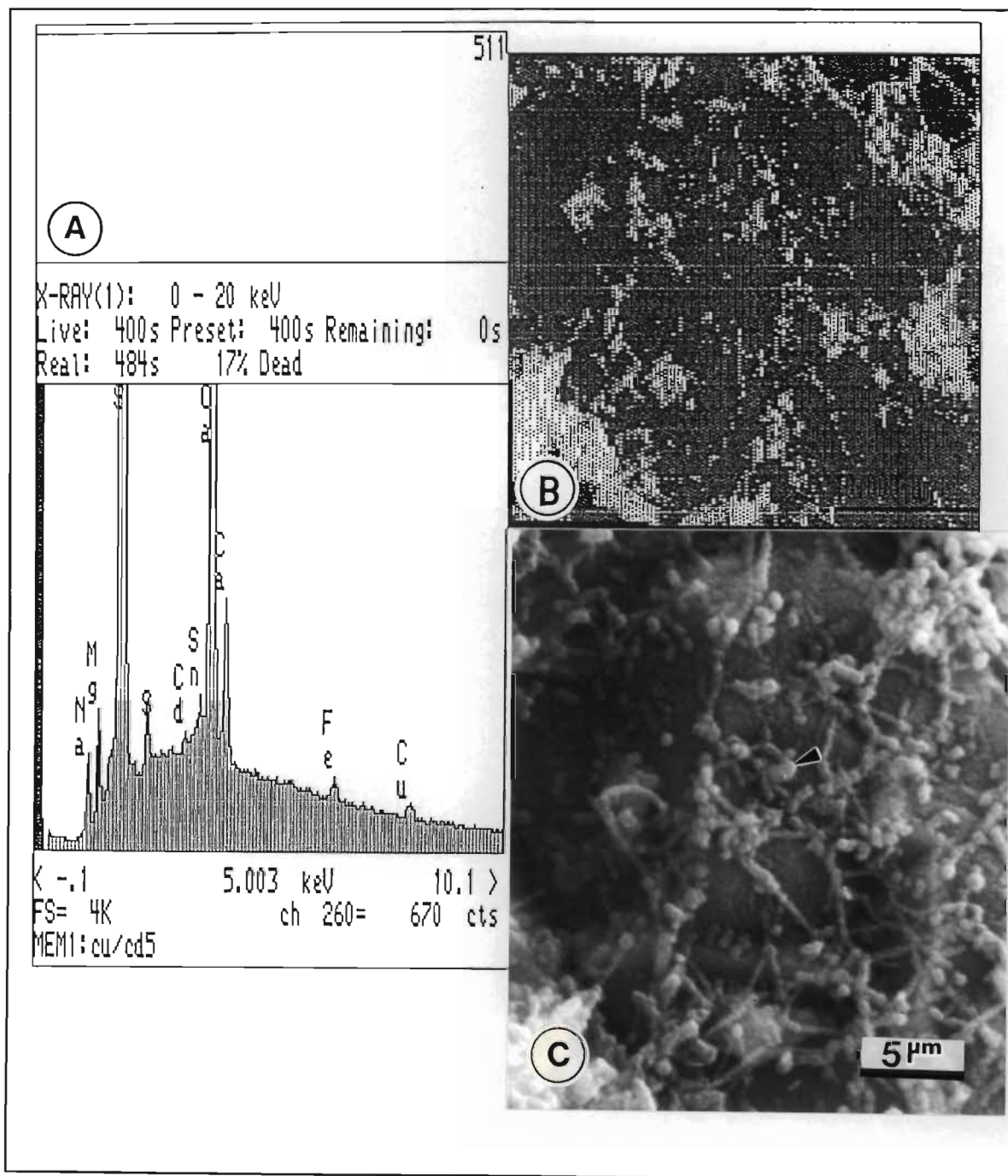
Figure 4.7 Spot analysis (A) of a cluster of bacteria (indicated by the + symbol in B) in the biofilm (C, cluster of bacteria indicated by the arrow) exposed for 24 hours to 50 mg l^{-1} Cu^{2+} and 200 mg l^{-1} Cd^{2+} in channel 5.

The EDX spot analysis technique is useful when investigating the presence of a specific metal in an area on a biofilm. The results of the HPLC analysis correlated with those obtained by the EDX spot analysis technique indicating that these techniques could be used in conjunction with one another to investigate a microbial metal uptake system.

4.3.2 Copper/Lead Combinations

Figure 4.8 shows the graphs indicating the uptake of Cu^{2+} and Pb^{2+} by the microbial associations, comprising both the free-living (flask culture experiment) and the ground glass attached microbial biofilm (model stream) populations. The free-living microorganisms exposed to 100 mg l^{-1} Cu^{2+} initially showed a fairly rapid uptake of Cu^{2+} (Fig. 4.8A), which was followed by a decrease and a subsequent final increase in uptake. The same pattern was shown by the attached cells in the model stream except that in this case the concentration of Cu^{2+} in solution increased again after 48 hours, probably due to evaporation from the system leading to concentration of the Cu^{2+} remaining in solution. In the cultures exposed to 100 mg l^{-1} Pb^{2+}, uptake of the metal was good with an initial rapid uptake which, although it slowed down, continued steadily (Fig. 4.8B). In the channel exposed to 100 mg l^{-1} of both Cu^{2+} and Pb^{2+}, uptake of Cu^{2+} was limited while that of Pb^{2+} by the attached cells was good (Fig. 4.8C). The free-living cells did not significantly take up either metal. Pb^{2+} at a concentration of 50 mg l^{-1} was completely removed by both the free-living and attached populations (Fig. 4.8D), while the uptake of Cu^{2+} was limited. The free-living cells appeared to take up no copper at all and the Cu^{2+} concentration remaining in solution increased possibly due to evaporation of the liquid from the flask. The populations exposed to 50 mg l^{-1} Cu^{2+} and 200 mg l^{-1} Pb^{2+} showed little uptake of Cu^{2+} or Pb^{2+} (Fig. 4.8E) with the free-living cultures taking up less metal than the attached cultures. It must be noted that when samples were analyzed for Pb^{2+} the concentrations were very low, due to the precipitation problems encountered.

Figure 4.8   Graphs of % metal remaining in solution in Cu^{2+}/Pb^{2+} combination after uptake by attached and free-living microorganisms; A. Channel/flask 1, biomass exposed to 100 mg l^{-1} Cu^{2+}; B. Channel/flask 2, biomass exposed to 100 mg l^{-1} Pb^{2+}; C. Channel/flask 3, biomass exposed to 100 mg l^{-1} of both Cu^{2+} and Pb^{2+}; D. Channel/flask 4, biomass exposed to 200 mg l^{-1} Cu^{2+} and 50 mg l^{-1} Pb^{2+}; E. Channel/flask 5, biomass exposed to 50 mg l^{-1} Cu^{2+} and 200 mg l^{-1} Pb^{2+}.
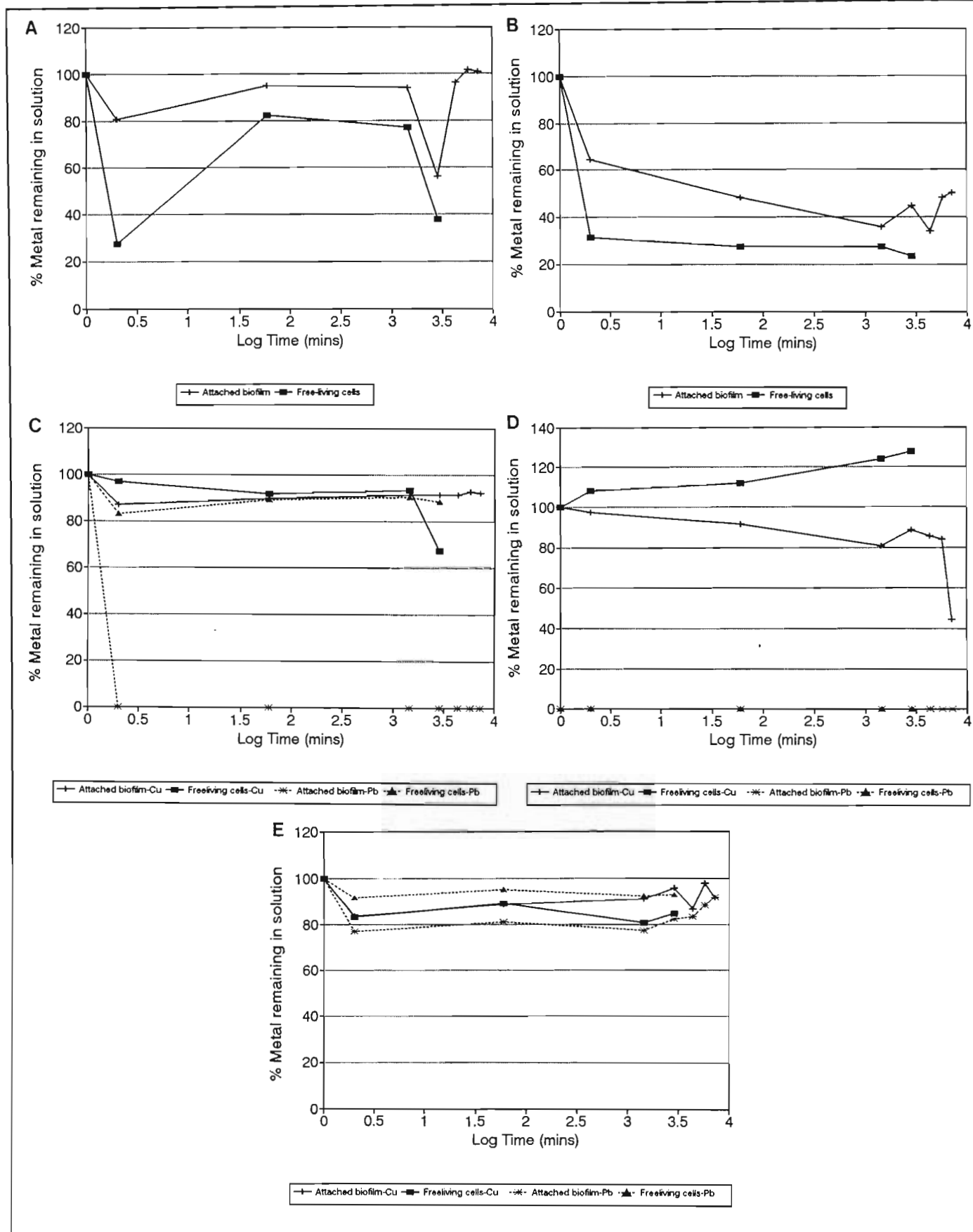
Table 4.2   Approximate dry weights of microorganisms comprising the free-living (flask cultures) and attached (model stream) microbial populations.

Culture	Dry weight (mg) of free-living cells in 50 ml flask cultures	Dry weight (mg) of biofilm in each entire stream channel
Cu^{2+} 100 (1)	35.5	39.1
Pb^{2+} 100 (2)	72.4	133.2
Cu^{2+} 100 Pb^{2+} 100 (3)	31.7	52.4
Cu^{2+} 200 Pb^{2+} 50 (4)	48.2	89.4
Cu^{2+} 50 Pb^{2+} 200 (5)	31.4	31.1

Numbers in parentheses indicate the different channels and flasks.

In general, the dry weights of the attached biofilms in the model stream were greater than the corresponding free-living populations, possibly explaining the greater capacity of the attached biofilms for metal uptake (Table 4.2).

Samples of ground glass plates were prepared for EDX analysis. Figure 4.9 depicts the EDX spectrum of the biofilm which developed on a ground glass plate in the Pb^{2+}/Cu^{2+}-containing channel (channel 3). It appeared that there was more lead than copper present, which correlates with data shown in Fig. 4.8C, which indicated little Cu^{2+} uptake had occurred. Lead has been shown to be preferentially taken up by a *Chlorella fusca* species (Wehrheim and Wettern 1994b) and an actinomycete system was also shown to be more effective in binding lead than copper, with the extracellular material playing a more important role in lead uptake than the cellular component of this system (Pradhan and Levine 1992a). It appeared that lead was also preferentially taken up over copper by the attached microbial biofilm in this experiment.
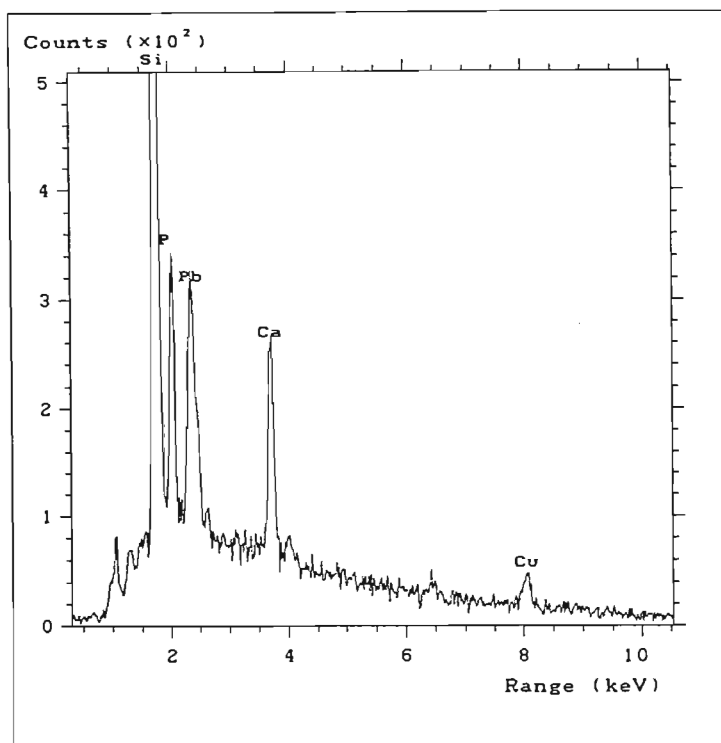
Figure 4.9   EDX spectrum of biofilm on ground glass surface exposed for 5 days to 100 mg l^{-1} of both Pb^{2+} and Cu^{2+}.

4.3.3 Cadmium/Lead Combinations

Uptake of metals by the microorganisms exposed to Cd^{2+}/Pb^{2+} combinations is shown in Fig. 4.10. Microorganisms exposed to 100 mg l^{-1} Cd^{2+} as both free-living and attached populations displayed little Cd^{2+} uptake (Fig. 4.10A). The attached cells were, however, more active in metal uptake than the corresponding free-living cells in the flask cultures. Pb^{2+} was efficiently taken up by both the attached and free-living cells, with the attached cultures once again displaying a better uptake of the metal than the free-living cells (Fig. 4.10B). When exposed to equal concentrations of Cd^{2+} and Pb^{2+} the uptake of the two metals varied. Pb^{2+} uptake by the biofilm in the model stream was especially efficient while Cd^{2+} uptake by the attached cells was inefficient (Fig. 4.10C). Cd^{2+} ions were not actively removed by the free-living cells, while Pb^{2+} ions were only removed to a certain extent (Fig. 4.10C). The same trend was observed in the presence of 200 mg l^{-1} Cd^{2+} and 50 mg l^{-1} Pb^{2+}, with the free-living cells once again showing a generally lower level of

Figure 4.10 Graphs of % metal remaining in solution in Cd^{2+}/Pb^{2+} combinations after uptake by attached and free-living microorganisms; A. Channel/flask 1, biomass exposed to 100 mg l^{-1} Cd^{2+}; B. Channel/flask 2, biomass exposed to 100 mg l^{-1} Pb^{2+}; C. Channel/flask 3, biomass exposed to 100 mg l^{-1} of both Cd^{2+} and Pb^{2+}; D. Channel/flask 4, biomass exposed to 200 mg l^{-1} Cd^{2+} and 50 mg l^{-1} Pb^{2+}; E. Channel/flask 5, biomass exposed to 50 mg l^{-1} Cd^{2+} and 200 mg l^{-1} Pb^{2+}.
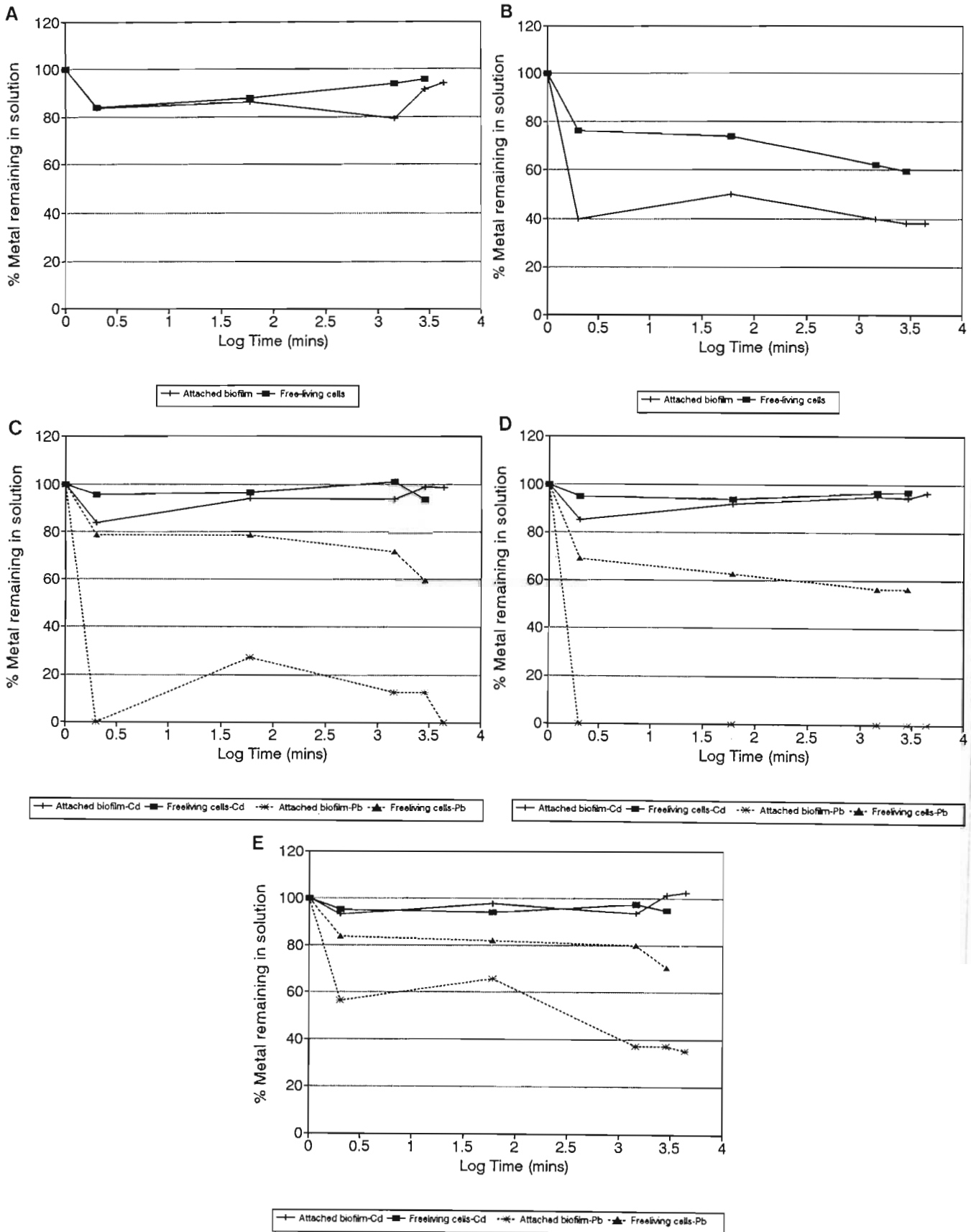
metal uptake than the attached population (Fig. 4.10D). The Pb^{2+} ions were rapidly and completely removed from solution by the attached population (Fig. 4.10D). The uptake of Cd^{2+} ions at 50 mg l^{-1} and Pb^{2+} at 200 mg l^{-1} was limited, except for the uptake of Pb^{2+} ions by the attached biofilm (Fig. 4.10E). In these cultures the general trend was once again towards better uptake of metals by the attached populations found in the biofilms on the ground glass surface than by the free-living flask cultures. Lead was also preferentially taken up over cadmium in this experiment.

Table 4.3   Approximate dry weights of microorganisms comprising the free-living (flask cultures) and attached (model stream) microbial populations.

Culture	Dry weight (mg) of free-living cells in 50 ml flask cultures	Dry weight (mg) of biofilm in each entire stream channel
Cd^{2+} 100 (1)	50.5	21.2
Pb^{2+} 100 (2)	32.7	35.4
Cd^{2+} 100 Pb^{2+} 100 (3)	19.0	27.8
Cd^{2+} 200 Pb^{2+} 50 (4)	6.2	49.6
Cd^{2+} 50 Pb^{2+} 200 (5)	25.5	57.8

Numbers in parentheses indicate the different channels and flasks.

In general, the biofilms had a greater biomass dry weight than the free-living flask populations, possibly explaining the advantage of using immobilised systems, as the greater surface area allowed for the establishment of a greater population for metal uptake (Table 4.3).

Some of the biofilms on the sample ground glass plates were investigated using the EDX technique to detect the presence of cadmium and lead. A plate from channel 3 exposed

to a medium containing 100 mg l^{-1} of both Cd^{2+} and Pb^{2+} showed the presence of both metals in the EDX spectrum (Fig. 4.11). Analysis of the associated supernatant by HPLC showed only a small decrease in the Cd^{2+} concentration, with a larger decrease in the Pb^{2+} concentration (Fig. 4.10C).
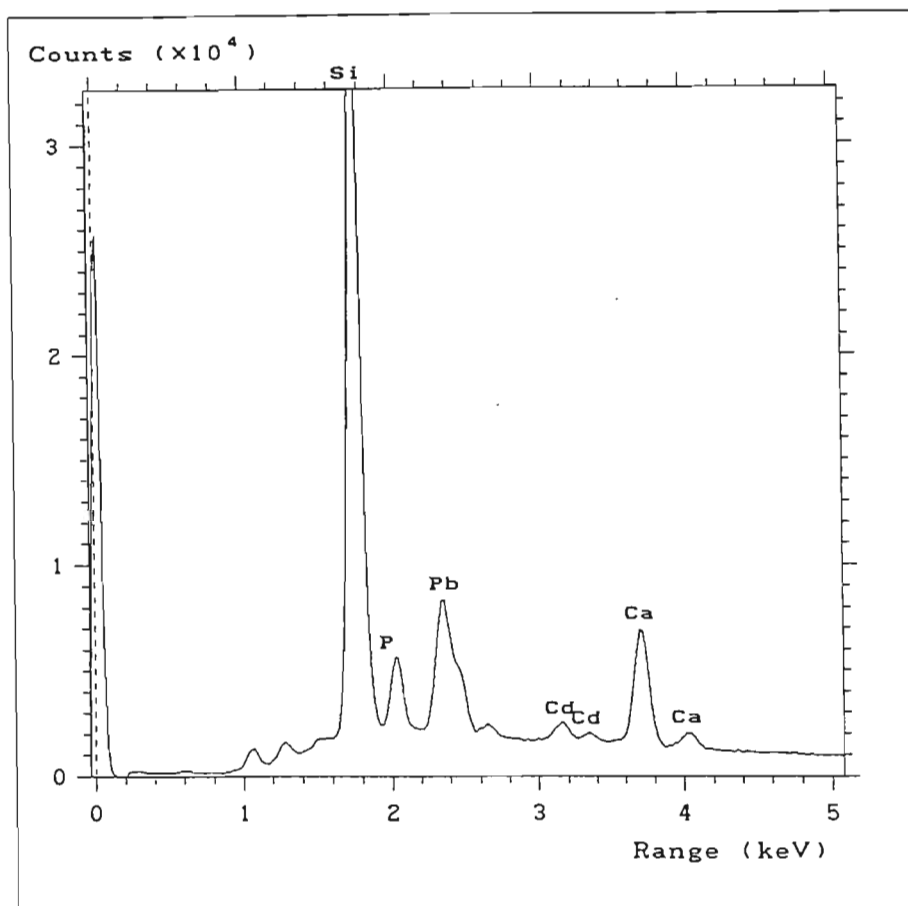
Figure 4.11 EDX spectrum of biofilm exposed for 96 hours to equal concentrations of Cd^{2+} (100 mg l^{-1}) and Pb^{2+} (100 mg l^{-1}).

In channel five a metal solution containing 50 mg l^{-1} Cd^{2+} and 200 mg l^{-1} Pb^{2+} was added initially. Lead was again detected within the area selected for EDX digital mapping. By increasing the number of frames from 10 (Fig. 4.12A) to 30 (Fig. 4.12B), and keeping the dwell time at 2 milliseconds, considerable improvements in the clarity of the map depicting the distribution of the lead, and of the secondary image were obtained. It can be clearly seen that the lead was concentrated in the area of the microbial mass in the centre of the

picture (Fig. 4.12C). Results of the supernatant analysis by HPLC again correlated, showing little Cd^{2+} uptake but good Pb^{2+} uptake (Fig. 4.10E).
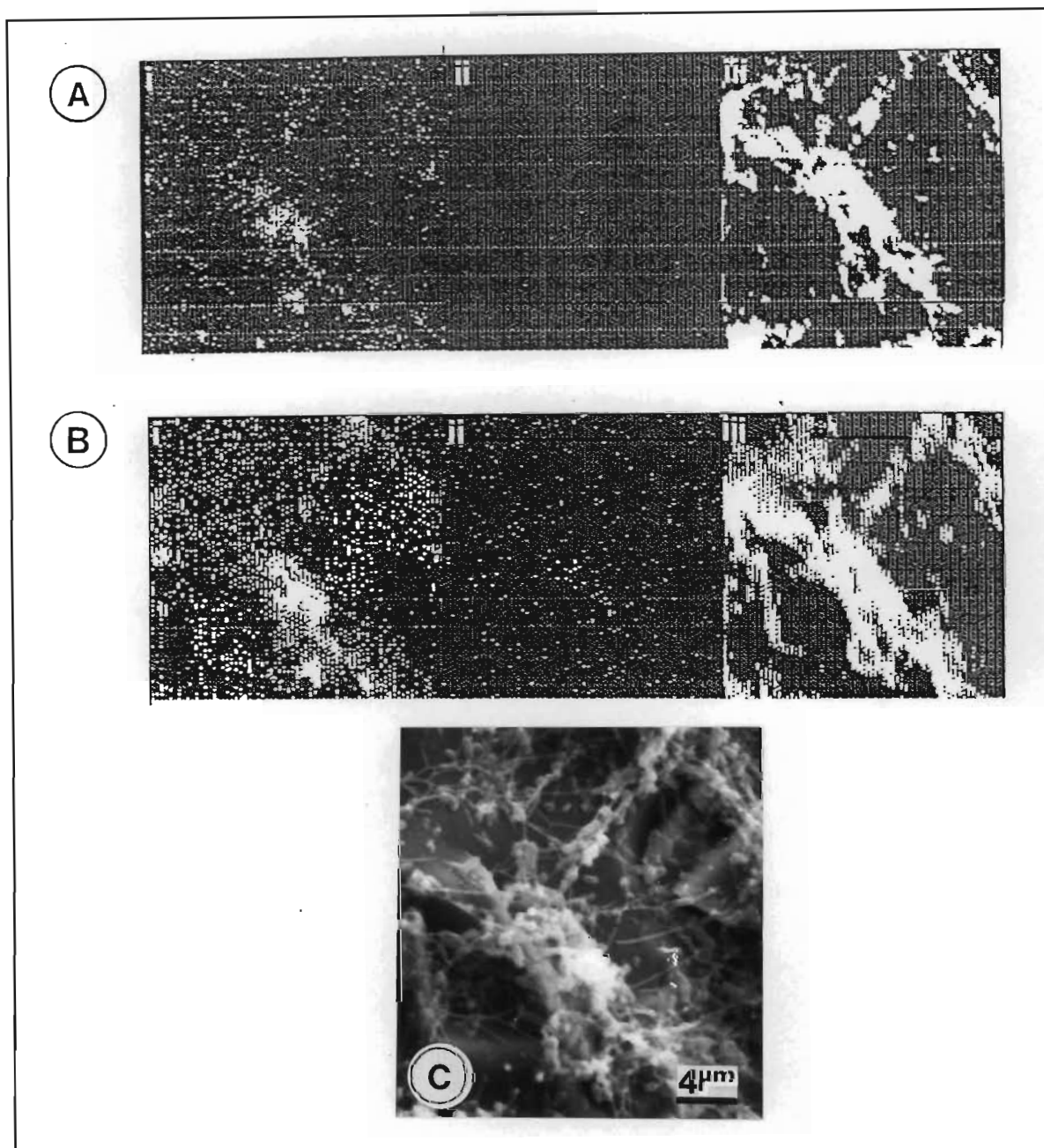
Figure 4.12   EDX digital maps of biofilms exposed for 96 hours to 50 mg l^{-1} Cd^{2+} and 200 mg l^{-1} Pb^{2+} and corresponding secondary images; A. map with 10 frames and a dwell time of 2 milliseconds (i. lead map; ii. cadmium map; iii. secondary image); B. map with 30 frames and a dwell time of 2 milliseconds (i. lead map; ii. cadmium map; iii. secondary image); C. electron micrograph corresponding to the secondary images.

Various different biofilms on ground glass were investigated by scanning electron microscopy to determine the effect of different metal combinations on the microbial populations (Plate 4.2). The biofilm formed when the microorganisms were exposed to a Cu^{2+}/Cd^{2+} combination consisted of clusters of cells (Plate 4.2A). Those formed in the presence of Cu^{2+}/Pb^{2+} and Cd^{2+}/Pb^{2+} consisted of layers which covered the entire surface of the plate (Plate 4.2B and C, respectively). Extracellular polysaccharide strands were visible in the biofilm formed in the presence of Cd^{2+}/Pb^{2+} (Plate 4.2C). Extracellular material has been found to play a role in the overall uptake of metal ions from an aqueous bimetallic solution (Pradhan and Levine 1992a), as discussed in Chapter 3, and may account for the more efficient metal uptake by the attached microorganisms compared to the free-living microorganisms.

Plate 4.2   Electron micrographs of biofilms formed on ground glass in different metal-containing solutions; clusters of cells in Cu^{2+}/Cd^{2+}-containing medium (A); layers of biofilm formed in Cu^{2+}/Pb^{2+}- (B) and Cd^{2+}/Pb^{2+}-containing media (C).
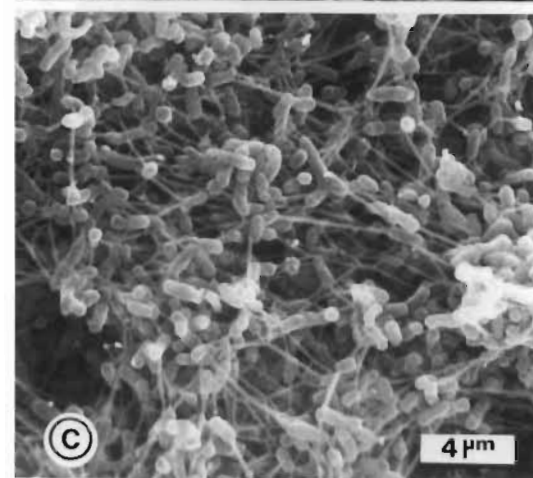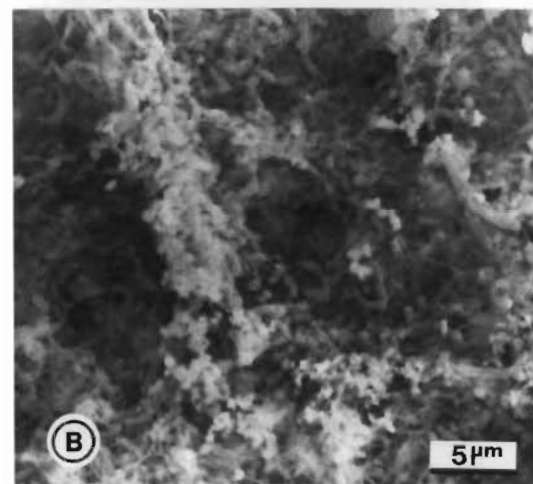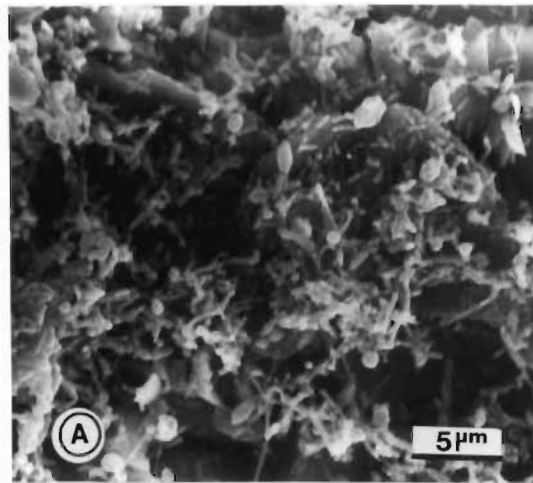
4.4 CONCLUSIONS

The free-living cultures displayed a much lower metal uptake ability than the attached biofilm microorganisms. This could have been due to the lower biomass in the former and/or their greater vulnerability to the toxic metal concentrations used. Dean-Ross and Mills (1989) reported that planktonic or free-living microorganisms display the lowest adaptation to heavy metals in natural systems. The presence of extracellular material in the microbial biofilms may also account for the better metal uptake by the attached microorganisms, as discussed in Chapter 3. Thus, attached microbial populations offer a more efficient system for the removal of heavy metals from solution than do free-living associations.

The selective accumulation of certain metals over others was again observed in these experiments, with the Pb^{2+} ions being preferentially taken up over the Cd^{2+} and Cu^{2+} ions. This selective accumulation may be due to the types of uptake sites available on the microbial surfaces and the intracellular uptake mechanisms in operation in the system. Different microbial associations are known to differ in their selective accumulation of various metal ions.

CHAPTER 5: EFFECT OF METAL COMBINATIONS AND VARIOUS PREGROWN INOCULUM PROPORTIONS ON THE UPTAKE OF METALS BY MICROBIAL ASSOCIATIONS

5.1 INTRODUCTION

Different cations inhibit others to varying degrees, as the potential of an atom to attract electrons to itself depends on its electronegativity; the higher its electronegativity, the stronger its ability to attract electrons (Emsley 1989). The inhibition of metal cation uptake by other metal ions may be related to ionic radii (Norris and Kelly 1977) although the situation may be complicated when toxic symptoms, such as membrane disruption occur (Gadd 1986).

The effect of the uptake of a metal in the presence of a second or third metal ion in the system depends on the actual metal species and on the environment (Tobin *et al.* 1988; Ting *et al.* 1991). The effect on uptake of specific metal ions from combinations of metals (Cu^{2+}/Pb^{2+}, Cu^{2+}/Cd^{2+}, Cd^{2+}/Pb^{2+} and $Cu^{2+}/Pb^{2+}/Cd^{2+}$) in different ratios and proportions was investigated using various pregrown inocula in flask cultures.

Various proportions of pregrown inocula were also used to investigate the effect of various the composition of the microbial association on metal-uptake.

5.2 MATERIALS AND METHODS

5.2.1 Effect of Combinations of Heavy Metals in Various Ratios on Metal Uptake by Microbial Associations

5.2.1.1 Copper\Cadmium combinations

Equal proportions of inoculum from Cu^{2+}- and Cd^{2+}-adapted microbial associations were used to initiate a series of flask cultures. Half-strength Nutrient Broth was used as the

growth medium and the cultures were incubated at 30°C on a rotary shaker (150 rpm) for five days. The metals were then added in various concentrations as indicated below:

Table 5.1 Cu^{2+} and Cd^{2+} combinations added to pregrown microbial associations in half-strength Nutrient Broth.

Culture	Cu^{2+} (mg l^{-1})	Cd^{2+} (mg l^{-1})
A	100	200
B	75	150
C	50	100
D	0	200
E	50	200
F	100	100
G	200	0

Samples (1.5 ml) were taken at various times (1, 15, 30, 60 minutes, and 24 and 48 hours) after addition of the metal(s). Each sample was centrifuged (11000 rpm x g for 3 minutes in an Eppendorf centrifuge, Model 5413) to remove the microorganisms and the supernatant was stored in the freezer (-4°C) until analyzed by HPLC (see section 2.2.4.1) for the metal ion content. Uninoculated flasks containing half-strength Nutrient Broth and the same metal combinations served as controls from which samples (1.5 ml) were taken to determine the initial concentrations of the metals in solution.

Culture samples (2 ml) from the inoculated flasks were withdrawn after 48 hours to determine the amount of metal associated with the microorganisms. These samples were prepared for semi-quantitative EDX investigations as follows:

Two millilitre volumes of the various metal-containing cultures were removed and centrifuged in an Eppendorf centrifuge, Model 5413 (11000 rpm x g for 3 minutes). The supernatant was removed and the pellet resuspended and washed several times in distilled

water. After further centrifugation (11000 rpm x g for 3 minutes in the Eppendorf centrifuge, Model 5413) the pellet was smeared onto a carbon stub and allowed to air dry. The sample was then viewed in an Hitachi S-570 SEM fitted with a Link eXL II EDX system. A semi-quantitative method was used which determined the relative percentages of each element in the sample. It should be noted that elements such as carbon, nitrogen, hydrogen and oxygen were not detected in this system as their Atomic Numbers are too low (Morgan 1985).

5.2.1.2 Copper/Lead combinations

The same experiment was repeated with pregrown Cu^{2+}- and Pb^{2+}-adapted microbial associations using copper and lead combinations at the same concentrations as shown in Table 5.1 for the copper/cadmium mixtures. Samples were taken, both for HPLC (see section 2.2.4.1) and semi-quantitative EDX metal analyses, as described in 5.2.1.1. Due to problems experienced with Pb^{2+} precipitation, supernatant samples were diluted by acidifying with 55% (v/v) nitric acid to a final acid concentration of 1 M before filtration and clean-up with Sep-Pak cartridges (Millipore) in preparation for HPLC analysis (see section 2.2.4.1).

5.2.1.3 Cadmium/Lead combinations

The experiment was repeated with pregrown Cd^{2+}- and Pb^{2+}-adapted microbial associations using the Cd^{2+}/Pb^{2+} concentrations shown in Table 5.2.

Table 5.2 Cd^{2+}/Pb^{2+} combinations added to pregrown microbial associations in half-strength Nutrient Broth.

Culture	Cd^{2+} (mg l^{-1})	Pb^{2+} (mg l^{-1})
A3	100	200
B3	200	100
C3	75	150
D3	0	200
E3	150	75
F3	100	100
G3	200	0
H3	200	50
I3	50	200

Microbial biomass samples were prepared for semi-quantitative EDX analysis (see section 5.2.1.1) and supernatant samples were collected for metal analysis by HPLC or AAS (see section 2.2.4). Here also, the samples were diluted and acidified before filtration and metal analysis.

5.2.2 Effect of Varying the Proportions of Pregrown Inocula on Uptake of Metals in Combination

A series of experimental flasks was set up to determine the effect on metal uptake of mixed inocula in different proportions. Inoculum comprising cultures previously exposed to the various metal solutions was used to inoculate half-strength Nutrient Broth medium. These cultures were grown at 30°C for one week and then mixed in different proportions, i.e. 30 ml of the one culture were combined with 15 ml of the other and *vice versa* for each of the metal combinations. Metal ion solutions were added immediately following combination of the various mixed metal-adapted inocula to obtain the initial metal ion concentrations listed in Table 5.3. Liquid samples (1.5 ml) were taken for metal analysis immediately after addition of the metal solutions and after 30 and 60 minutes, 24 and 48

hours, and 5 days. Corresponding uninoculated controls containing medium and the appropriate metal concentrations were set up to determine the initial metal concentrations. After 5 days microbial biomass samples were withdrawn and prepared as described above (5.2.1.1) to obtain pellets for semi-quantitative EDX investigations.

Table 5.3   Metal concentrations used for each metal combination, Cu^{2+}/Cd^{2+}; Cu^{2+}/Pb^{2+}; Cd^{2+}/Pb^{2+}.

Culture	Metal 1 conc (mg l^{-1})	Metal 2 conc (mg l^{-1})
A	100	100
B	100	0
C	0	100
D	200	100
E	100	200
F	50	200
G	200	50

5.2.3 Effect of Triple Combinations of Metals on Microbial Metal Uptake

The uptake of the different metal ions from a combination of Pb^{2+}-, Cd^{2+}- and Cu^{2+}-containing solutions was investigated using microbial associations which had been previously adapted to grow in the various metal-containing solutions. These microbial associations were either cultured together in mixed cultures, or grown up separately in individual cultures and then mixed together.

5.2.3.1 Different metal-adapted microbial associations cultured together

An inoculum comprising 2.5 ml each of pregrown cultures of Cu^{2+}-, Cd^{2+}- and Pb^{2+}-adapted microorganisms was added to 41 ml half-strength Nutrient Broth (NB) in a 100 ml Erlenmeyer flask and incubated for one week at 30ºC on a rotary shaker (150 rpm).

Aliquots of the concentrated metal solutions were then added to obtain a final concentration of 100 mg l^{-1} of each metal and samples (1.5 ml) of the solution were taken for metal analysis at various times (immediately after metal addition and 15, 30, 60, 120 minutes, 24 and 48 hours, and 5 days thereafter). These samples were centrifuged (11000 x g rpm for 3 minutes in an Eppendorf Model 5413 Centrifuge) and the supernatant frozen until analyzed by HPLC (see section 2.2.4.1). Samples (2 ml) of the cultures were taken on day 5 and centrifuged (11000 rpm x g for 3 minutes) to pelletize the microorganisms for semi-quantitative EDX analysis (see section 5.2.1.1).

5.2.3.2 Different metal-adapted microbial associations cultured separately and then mixed together

Each of the Cu^{2+}-, Cd^{2+}- and Pb^{2+}-adapted mixed cultures were cultured separately in half-strength Nutrient Broth at 30ºC for one week prior to mixing. Fifteen ml of Cu^{2+}-adapted culture + 15 ml Cd^{2+}-adapted culture + 15 ml Pb^{2+}-adapted culture + 0.5 ml each of concentrated Cu^{2+}, Cd^{2+} and Pb^{2+} solutions (APPENDIX A) were mixed to obtain final metal concentrations in the mixed culture of 100 mg l^{-1} each + 3.5 ml half-strength Nutrient Broth. Sampling was carried out as described in section 5.2.3.1, for both metal analyses of the solutions and semi-quantitative EDX analyses of the pelletized microorganisms.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of Combinations of Heavy Metals in Various Ratios on Metal Uptake by Microbial Associations

5.3.1.1 Copper/Cadmium combinations

The semi-quantitative EDX analysis results obtained for the pelletized microorganisms exposed to the various ratios of Cu^{2+}/Cd^{2+} in combination are shown in Table 5.4.

Table 5.4   Element % of copper and cadmium on or in microbial biomass in Cu^{2+}/Cd^{2+} combinations.

Culture	Cu^{2+} added (mg l^{-1})	Element % on/in cells - copper	Cd^{2+} added (mg l^{-1})	Element % on/in cells - cadmium
A	100	14.8	200	70.3
B	75	0	150	85.4
C	50	7.3	100	78.9
D	0	0	200	87.0
E	50	9.1	200	77.9
F	100	16.8	100	69.5
G	200	74.0	0	11.5

The element % is the percentage of copper and cadmium found on or in the cells in comparison to the other elements detected. It must be noted that it is not the percentage per mass of the biomass as carbon, hydrogen, nitrogen and oxygen are not detected by this method. It is merely a proportional relationship between the metals in the different combinations. It must be kept in mind that the element % or signal intensity (counts) is a relative concentration of the elements and is subject to a number of physical factors such as orientation and topography of the specimen (Chandler 1978). Elements which form peaks towards the left hand side of the spectrum are also generally more easily detected than those that form peaks towards the right hand side of the spectrum (Chandler 1978).

A small percentage of cadmium was found to be associated with the culture which received no Cd^{2+} ions initially (culture G, Table 5.4). This may have been due to residual cadmium left on or in the biomass when it was originally exposed to a Cd^{2+}-containing solution. When this mixture of metal-adapted microbial associations was exposed to equal concentrations of Cu^{2+} (100 mg l^{-1}) and Cd^{2+} (100 mg l^{-1}) it appeared that more cadmium than copper was associated with the cells (culture F, Table 5.4). The spectrum of this sample is shown in Fig. 5.1.
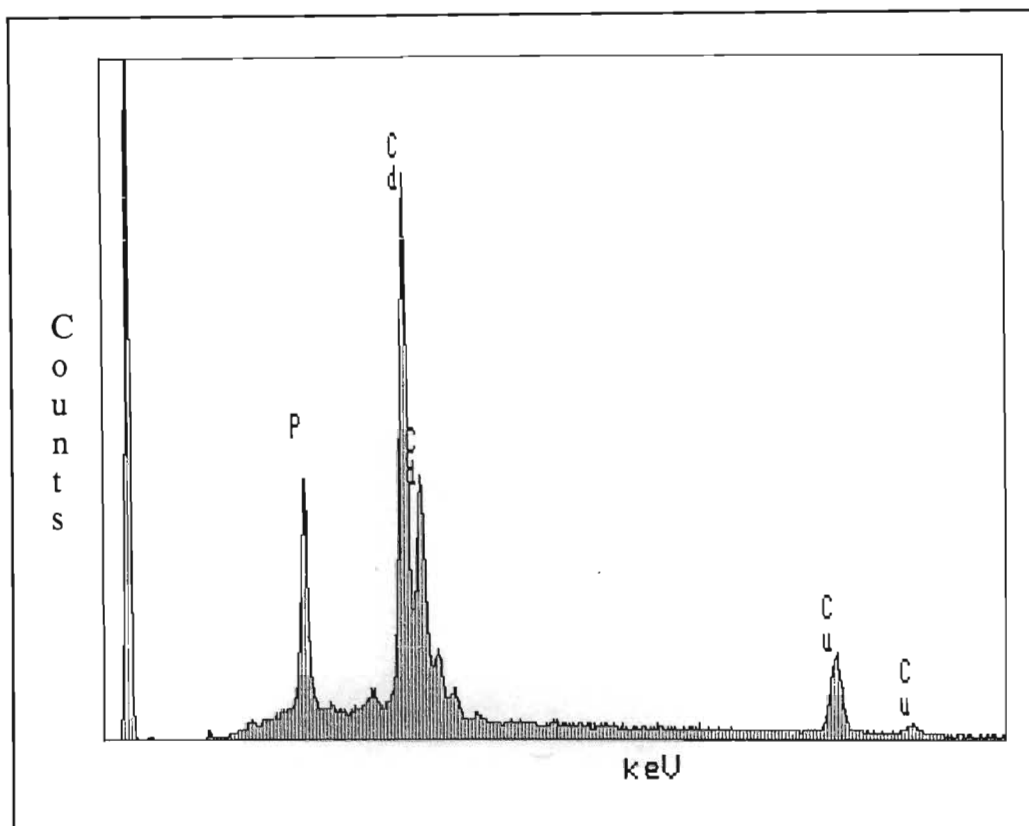
Figure 5.1 EDX Spectrum of culture F where the respective metal-adapted microbial associations were together exposed to equal concentrations of Cu^{2+} (100 mg l^{-1}) and Cd^{2+} (100 mg l^{-1}).

The P peak of the spectrum originated from the phosphorus found within the wall structure, nucleic acids and other components of the microorganisms, and possibly from the culture medium although the cells had been washed.

The liquid medium samples were analyzed by HPLC (see section 2.2.4.1) to determine what percentage of the metal ions remained in solution after uptake by the microbial biomass, and thus the % metal taken up by the biomass.

Copper uptake in this combination varied from 9 to 52 % (Table 5.5), depending on the initial concentration of the metal. The concentration of Cu^{2+} in cultures A, B and particularly F and G increased between the time 0 and 48 hour sampling; this may have been due to desorption of the copper from the biomass or possibly due to evaporation of the medium leading to concentration of the copper remaining in solution.

Table 5.5 Metal concentrations remaining in the Cu^{2+}/Cd^{2+} containing media after 48 hours contact with the microbial biomass.

Culture	$Cu^{2+}/$ Cd^{2+} ratios $(mg\ l^{-1})$	Initial metal ion conc * $(mg\ l^{-1})$	Metal ion conc immediately after inoculation $(mg\ l^{-1})$	Metal ion conc after 48 hours $(mg\ l^{-1})$	% metal uptake after 48 hours
A	100 Cu^{2+}	90.8	39.8	43.5	52
	200 Cd^{2+}	32.7	0.8	0.6	98.3
B	75 Cu^{2+}	52.2	35.7	36.4	30.2
	150 Cd^{2+}	23.7	0	0.6	97.6
C	50 Cu^{2+}	24.0	22.2	21.9	9
	100 Cd^{2+}	15.4	0	0.8	94.6
D	200 Cd^{2+}	18.8	1.4	1.3	93
E	50 Cu^{2+}	16.0	14.8	12.7	20.6
	200 Cd^{2+}	29.0	0.2	0.5	98.3
F	100 Cu^{2+}	83.4	38.8	60.0	28
	100 Cd^{2+}	19.9	0.5	0.7	96.7
G	200 Cu^{2+}	45.4	18.9	30.7	32.4

* = metal concentration of corresponding uninoculated controls.

Cadmium uptake appeared to be high (an average uptake of 96.4%; Table 5.5). However, this apparent high uptake may have been due to precipitation of the cadmium, with the result that it would have been removed from the samples during the filtering process which preceded HPLC analysis (see section 2.2.4.1). It should be noted that the actual initial Cd^{2+} concentration analyzed was considerably lower than the theoretical amount of cadmium added. For example, in culture A, 200 mg l^{-1} Cd^{2+} were theoretically added but the analyzed concentration was only 32.7 mg l^{-1} (Table 5.5). This discrepancy was probably due to precipitation of the Cd^{2+} ions. Insoluble complexes may also have been formed by substances released by the microorganisms. Macaskie and Dean (1987)

determined that a *Citrobacter* species, which precipitated Cd^{2+}, liberated inorganic phosphate from glycerol-2-phosphate. This process was mediated by a cell-bound phosphatase (Macaskie and Dean 1987). The same process may have occurred in the microbial association under investigation here. Differences in the Cd^{2+} concentrations of the cultures immediately after inoculation and after 48 hours were small and were not taken into account, as they were probably outside the detection limits of the method. In these experiments it appeared that Cd^{2+} ions rather than Cu^{2+} ions were preferentially taken up. Antagonistic competition between metal ions for uptake sites was reported to be competition for adsorptive sites on the cell surfaces and/or competition for transport across membranes (Ting *et al.* 1991).

5.3.1.2 Copper/Lead combinations

The semi-quantitative EDX results obtained from the pelletized microorganisms exposed to various ratios of Cu^{2+}/Pb^{2+} in combination are shown in Table 5.6.

Table 5.6 Element % of copper and lead on or in microbial biomass in Cu^{2+}/Pb^{2+} combinations.

Culture	Cu^{2+} added (mg l^{-1})	Element % on/in cells - copper	Pb^{2+} added (mg l^{-1})	Element % on/in cells - lead
A2	100	56.3	200	20.9
B2	75	63.9	150	17.0
C2	50	10.2	100	78.2
D2	0	0	200	88.0
E2	50	20.0	200	64.9
F2	100	55.8	100	32.9
G2	100	77.3	0	0

When mixed inocula, comprising Cu^{2+}- and Pb^{2+}-adapted cultures, were cultured together more copper appeared to be associated with the cells than lead, even though equal

concentrations of Pb^{2+} (100 mg l^{-1}) and Cu^{2+} (100 mg l^{-1}) were added initially (culture F2, Table 5.6). The spectrum of this culture is presented in Fig. 5.2.
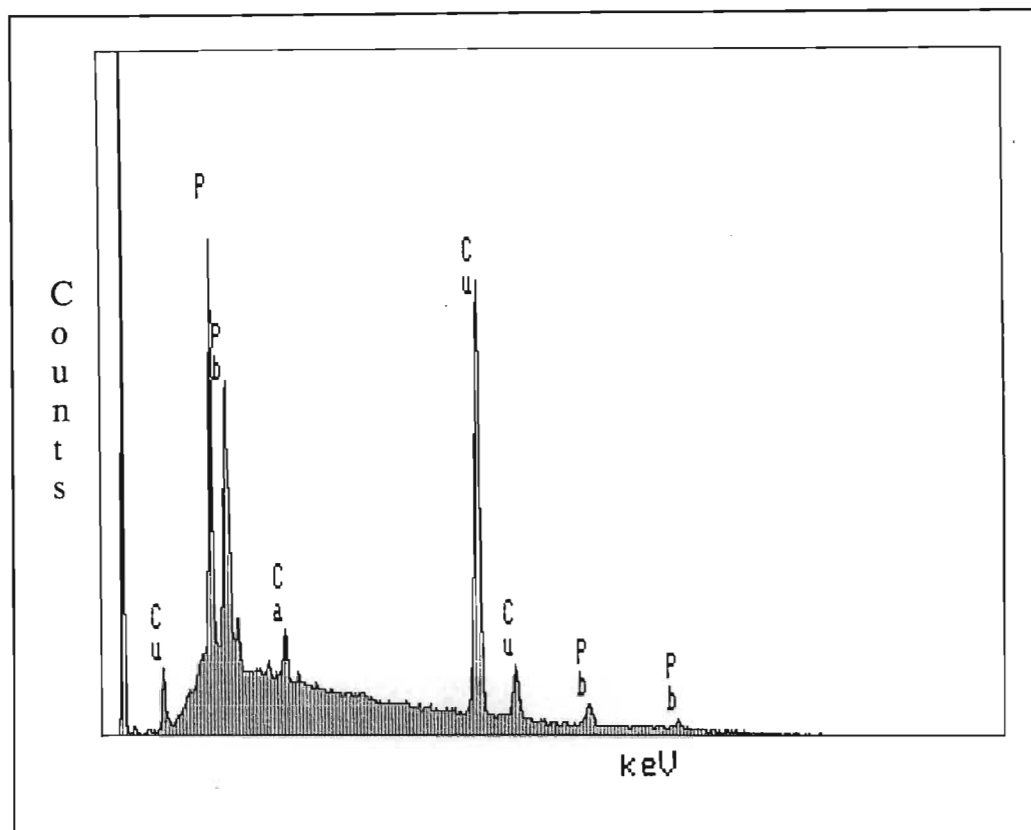
Figure 5.2 EDX spectrum of culture F2 where the respective metal-adapted microbial associations were together exposed to equal concentrations of Cu^{2+} (100 mg l^{-1}) and Pb^{2+} (100 mg l^{-1}).

No results were obtained for the concentrations of Cu^{2+} and Pb^{2+} in the supernatant due to analytical problems. Even on acidification the Pb^{2+} concentrations were still low, almost negligible, and results could not be obtained. Acidification appeared to affect the time of elution of the metals and the Cu^{2+} and Pb^{2+} peaks were not separated in the column during HPLC analysis.

5.3.1.3 Cadmium/Lead combinations

The semi-quantitative EDX results obtained for the pelletized microorganisms exposed to various proportions of Cd^{2+}/Pb^{2+} are presented in Table 5.7.

Table 5.7 Element % of cadmium and lead on or in microbial biomass in Cd^{2+}/Pb^{2+} combinations.

Culture	Cd^{2+} added (mg l^{-1})	Element % on/in cells - cadmium	Pb^{2+} added (mg l^{-1})	Element % on/in cells - lead
A3	100	33.2	200	58.3
B3	200	54.9	100	30.0
C3	75	32.2	150	57.4
D3	0	4.5	200	86.7
E3	150	52.3	75	34.9
F3	100	47.7	100	43.2
G3	200	80.0	0	7.9
H3	200	67.9	50	21.1
I3	50	23.8	200	68.3

When mixed inocula, comprising Cd^{2+}- and Pb^{2+}-adapted cultures, were grown together and exposed to equal concentrations of Cd^{2+} (100 mg l^{-1}) and Pb^{2+} (100 mg l^{-1}), approximately equal amounts of Cd^{2+} and Pb^{2+} were associated with the cells (culture F3, Table 5.7). The EDX spectrum of this culture is shown in Fig. 5.3. Small percentages of cadmium and lead were associated with the biomass of cultures which were not exposed to these metals (cultures D3 and G3, Table 5.7). This may have been due to residual metal which was associated with the cells when they were originally exposed to the respective metal solutions.
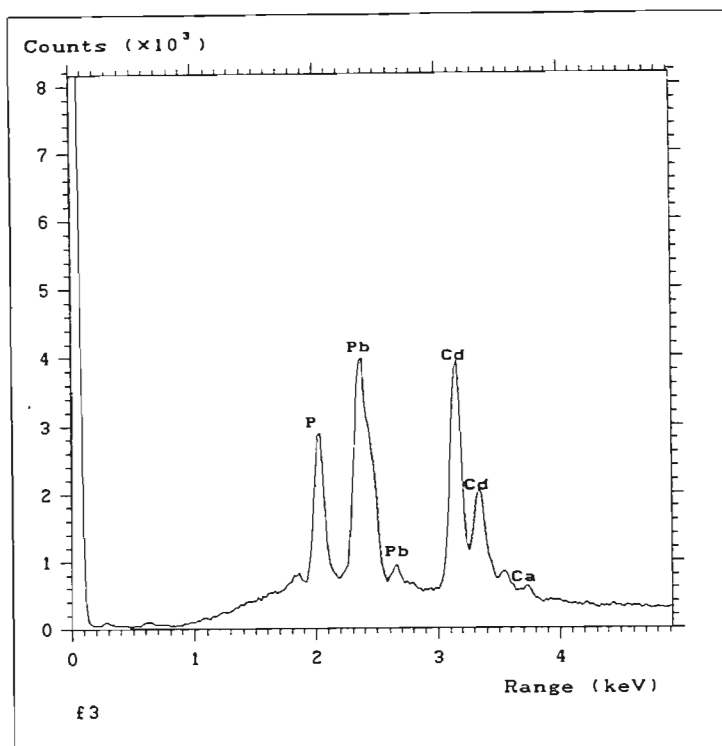
Figure 5.3 EDX spectrum of culture F3 where the respective metal-adapted microbial associations were together exposed to equal concentrations of Cd^{2+} (100 mg l^{-1}) and Pb^{2+} (100 mg l^{-1}).

Problems were experienced during HPLC analysis of these Cd^{2+}/Pb^{2+} combination samples. Cd^{2+} and Pb^{2+} concentrations remained low even after acidification. These low values were possibly the result of metal loss through precipitation - the precipitate having been removed during centrifugation of the samples to remove the microbial cells.

In direct competition studies, it has been shown that certain fractions of the uptake sites preferentially bind particular cations (Tobin *et al.* 1988). The effect of the presence of a second metal ion in a system, on the uptake of a heavy metal, depends on the actual metal species and on the environment in which they exist (Ting *et al.* 1991). In uptake experiments with both zinc and cadmium present, the removal of zinc from solution was greater than that of cadmium (Ting *et al.* 1991). This could be due to the essential and non-essential nature of zinc and cadmium, respectively (Ting *et al.* 1991). The presence of Pb^{2+} ions in solution inhibited the overall metal removal capacity of *Pseudomonas*

putida, while anions such as borate, carbonate, chloride and sulphate had no effect (Wong *et al.* 1993). Simple cation competition for anionic sites was also observed with *Rhizopus arrhizus* biomass (Tobin *et al.* 1988; Collins and Stotzky 1989). Nakajima and Sakaguchi (1986) suggested that the selective accumulation of heavy metals was determined by interionic competition. In the present study metals were selectively accumulated from mixed metal solutions, for example, Cd^{2+} ions were preferentially taken up over Cu^{2+} ions.

5.3.2 Effect of Varying the Proportions of Pregrown Inocula on Uptake of Metals in Combination

Multiple metal resistance may exist but, generally, resistance to one heavy metal does not automatically entail resistance to another (Doelman 1985). Coupling of resistance against heavy metals suggests that either the site of action of these metals or the resistance mechanisms is the same (Doelman 1985). In fungi co-tolerance to heavy metals (the simultaneous development of tolerance to several metals during training to only one metal) has been observed and shows that the cellular mechanism conferring tolerance is not unique to any one metal (Garcia-Toledo *et al.* 1985). This phenomenon has also been noted with bacteria and algae (Garcia-Toledo *et al.* 1985). Both *Rhizopus stolonifer* and *Cunninghamella blakesleeana*, trained to tolerate copper, were also more tolerant of cadmium, cobalt, nickel and lead than the untrained parentals (Garcia-Toledo *et al.* 1985).

Various proportions of pregrown inocula were investigated to determine if the microorganisms previously exposed to a specific metal were also able to accumulate other metals from mixed metal solutions.

The semi-quantitative EDX results obtained for the microbial association comprising various proportions of different pregrown inocula are shown in Table 5.8.

Table 5.8 Element % of copper and cadmium on or in microbial biomass found in Cu^{2+}/Cd^{2+} combination media with various inoculum compositions.

Culture	Cu^{2+} added (mg l^{-1})	Element % on/in cells - copper	Cd^{2+} added (mg l^{-1})	Element % on/in cells - cadmium	Inoculum composition
A1	100	21.7	100	65.1	30 ml Cu^{2+}-adapted inoculum + 15 ml Cd^{2+}-adapted inoculum
B1	100	56.8	0	27.9	
C1	0	0	100	85.4	
D1	200	50.6	100	35.7	
E1	100	24.1	200	63.7	
F1	50	6.5	200	80.0	
G1	200	62.4	50	23.1	
A2	100	15.5	100	67.7	15 ml Cu^{2+}-adapted inoculum + 30 ml Cd^{2+}-adapted inoculum
B2	100	39.3	0	42.9	
C2	0	0	100	85.5	
D2	200	46.3	100	40.2	
E2	100	11.7	200	74.6	
F2	50	5.5	200	80.9	
G2	200	57.1	50	26.7	

Comparing samples from cultures with various inoculum compositions (Table 5.8) it was found that when more Cu^{2+}-adapted microorganisms were present there was more copper associated with the microorganisms compared to the amount of copper associated with the inoculum with less Cu^{2+}-adapted microorganisms (for example, compare the element % copper of cultures A1 and A2, Table 5.8). The same trend occurred with the Cd^{2+}-adapted microorganisms and Cd^{2+}-uptake, but this was not as marked. There was a fairly high element % of cadmium present in the cultures which were not exposed to the Cd^{2+}-containing solution (cultures B1 and B2, Table 5.8); the reason for this is not known but may have been due to residual cadmium associated with the microorganisms from when

they were previously cultured in Cd^{2+}-containing media. As this phenomenon was not observed with copper, it suggested that Cd^{2+} ions were more firmly bound to the biomass than were the Cu^{2+} ions.

When equal concentrations of Cu^{2+} (100 mg l^{-1}) and Cd^{2+} (100 mg l^{-1}) were present, it appeared that there was more cadmium than copper associated with the cells, eg. cultures A1 and A2, Table 5.8). This result correlates with the results shown in Table 5.4. Some samples were analyzed by AAS (see section 2.2.4.2) to determine the amount of metal remaining in solution. These results are presented in Table 5.9.

Table 5.9 Cu^{2+} concentrations remaining in supernatants of Cu^{2+}/Cd^{2+}-adapted combination cultures mixed in various inoculum proportions.

Culture	Initial metal ion conc * (mg l^{-1})	Metal ion conc on day 0 (mg l^{-1})	Metal ion conc on day 5 (mg l^{-1})	% metal uptake after 5 days	Inoculum composition
A1	109.5	58.5	34.2	68.8	30 ml Cu^{2+}-adapted inoculum + 15 ml Cd^{2+}-adapted inoculum
B1	105.2	62.9	44.5	57.7	
C1	0	0	0	0	
D1	199.5	50.9	60.7	69.6	
E1	106.8	48.2	32.5	69.6	
F1	52.0	43.6	16.5	68.3	
G1	194.1	65.1	48.8	74.9	
A2	109.5	59.6	35.8	67.3	15 ml Cu^{2+}-adapted inoculum + 30 ml Cd^{2+}-adapted inoculum
B2	105.2	66.1	40.1	61.9	
C2	0	0	0	0	
D2	199.5	64.0	75.9	62.0	
E2	106.8	52.6	32.5	69.6	
F2	52.0	40.7	19.8	61.9	
G2	194.1	67.2	43.4	77.6	

* = Cu^{2+} concentration in corresponding uninoculated controls.

The day 0 sample was taken immediately after mixing the metals with the microorganisms, and the day 5 sample at the time when the microorganisms were removed for semi-quantitative EDX analysis. In Table 5.9 it can be seen that the copper was removed from solution by the mixed microbial association, with an average uptake of 68% when the proportion of Cu^{2+}-adapted microorganisms was greater, and 66 % when there was a greater proportion of Cd^{2+}-adapted microorganisms. Obviously the uptake of copper was not effected to any great extent by the composition of the microbial association. Cultures D1 and D2 showed increases in the copper concentrations between day 0 and day 5; this may have been due to desorption of the Cu^{2+} ions from the biomass or due to evaporation leading to concentration of the metals in solution. Problems were experienced with Cd^{2+} analysis, and no results were obtained except for the controls where the concentrations were very low (results not shown). Obviously, precipitation affected the concentration of Cd^{2+} in solution.

The semi-quantitative EDX results obtained for the microorganisms exposed to various ratios of Cu^{2+}/Pb^{2+} are presented in Table 5.10.

Table 5.10 Element % of copper and lead on or in the microbial biomass found in Cu^{2+}/Pb^{2+} combination media with various inoculum compositions.

Culture	Cu^{2+} added (mg l^{-1})	Element % on/in cells - copper	Pb^{2+} added (mg l^{-1})	Element % on/in cells - lead	Inoculum composition
A3	100	7.1	100	78.2	15 ml Cu^{2+}-adapted inoculum + 30 ml Pb^{2+}-adapted inoculum
B3	100	39.6	0	43.1	
C3	0	0	100	87.7	
D3	200	38.2	100	49.4	
E3	100	6.6	200	81.6	
F3	50	3.6	200	83.2	
G3	200	53.2	50	31.9	
A4	100	11.7	100	72.3	30 ml Cu^{2+}-adapted inoculum + 15 ml Pb^{2+}-adapted inoculum
B4	100	47.3	0	30.0	
C4	0	0	100	89.	
D4	200	48.2	100	39.3	
E4	100	8.0	200	80.9	
F4	50	7.6	200	81.8	
G4	200	59.5	50	26.7	

When comparing samples of cultures with various inoculum proportions exposed to Cu^{2+}/Pb^{2+} combinations (Table 5.10), it was found that there was generally a greater association of copper with the biomass which contained the greater proportion of Cu^{2+}-adapted microorganisms than with the biomass with less Cu^{2+}-adapted microorganisms (for example, compare the element % of copper on or in cells in cultures B3 and B4). The same trend was observed with Pb^{2+}-adapted microorganisms and their accumulation of Pb^{2+}. A similar trend occurred with the $Cu^{2+}\backslash Cd^{2+}$ combination (Table 5.8). When comparing the uptake of Pb^{2+} and Cu^{2+} ions it was found that more lead than copper was associated with the cells when equal concentrations of both metals were added (Table 5.10, cultures A3 and A4, 100:100 mg l^{-1} ratio; Figs 5.4 and 5.5).

An actinomycete system has also been found to be more effective in binding lead than copper (Pradhan and Levine 1992a). Once again the cultures which received no Pb^{2+}-containing solution (Table 5.10, cultures B3 and B4) had some lead associated with the biomass, due to residual lead adsorbed to or taken up by the cells during the adaptation period.
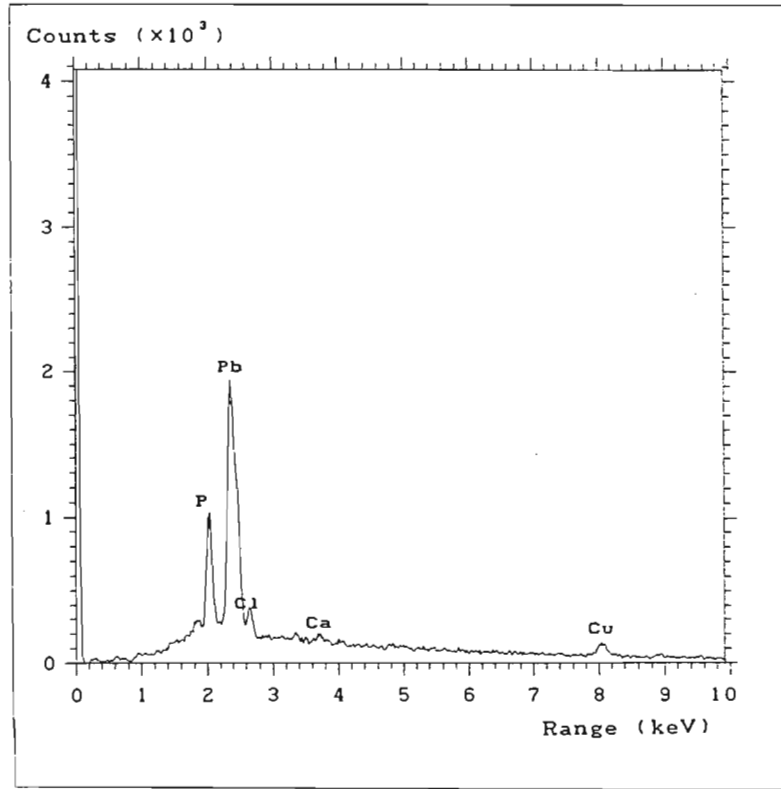
Figure 5.4 EDX spectrum of culture A3 exposed for 5 days to 100 mg l^{-1} Cu^{2+} and 100 mg l^{-1} Pb^{2+}.
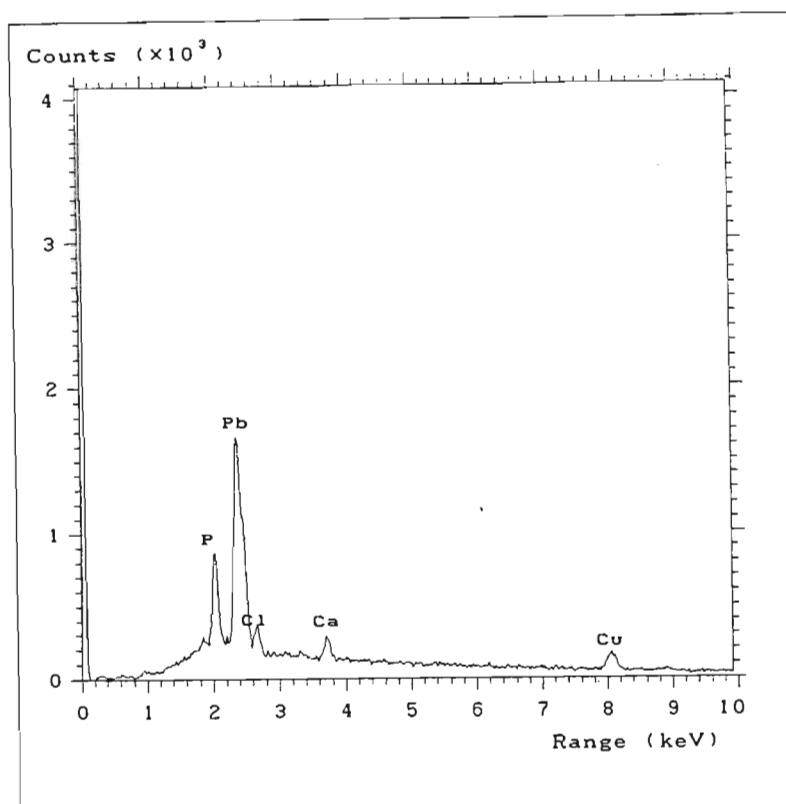
Figure 5.5 EDX spectrum of culture A4 exposed for 5 days to 100 mg l^{1} Cu^{2+} and 100 mg l^{1} Pb^{2+}.

Once again only results for Cu^{2+} uptake were obtained as problems were encountered with Pb^{2+} analysis. The microbial association with a larger proportion of Cu^{2+}-adapted microorganisms appeared to remove on average, 59.6% of the Cu^{2+} over the 5 day experimental period, while the population with a lower proportion of Cu^{2+}-adapted microorganisms only took up 54.8% of the Cu^{2+} over the same time period. Culture G4 showed an increase in the Cu^{2+} concentration of the solution after 5 days, perhaps due to desorption of the metal ions or, possibly, evaporation of the liquid leading to concentration of the copper remaining in solution. Cu^{2+} uptake from multi-ion solutions (average uptake of 57.8%; Table 5.11) and from pure Cu^{2+} solutions (average uptake of 53.9%; Table 5.11) was found to be similar, i.e. Cu^{2+} was taken up with similar efficiency irrespective of the presence of other metals in solution. It has been reported that metal uptake from single ion solutions was higher than the accumulation of ions from a solution containing combinations of metals at the same concentrations (Wehrheim and Wettern 1994b). However this was not the case with Cu^{2+} ions in the present study. The uptake of lead from a multi-ion solution was reported to be less affected than Cd^{2+} and Co^{2+} accumulation in *Chlorella fusca* (Wehrheim and Wettern 1994b), which appeared to also be the case in the present study as Pb^{2+} was efficiently taken up in all cases.

Table 5.11 Cu^{2+} concentrations remaining in supernatants of Cu^{2+}/Pb^{2+}-adapted combination cultures mixed in various inoculum proportions.

Culture	Initial metal ion conc * (mg l^{-1})	Metal ion conc on day 0 (mg l^{-1})	Metal ion conc on day 5 (mg l^{-1})	% metal uptake after 5 days	Inoculum composition
A3	100.8	61.3	41.2	59.1	15 ml Cu^{2+}-adapted inoculum + 30 ml Pb^{2+}-adapted inoculum
B3	105.2	76.4	63.4	39.7	
C3	0	0	0	0	
D3	184.3	58.8	56.4	69.4	
E3	94.3	60.2	47.7	49.4	
F3	37.4	46.4	22	41.2	
G3	199.5	65.1	59.6	70.1	
A4	100.8	67.2	41.2	59.1	30 ml Cu^{2+}-adapted inoculum + 15 ml Pb^{2+}-adapted inoculum
B4	105.2	81.9	33.6	68.1	
C4	0	0	0	0	
D4	184.3	66.1	44.5	75.9	
E4	94.3	46.1	43.9	53.4	
F4	37.4	42.8	27.1	27.5	
G4	199.5	46.6	53.1	73.4	

* = Cu^{2+} concentration of corresponding uninoculated controls.

The semi-quantitative EDX results obtained for the microorganisms exposed to various ratios of Cd^{2+}/Pb^{2+} are presented in Table 5.12.

Table 5.12 Element % of cadmium and lead on or in the microbial biomass found in Cd^{2+}/Pb^{2+} combination media with various inoculum compositions.

Culture	Cd^{2+} added (mg l^{-1})	Element % on/in cells - cadmium	Pb^{2+} added (mg l^{-1})	Element % on/in cells - lead	Inoculum composition
A5	100	44.9	100	44.1	15 ml Cd^{2+}-adapted inoculum + 30 ml Pb^{2+}-adapted inoculum
B5	100	81.9	0	0	
C5	0	5.3	100	83.5	
D5	200	57.6	100	30.8	
E5	100	34.1	200	55.3	
F5	50	21.8	200	68.2	
G5	200	62.1	50	25.6	
A6	100	44.9	100	43.7	30 ml Cd^{2+}-adapted inoculum + 15 ml Pb^{2+}-adapted inoculum
B6	100	70.2	0	15.5	
C6	0	9.7	100	79.4	
D6	200	55.5	100	32.3	
E6	100	36.1	200	53.5	
F6	50	23.4	200	66.1	
G6	200	63.7	50	23.8	

On comparing the semi-quantitative EDX results of the microbial biomass from cultures with various inoculum proportions exposed to Cd^{2+}/Pb^{2+} combinations (Table 5.12), it was seen that both the microbial inoculum compositions were associated with similar amounts of both cadmium and lead (for example, compare the element % cadmium in culture A5 and A6, and the element % lead in cultures A5 and A6; Figs. 5.6 and 5.7). Both metals also appeared to be taken up to the same extent, as was observed in Table 5.7. Some residual metal was again detected in the cultures which did not receive those metals (cultures B6, C5 and C6, Table 5.12). The different proportions of pregrown inoculum

did not seem to affect the uptake of either metal to any great extent. Obviously the metal uptake sites for Cd^{2+} and Pb^{2+} are similar.
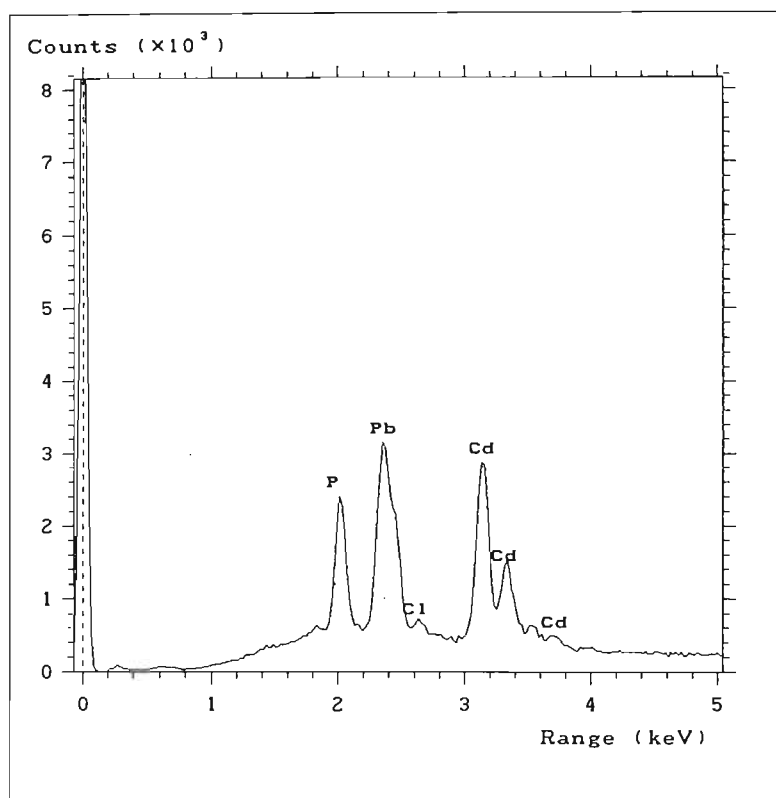
Figure 5.6 EDX spectrum of culture A5 exposed for 48 hours to 100 mg l^{-1} Cd^{2+} and 100 mg l^{-1} Pb^{2+}.
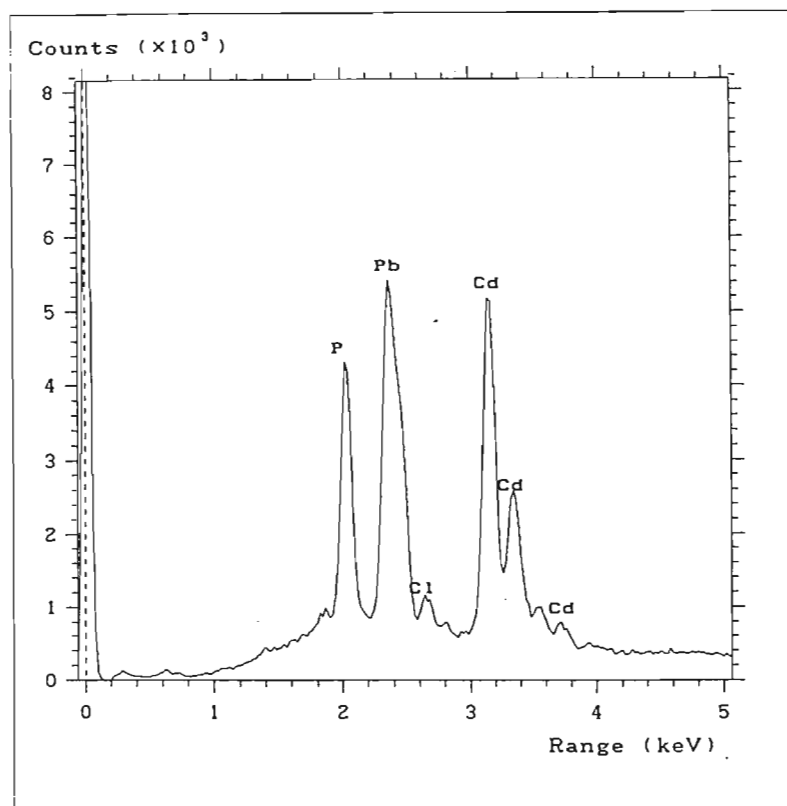
Figure 5.7 EDX spectrum of culture A6 exposed for 48 hours to 100 mg l^1 Cd^{2+} and 100 mg l^1 Pb^{2+}.

No results were obtained on analysis of the supernatant samples for the concentration of each metal remaining in solution due to the problems experienced with Cd^{2+} and Pb^{2+} precipitation.

5.3.3 Triple Combinations of Metals

5.3.3.1 Different metal-adapted microbial associations cultured together

These semi-quantitative EDX results (Table 5.13) indicate that more lead was associated with the biomass than cadmium and copper. HPLC results for the concentrations of the respective metals remaining in the medium can be seen in Fig. 5.8. These results indicate that both Cu^{2+} and Cd^{2+} were taken up to a large extent by the microbial cells. No results were obtained for the concentration of Pb^{2+} ions remaining in solution. When investigating metal uptake from an artificial effluent by a *Citrobacter* species, it was observed that the removal of copper was the most sensitive to the presence of other

components (lead, cadmium, zinc, arsenic and cyanide), while the removal of lead was the least sensitive (Macaskie and Dean 1984b). A similar effect was observed in the present study. Heavy metal uptake by this *Citrobacter* species was non-specific with respect to the heavy metal cation forming the challenge. Uptake of Cd^{2+} and Pb^{2+} was comparable and occurred at a wide range of metal concentrations (Macaskie and Dean 1984b). However, Cu^{2+} accumulation was concentration dependent as at high concentrations Cu^{2+} accumulation decreased (Macaskie and Dean 1984b).

Table 5.13 Semi-quantitative EDX results of triple metal combinations (each at the same initial concentration) exposed to the different metal-resistant microbial associations cultured together.

Culture	Element % on/in cells - copper	Element % on/in cells - cadmium	Element % on/in cells - lead
A1	6.9	31.2	55.7
A2	6.8	29.9	57.6
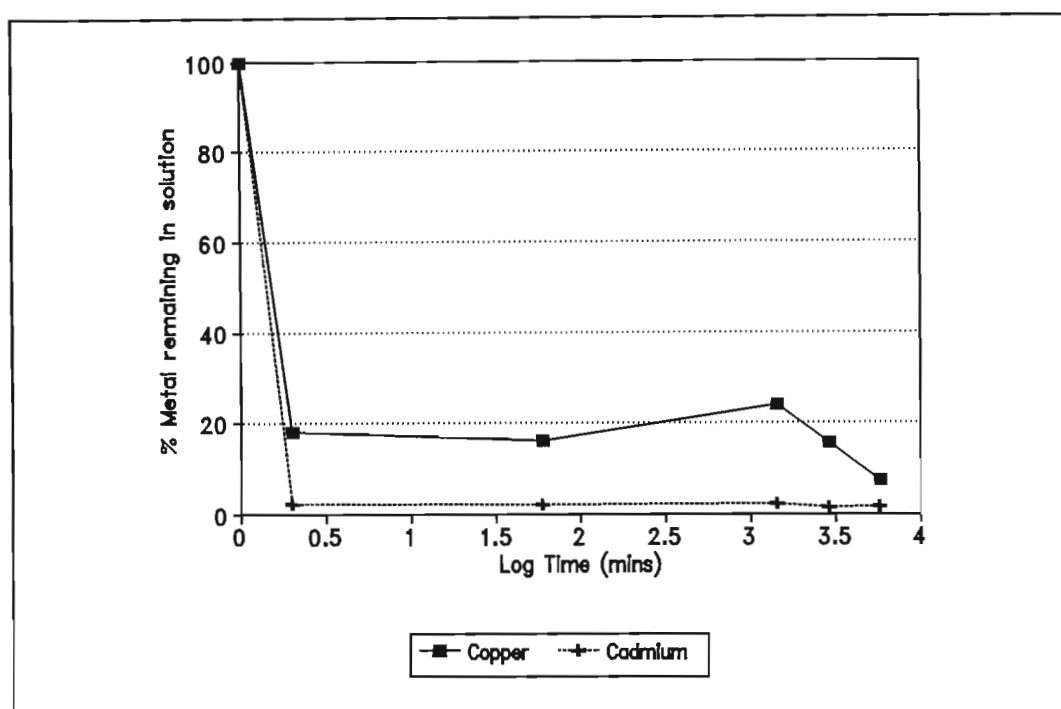
Figure 5.8 Percentage Cu^{2+} and Cd^{2+} in the supernatant of the triple metal containing medium (each at the same initial concentration) inoculated with the different metal-resistant microbial associations (metal-adapted inocula cultured together).

5.3.3.2 Different metal-adapted microbial associations cultured separately and then mixed together

Table 5.14 Semi-quantitative EDX results of triple metal combinations (each at the same initial concentration) exposed to the different metal-resistant microbial associations cultured separately and then mixed.

Culture	Element % on/in cells - copper	Element % on/in cells - cadmium	Element % on/in cells - lead
B1	3.71	27.80	62.80
B2	3.35	29.15	61.39

Once again, it appeared that more lead than copper or cadmium was associated with the microbial biomass. The concentrations of metals remaining in the medium were determined by HPLC (see section 2.2.4.1) and are shown in Fig. 5.9. In this case, Cd^{2+} uptake was better than that of Cu^{2+}. No results were obtained for the concentration of Pb^{2+} in solution.
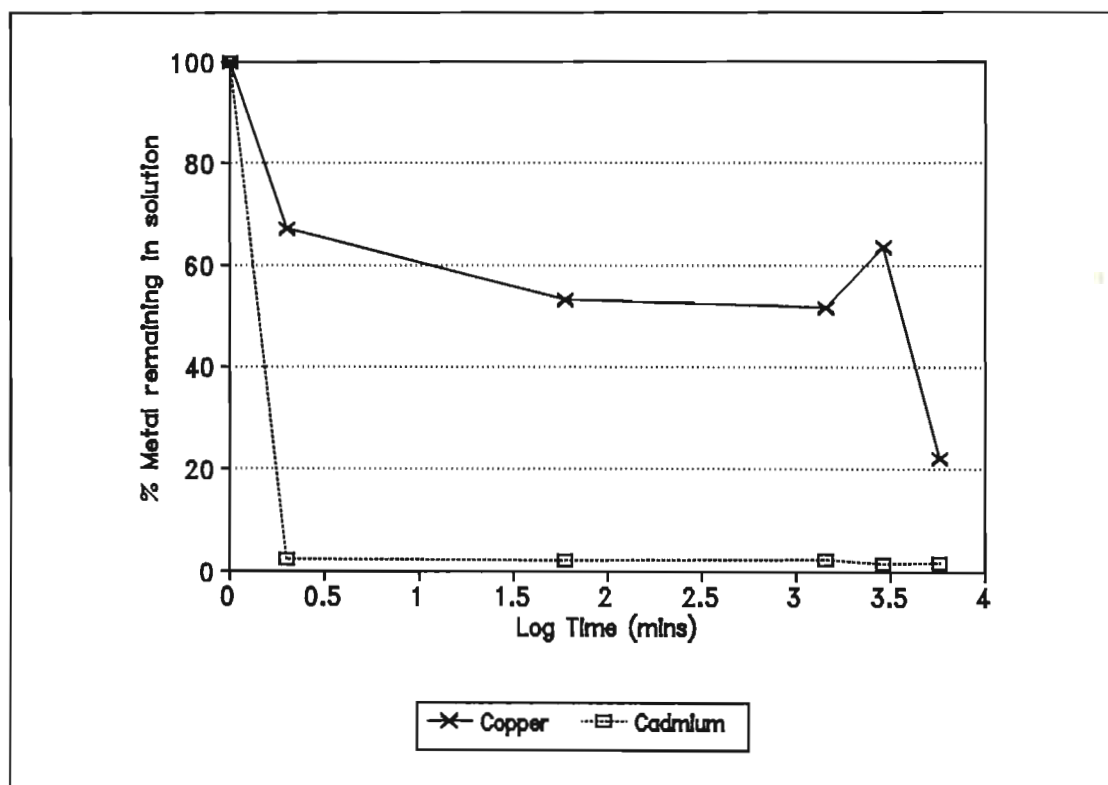
Figure 5.9 Percentage Cu^{2+} and Cd^{2+} in the supernatant of the triple metal-containing medium (each at the same initial concentration) inoculated with the different metal-resistant microbial associations (metal-adapted inocula cultured separately and then mixed).

When the different metal-resistant microbial associations were cultured together there appeared to be a greater uptake of Cu^{2+} and Cd^{2+} than when they were cultured separately and then mixed together. The uptake of Pb^{2+}, however, was not affected by the nature of the inoculum.

5.4 CONCLUSIONS

The effects of combinations of heavy metals on their uptake by the microbial associations varied. In general, uptake of a particular metal was better by those microorganism which had previously been exposed to that metal. In addition, those microorganisms which had been exposed to a specific metal, also had enhanced ability to take up other metals from solution.

In the various combinations tested, the uptake of Pb^{2+} seemed to be generally higher than that of Cu^{2+} and Cd^{2+}, and in most cases cadmium was taken up more readily than copper. Varying the proportions of the different metal-resistant microbial associations used as inocula did not affect the specific uptake of the metals to any large extent. The uptake of these metals in combination depended on the environmental conditions and on the microorganisms present in the microbial associations.

CHAPTER 6: EFFECT OF pH ON COPPER UPTAKE BY A MICROBIAL ASSOCIATION

6.1 INTRODUCTION

Of all the environmental factors affecting metal uptake, pH has been shown to be the most significant (Kuyucak and Volesky 1988). The optimum pH for metal uptake depends on the microbial association, environmental factors and the metal of interest.

The pH of a metabolising culture is known to vary with time. These changes are associated with the uptake of various anions and cations, the metabolism of carbohydrates and hydrocarbons which yields organic acids, and the degradation of proteins and other nitrogenous compounds yielding ammonia or other alkaline products (Munro 1970). A buffer should, therefore, be used which can counteract any changes in pH caused by the production of acids or bases. Good buffers should be soluble in water and should not complex with cations, or they should form soluble complexes (Munro 1970).

Thus, various microbial metal-uptake processes require a specific pH for optimum efficiency. Various buffers were used and acid-base adjustments made to investigate the effect of pH on copper uptake by a microbial association adapted to grow in the presence of 100 mg l^{-1} Cu^{2+}.

6.2 MATERIALS AND METHODS

6.2.1 Initial Experiments with Sodium-Phosphate Buffer and Acid\Base Treatments

Copper, at a concentration of 100 mg l^{-1}, was used in these experiments as fewer precipitation problems were experienced with the Cu^{2+} ion, than with Cd^{2+} and Pb^{2+} ions.

Three initial pH values were chosen, *viz.* 5.8, 7.0 and 8.0. A series of six experimental flasks was set up at each of these pH values. In each series: one culture was buffered with sodium phosphate buffer (0.2M $Na_2HPO_4.12H_2O/NaH_2PO_4.2H_2O$; the volume of each

buffer salt solution used is shown in APPENDIX E, Table 1); a second culture was periodically treated with 1M HCl or 1M NaOH to maintain the desired pH; and the third culture acted as a control with no buffer or acid-base adjustments. Each of these three treatments was set up in duplicate with one flask acting as the test (i. e. inoculated with 5 ml of inoculum from Cu^{2+}-adapted cultures) and the second flask as the uninoculated control. A concentrated (10000 mg l^{-1}, APPENDIX A) copper solution (0.5 ml) was added to all six flasks in each series and the volume made up to 50 ml with half-strength Nutrient Broth, to obtain a final Cu^{2+} concentration in the media of 100 mg l^{-1}.

The cultures and controls adjusted with acid or base were carefully monitored and the pH maintained at the desired value by addition of 1M NaOH or 1M HCl.

The pH was measured at various times after inoculation (immediately and after 30, 60, 120 minutes, 24 and 48 hours) and samples of the liquid medium (1.5 ml) were taken at the same times to monitor metal uptake. These samples were centrifuged (11000 rpm x g for 3 minutes in an Eppendorf Centrifuge Model 5413) to remove the microorganisms, filtered (0.22μm) and diluted for analysis by AAS (see section 2.2.4.2).

Each of these series of six experimental flasks was set up in duplicate with one set agitated on a rotary shaker (Thermolyne Big Bill Rotary shaker) at 200 rpm and the other kept stationary. All the cultures were incubated at 30°C.

6.2.2 Citric Acid/Na$_2$HPO$_4$.12H$_2$O Buffer

A second set of six shake flasks was set up with a different buffer system to obtain lower pH values. In this case 0.1M citric acid and 0.2M Na$_2$HPO$_4$.12H$_2$O were used to obtain media with pH values of 2.6, 3.0, 4.0, 5.0 and 5.8 by mixing together different volumes of the two buffer salt solutions (APPENDIX E, Table 2). The sixth flask was a control with no buffer added. Five ml of a Cu^{2+}-adapted culture were added to each of these 6 flasks containing 25 ml of the buffer components (APPENDIX E, Table 2) or half-strength Nutrient Broth in the case of the control flask, together with 0.5 ml of a concentrated Cu^{2+} solution (APPENDIX A) and additional half-strength Nutrient Broth to make up the

volume to 50 ml. The final Cu^{2+} concentration in the medium was 100 mg l^{-1}. Each of these treatments was duplicated but in each case the second flask was not inoculated and served as a control.

The pH was monitored and samples of the media taken as described above to determine their metal concentrations by AAS (see section 2.2.4.2).

6.2.3 Sodium-Phosphate Buffer

The experiment with sodium-phosphate buffer was repeated but at pH values near to neutrality, *viz.* 5.8, 6.4, and 7.0. These pH values were chosen because it has been shown by Ferris *et al.* (1989) that microbial biofilms are more efficient at metal uptake under neutral conditions than at alkaline pH's. The volumes of the buffer salt solutions used are shown in APPENDIX E (Table 3). A control containing an equivalent volume of half-strength Nutrient Broth instead of buffer was also prepared. The cultures were set up with 5 ml inoculum from a Cu^{2+}-adapted microbial association, 25 ml of the buffer components (APPENDIX E, Table 3) or half-strength Nutrient Broth in the control, 0.5 ml of a concentrated Cu^{2+} solution (APPENDIX A) and the volume made up to 50 ml with half-strength Nutrient Broth. The final Cu^{2+} concentration in each flask was 100 mg l^{-1}. Each treatment was duplicated except that the duplicate flask remained uninoculated and served as a control to determine the effect of the buffers on the Cu^{2+} concentration remaining in solution.

The pH was monitored and samples of the medium taken for metal analysis by AAS (see section 2.2.4.2) as described above.

6.3 RESULTS AND DISCUSSION

6.3.1 Initial Experiments with Sodium-Phosphate Buffer and Acid\Base Treatments

No significant differences were detected between the stationary and shake cultures and, therefore, the data shown are average values of the two treatments. All subsequent

experiments were carried out on the rotary shaker (200 rpm) to ensure sufficient mixing and contact between the microorganisms and the metal solutions.

Figure 6.1 shows the changes in pH and Cu^{2+} concentrations recorded for the initial experiment with the sodium phosphate buffered medium. After 48 hours exposure to copper the cultures at both pH 5.8 and pH 7.0 showed a decrease in the copper ion concentration in solution, i.e. 60% and 54% Cu^{2+} remaining in solution, respectively (Fig. 6.1A, B). The copper concentration in solution rose to above 100% in both the inoculated cultures and their corresponding controls at all three pH values. This may have been due to errors which occurred in the preparation and analyses of these samples. No results were obtained for the inoculated culture exposed to copper at a pH of 8.0 (Fig. 6.1C). In the controls with neither buffer additions nor acid-base adjustments (Fig. 6.1D) the copper concentration remaining in solution was 50% of the initial concentration after 48 hours exposure to the microbial association. However, the reduction may have been due to precipitation of the copper since the pH increased from an initial value of 6.5 to over 8 due to metabolic activity of the microorganisms. There also appeared to be a cycle of adsorption and desorption of the Cu^{2+} ions in the unbuffered control.

In the cultures buffered with sodium-phosphate the pH remained relatively constant over time, with the pH of the controls being slightly lower than those of the inoculated cultures (Fig. 6.1A-C). The increase in pH at 24 hours in the uninoculated control with no buffer or acid-base adjustments (Fig. 6.1D) was possibly due to contamination of the controls during sampling and pH measurements. The increase in the pH of the inoculated control at 120 minutes (Fig. 6.1D) showed the tendency for the microorganisms to increase the pH of the medium over time through metabolic activity (Munro 1970).
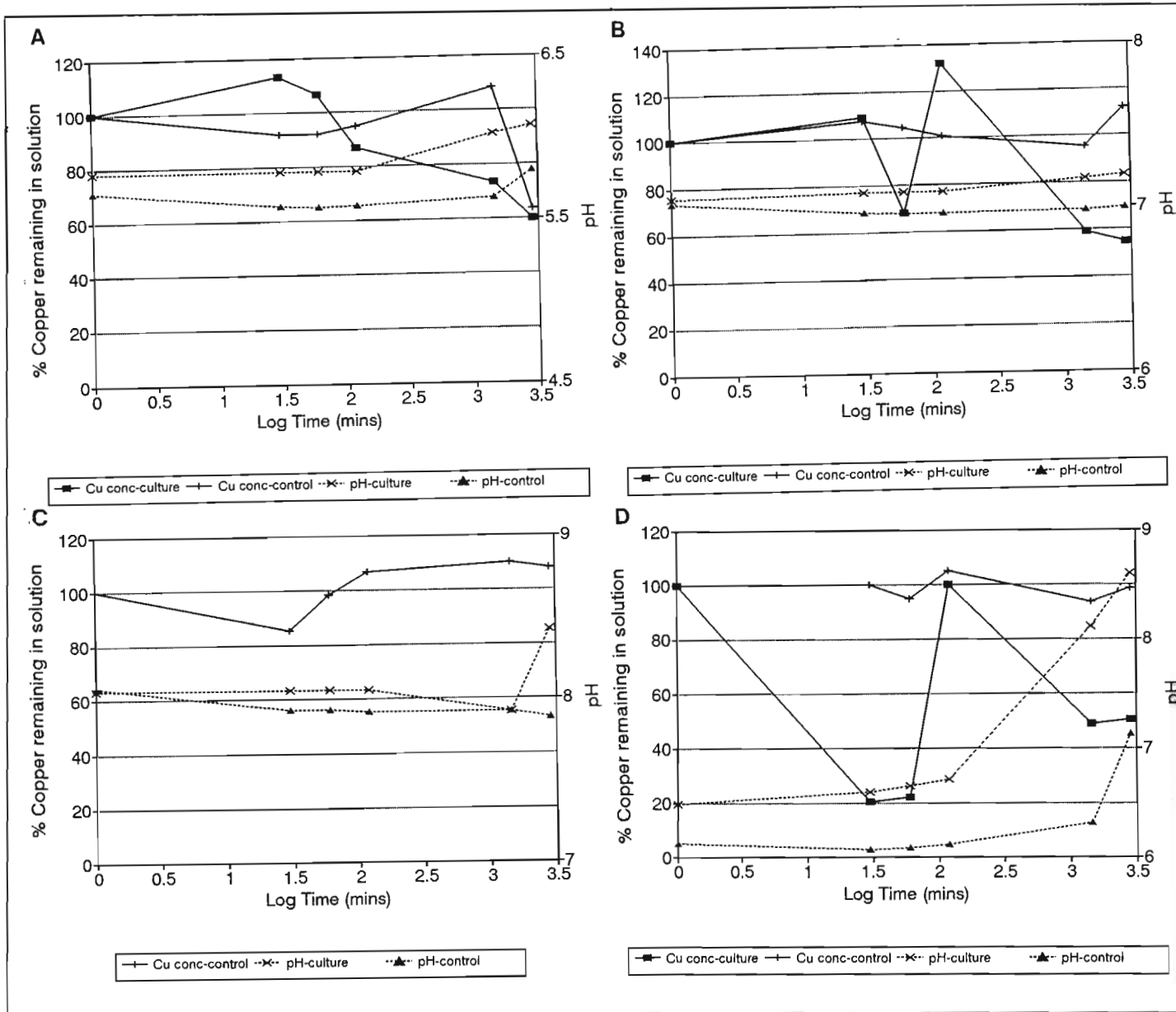
Figure 6.1   Percentage Cu^{2+} remaining in solutions buffered at initial pH 5.8 (A); initial pH 7.0 (B); and initial pH 8.0 (C) with sodium phosphate buffer; and in the unbuffered control (D), and changes in pH of the media after various periods of Cu^{2+} uptake.

Figure 6.2 shows the copper concentrations and changes in pH of the cultures and controls adjusted with 1M HCl and/or 1M NaOH. The Cu^{2+} concentrations in the inoculated cultures decreased only after 120 minutes for all the initial pH values tested, namely, 5.8, 7.0 and 8.0. At the end of the experimental period (48 hours) the percentage Cu^{2+}

remaining in solution was 54% at pH 5.8 (Fig. 6.2A); 82% at pH 7.0 (Fig. 6.2B) and 76% at pH 8.0 (Fig. 6.2C). It should be noted that the Cu^{2+} concentration in the culture adjusted to pH 5.8 (Fig. 6.2A) only decreased as the pH started to increase, perhaps indicating that the increase in pH led to precipitation of the Cu^{2+} ions. Brady and Duncan (1994) reported that insoluble metal oxides, hydroxides and carbonates tend to form near and above neutrality. The small decreases in copper concentrations in the uninoculated control flasks were probably due to contamination. The pH remained stable for only short periods following the addition of HCl or NaOH and therefore needed to be continually monitored and adjusted with acid or base. After 24 hours the pH had increased substantially in the inoculated cultures at initial pH values of 5.8 (Fig. 6.2A) and 7.0 (Fig. 6.2B). According to Munro (1970) this rise in pH may have been due to metabolism by the microorganisms. The pH increase observed in the controls was probably due to contamination introduced during sampling.

Figure 6.2   Percentage Cu^{2+} remaining in solutions adjusted to initial pH 5.8 (A); initial pH 7.0 (B); and initial pH 8.0 (C) with NaOH and HCl; and changes in pH of the media after various periods of Cu^{2+} uptake.
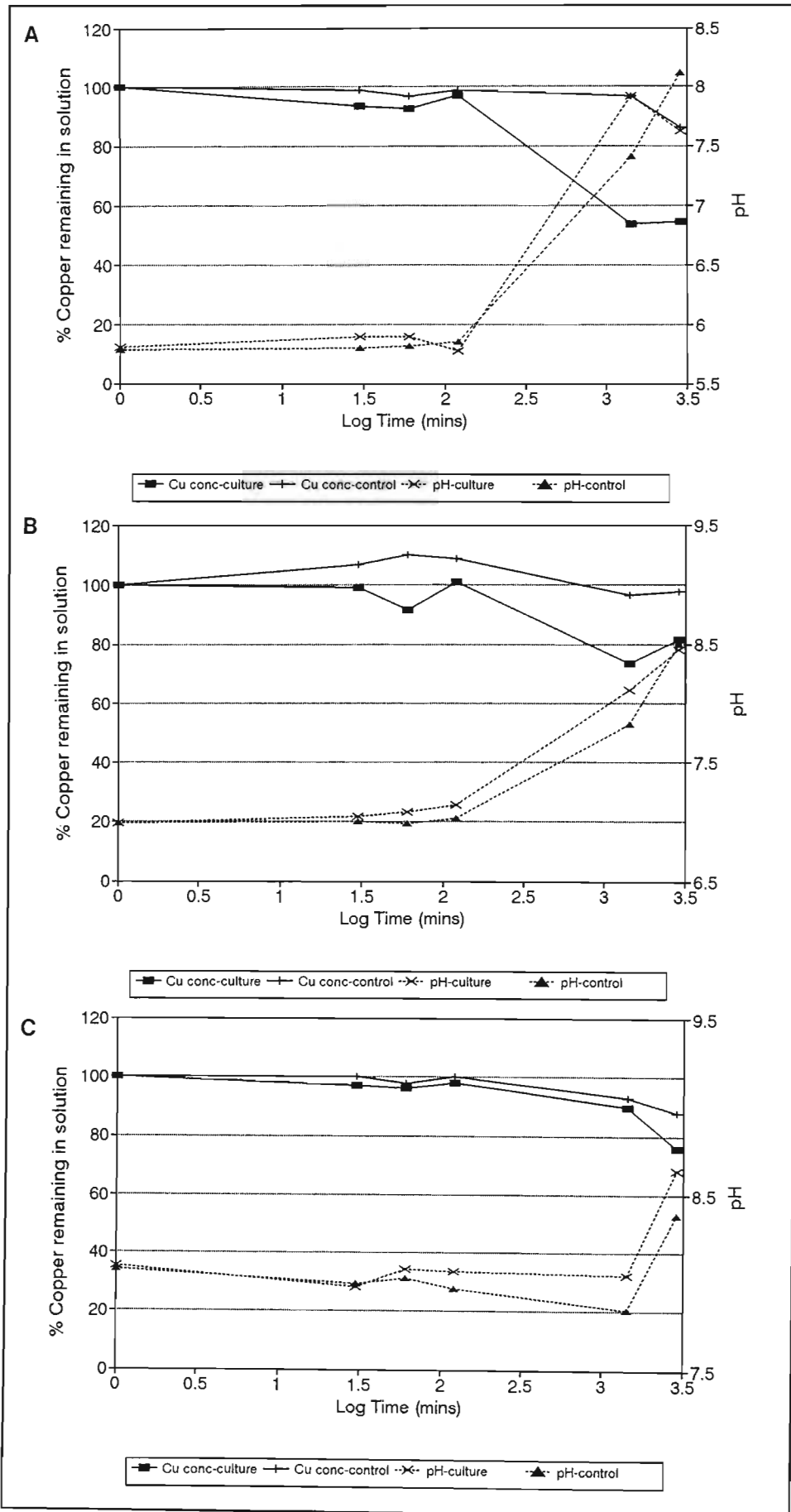
6.3.2 Citric Acid/$Na_2HPO_4.12H_2O$ Buffer

The different initial pH values used in this experiment affected the uptake of Cu^{2+} by the microbial association and the results are shown in Figure 6.3.

The amounts of copper remaining in solution after exposure to the microbial association after 48 hours at each pH value were: 71% at pH 2.6 (Fig. 6.3A); 74% at pH 3.0 (Fig. 6.3B); 90% at pH 4.0 (Fig. 6.3C); 72% at pH 5.0 (Fig. 6.3D); 74% at pH 5.8 (Fig. 6.3E); and 79% in the unbuffered control (Fig. 6.3F). Most Cu^{2+} was taken up by the cultures tested at pH 2.6 and pH 5.0 followed by the cultures tested at pH 5.8 and pH 3.0, with little Cu^{2+} taken up by the microbial association tested at pH 4.0. In general, Cu^{2+} uptake at these pH values with this buffer system was poor, possibly due to competition between the Cu^{2+} ions and the high concentration of H^+ ions present in these more acidic conditions.

The pH's set initially at 2.6, 3.0 and 4.0 remained relatively stable throughout the experiment (Figs 6.3A, B and C), while the pH of the medium initially adjusted to pH 5.0 increased to 6.5 after 24 hours (Fig. 6.3D), and the pH of the medium set initially at pH 5.8 increased to 7.5 after 24 hours (Fig. 6.3E). The pH of the unbuffered medium also increased, and rose to above 8.5 in 48 hours. These increases in pH in the initially less acidic media were possibly due to microbial metabolism, as reported by Munro (1970).

Figure 6.3   Percentage Cu^{2+} remaining in culture solutions buffered to initial pH 2.6 (A); initial pH 3.0 (B); initial pH 4.0 (C); initial pH 5.0 (D); and initial pH 5.8 (E) with citric acid/$Na_2HPO_4.12H_2O$ and in the unbuffered control (F); and changes in pH of the media after various periods of Cu^{2+} uptake.
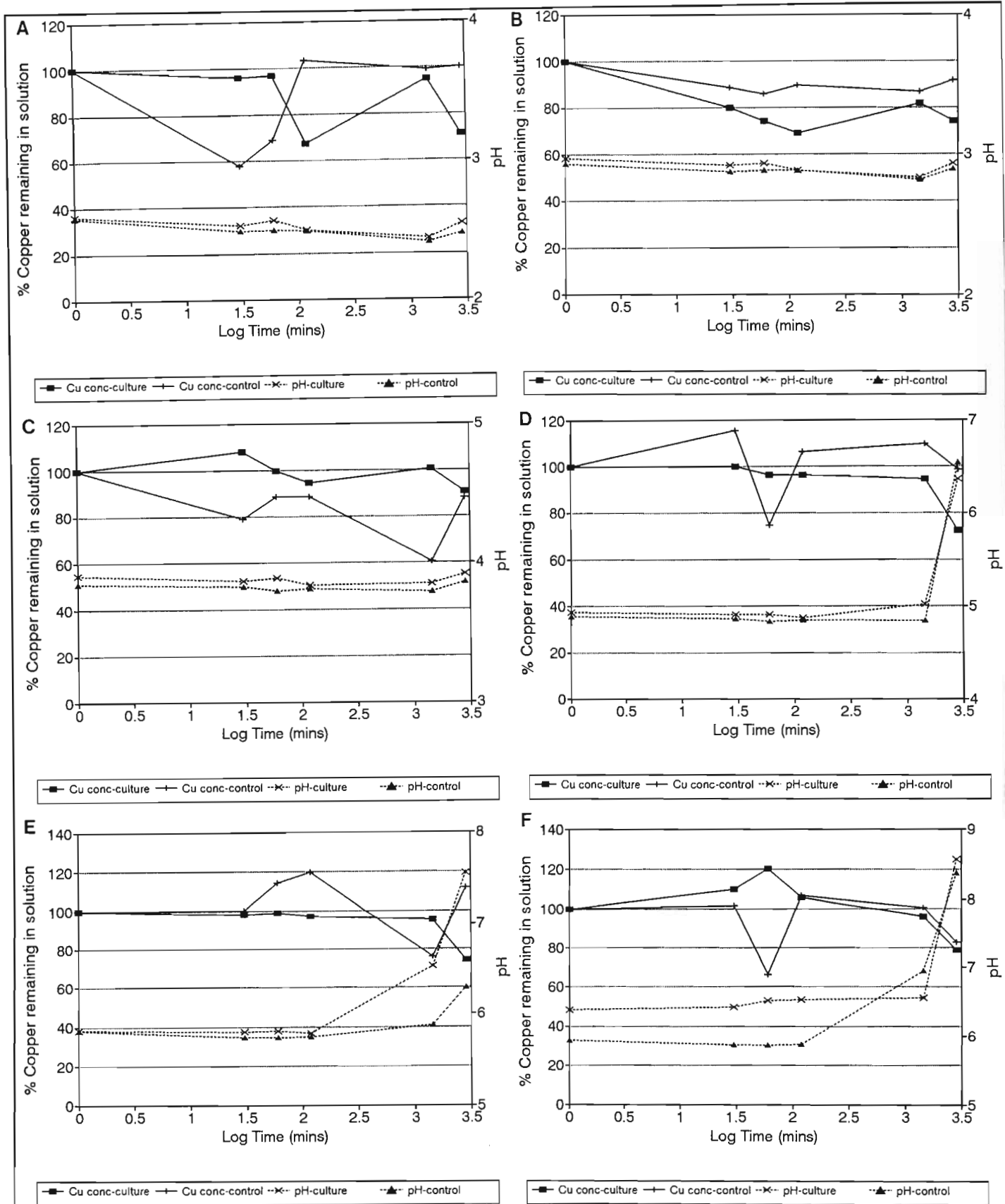
6.3.3 Sodium-Phosphate Buffer

When using media with pH values adjusted close to neutrality with sodium-phosphate buffer, it was found that Cu^{2+} uptake by the microbial association varied depending on the initial pH of the medium. The percentage Cu^{2+} remaining in solution after exposure to the microorganisms at pH 5.8 was 20% (Fig. 6.4A); 14% at pH 6.4 (Fig. 6.4B); 17% at pH 7.0 (Fig. 6.4C); and 82% in the unbuffered control (Fig. 6.4D). The cultures at pH 6.4 appeared to take up the highest concentration of Cu^{2+}, closely followed by the cultures at pH 7.0 and 5.8, with little copper uptake occurring in the unbuffered control.

The pH seemed to remain fairly constant, except in the unbuffered control (Fig. 6.4D). In the latter case the pH increased from below 6 to above 8 in the inoculated control and to above 7 in the uninoculated control (Fig. 6.4D).
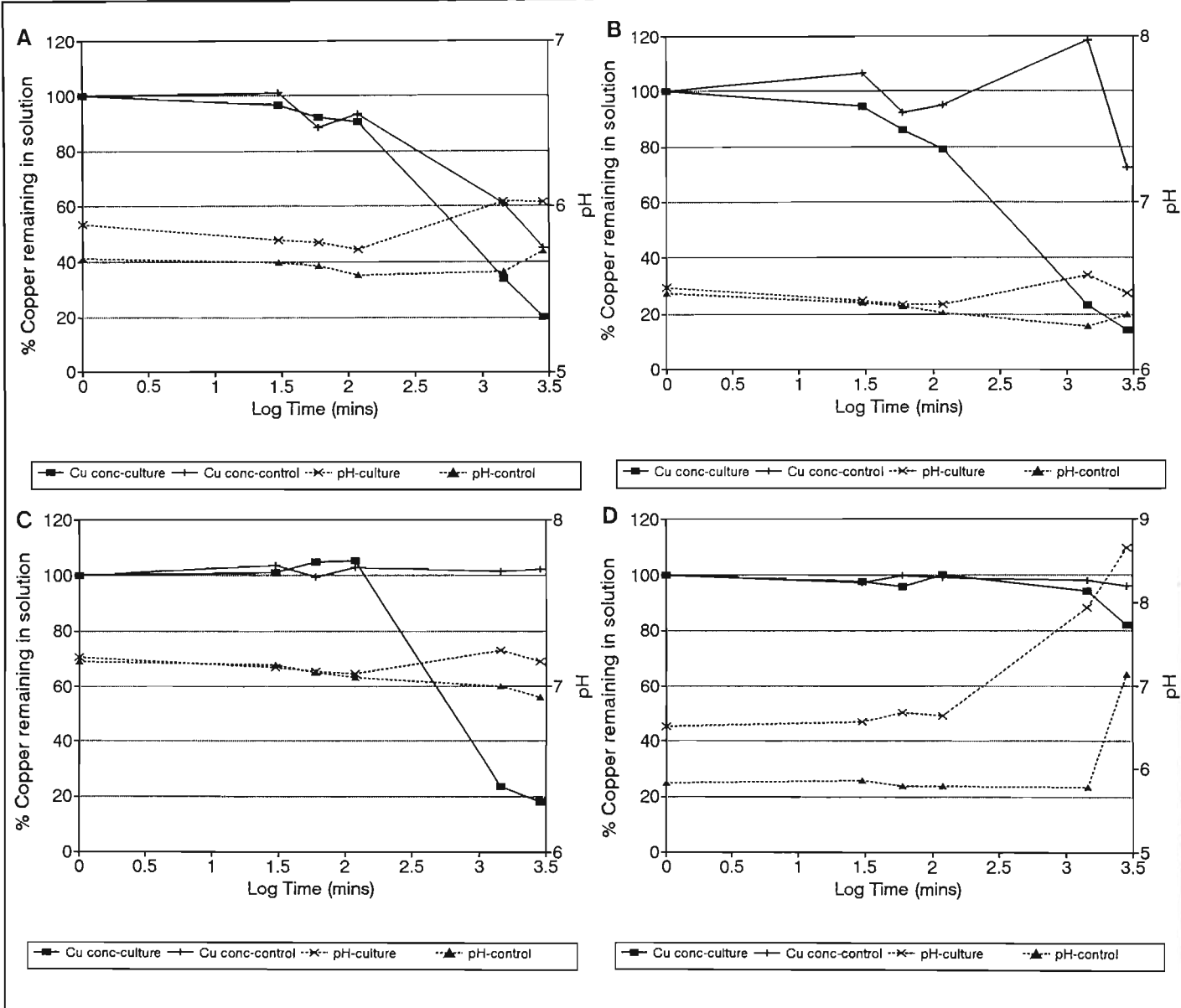
Figure 6.4   Percentage Cu^{2+} remaining in solutions buffered at initial pH 5.8 (A); initial pH 6.4 (B); and initial pH 7.0 (C) with sodium-phosphate buffer and in the unbuffered control (D); and changes in pH of the media after various periods of Cu^{2+} uptake.

6.3.4 Optimum pH for Cu^{2+} uptake by the microbial association

Table 6.1 is a summary of the percentage Cu^{2+} remaining in solution after uptake by the copper-resistant microbial association tested in media of various pH values maintained by buffer or acid-base adjustments.

Table 6.1   Summary of percentage Cu^{2+} remaining in solution after uptake by the microbial association cultured at various initial pH values in different buffer/acid-base controlled media.

pH	% Cu^{2+} remaining in solution			
	NaOH/HCl	Buffer 1 (Exp 1)	Buffer 2	Buffer 1 (Exp 2)
2.6	-	-	71	-
3.0	-	-	74	-
4.0	-	-	90	-
5.0	-	-	72	-
5.8	54	60	74	20
6.4	-	-	-	14
7.0	82	54	-	17
8.0	76	-	-	-
No pH control	50	50	79	82

Buffer 1 - 0.2M $Na_2HPO_4.12H_2O/NaH_2PO_4.2H_2O$

Buffer 2 - 0.1M Citric Acid and 0.2M $Na_2HPO_4.12H_2O$

In the experiments with phosphate buffer, large discrepancies were observed in the concentrations of Cu^{2+} ions remaining in solution (Table 6.1). At both pH values (5.8 and

7.0) considerably lower Cu^{2+} concentrations were obtained in the second experiment after 48 hours. This could be due to a higher population of microorganisms in the initial inoculum source used in the second experiment, or this microbial association had a greater proportion of Cu^{2+}-adapted microorganisms. The latter is possible since this experiment was carried out some time after the first experiment and the association was thus exposed to the Cu^{2+} for a longer period of time, which could have selected for more Cu^{2+}-adapted cells.

Differences in the Cu^{2+} concentrations remaining in solution in the buffered solutions and those adjusted with HCl or NaOH may have been due to greater concentrations of H^+ ions in the HCl/NaOH adjusted solutions blocking potential Cu^{2+}-uptake sites on the cell surfaces.

The buffer system used also affected copper uptake, with more copper remaining in solution in the medium buffered with citrate (pH 5.8; Table 6.1). It appears that citrate ions interfere with the uptake of Cu^{2+} ions, presumably by blocking potential copper binding sites. In these experiments phosphate buffer could be used to provide optimum pH conditions for efficient Cu^{2+} uptake by the microbial association.

The pH range for optimum Cu^{2+} uptake by the microbial association was 5.8-7.0 in phosphate buffered medium and 5.0-5.8 in citrate buffered medium (Table 6.1). This is similar to that found by Sahoo et al. (1992) who determined that the optimum pH for copper and cadmium uptake in Bacillus circulans was between 5 and 6. Wong et al. (1993) also used a pH of 5.5 for copper uptake by a Pseudomonas putida strain. As a mixed microbial association, containing Bacillus species amongst others, was used in the present investigation, a similar but wider pH range could be expected for optimum Cu^{2+} uptake. Saccharomyces cerevisiae was found to adsorb Cu^{2+} best in the pH range 5 to 7 (Huang et al. 1990). It was thought that the increase in Cu^{2+} uptake observed in this range was due to the ionisation of functional groups on the surface of the microorganism which served as binding sites for the metals, and that the Cu^{2+} ions became more competitive with the H^+ ions present in the medium (Huang et al. 1990).

6.4 CONCLUSIONS

Addition of HCl or NaOH to maintain the culture at the desired pH was not satisfactory as the cultures had to be monitored constantly to prevent the pH from increasing due to metabolic activity of the microorganisms. An automatic pH controlling device would need to be set up. Although buffers seemed to offer an alternative means of stabilising the pH, the buffer must be chosen very carefully. Sodium phosphate buffers would not be a good choice when Pb^{2+} or Cd^{2+} ions are present as both these ions are known to form insoluble complexes with phosphate (Hughes and Poole 1991).

The pH range for optimum Cu^{2+} uptake by the microbial association was found to be from 5.8- 7.0. However, every microbial system should be tested to determine the optimum pH for metal uptake as pH is one of the major factors affecting the removal of heavy metals from solution by microbial associations.

CHAPTER 7: THE USE OF DEAD MICROBIAL BIOMASS AND FUNGI FOR METAL UPTAKE[*]

7.1 INTRODUCTION

Both living and dead microorganisms have been found to accumulate heavy metals, and both systems have various advantages and disadvantages (Gadd 1988). Only non-living microbial cells were considered here. Dead or killed material has the advantage that it is not subject to metal toxicity, needs no nutrient supply and merely acts as an ion-exchanger (Gadd 1990). Experiments were set up to determine the metal uptake capacity of killed microbial associations.

Fungi are known to have good metal uptake systems (Gadd 1986). They often have greater tolerance than bacteria and algae towards metals and other adverse external conditions, such as low pH (Gadd 1986). Certain fungi, eg. *Aspergillus*, *Penicillium* and *Rhizopus*, species produce spherical mycelial pellets, have high metal uptake levels and good desorption characteristics (Tobin *et al.* 1994). Immobilized *Rhizopus arrhizus* was found to effectively remove low concentrations of Cu^{2+} ions from aqueous solutions (Zhou & Kiff 1991). A *Penicillium* sp. isolated from soil was found to accumulate large amounts of copper on the cell surface (Mitani & Misic 1991). Waste mycelium (*Aspergillus niger*, *Penicillium chrysogenum*, and *Claviceps paspali*) from several industrial fermentation plants has been used to remove zinc ions from aqueous solutions (Luef *et al.*1991).

Biomass related technologies will not necessarily replace existing metal ion removal treatments but may complement them in multi-disciplinary optimised processes. A fungus isolated from a copper-containing medium was investigated to determine its metal uptake capacity. Dried mycelial pellets were also used to investigate the effect of biomass concentration on metal uptake.

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[*]Part of this chapter has been submitted for publication to Applied Microbiology and Biotechnology as a paper titled "The use of *Aspergillus niger* biomass for lead uptake from aqueous systems"

Energy Dispersive X-ray microanalysis was used to investigate the metal uptake potential of the fungal biomass. The quantitative and qualitative results obtained from a combination of EDX and AAS or HPLC techniques should make it possible to calculate the amount of biomass required to efficiently remove the metal ions present at known concentrations in a given volume of effluent.

7.2 MATERIALS AND METHODS

7.2.1 Use of Dead Microbial Biomass

7.2.1.1 Dead microbial biomass in Nutrient Broth

Aliquots of cultures previously grown in the various heavy metal-containing nutrient solutions were used to inoculate 50 ml half-strength Nutrient Broth. These cultures were incubated for five days at 30°C on a rotary shaker (200 rpm). The flasks were then autoclaved (121°C/15 psi for 10 minutes) to kill the microorganisms and various aliquots of the concentrated metal solutions (APPENDIX A) were added to each of the cultures to obtain the desired metal concentrations, *viz.* Cu^{2+} - 200 mg l^{-1}, Cd^{2+} - 200 mg l^{-1}, Pb^{2+} - 200 mg l^{-1} and Cr^{3+} - 200 mg l^{-1}. Liquid samples (1.5 ml) were taken immediately, and then 10, 20, 30, 45 and 60 minutes after addition of the metal solutions, to determine if any adsorption of the metals to the dead biomass had occurred. During exposure to the metal ions the dead cultures were left on the rotary shaker (200 rpm) to ensure efficient mixing and contact between the microorganisms and metals. Samples were analyzed by HPLC (see section 2.2.4.1) after clean-up. Uninoculated controls containing half-strength Nutrient Broth and the corresponding metal concentrations were used to determine the initial metal concentrations.

7.2.1.2 Dead microbial biomass in Ringers Solution

A second experiment was undertaken with dead microbial biomass. Cultures capable of growing in the presence of heavy metals were cultured in 50 ml half-strength Nutrient Broth for three days at 30°C on a rotary shaker (150 rpm).

These cultures were autoclaved (121°C/15 psi for 15 minutes) to kill the microorganisms, and then centrifuged for 10 minutes at 5000 rpm x g (BHG Hermle Z-380 centrifuge), washed with quarter-strength Ringers solution and then recentrifuged for the same time and at the same speed. The supernatant was decanted and known volumes of Ringers and metal salt solutions were added to the pelletized biomass to obtain final metal-ion concentrations of: Cu^{2+} - 100 mg l^{-1}; Cd^{2+} - 200 mg l^{-1}; Pb^{2+} - 300 mg l^{-1} and Cr^{3+} - 200 mg l^{-1}.

Samples (1.5 ml) of the supernatant liquid were removed for metal ion analysis before exposure to the metals, immediately after metal addition and then at 15, 30, 60, 90, 120 minutes, 24 and 72 hours thereafter. All samples were centrifuged (11000 rpm x g for 3 minutes), filtered through 0.22 μm cellulose acetate membrane filters and then analyzed by HPLC (see section 2.2.4.1).

7.2.2 Use of Living Fungal Biomass

7.2.2.1 Isolation and identification of the fungus

A fungus was observed growing in a Cu^{2+}-containing culture. This fungus was initially inoculated on to half-strength Nutrient Agar plates which contained various concentrations of copper (100, 200 mg l^{-1}) and on a control plate containing no copper. Streak plating was continued until a monoculture of the fungal isolate was obtained. This was then inoculated into Malt Extract Broth (APPENDIX F). A sample of the mycelium was observed under the light microscope to identify the fungus.

7.2.2.2 Initial metal uptake by the fungus

Aliquots (50 ml) of half-strength Malt Extract Broth (APPENDIX F) containing various Cu^{2+} concentrations (50, 100, 150 mg l^{-1}) were dispensed into 100 ml Erlenmeyer flasks. A control containing no Cu^{2+} solution was also set up. Purified inoculum was obtained from the isolation experiment and a loopful of fungal mycelium was added to each flask.

These flasks were incubated stationary at 30°C and samples were taken for HPLC metal analysis (see section 2.2.4.1) immediately after inoculation (day 0) and on day 5. Corresponding uninoculated controls were established to determine the initial metal concentrations of the media.

7.2.2.3 Cu^{2+} uptake by pregrown fungal mycelium

To test the uptake of metals by an already growing fungal mass, four cultures were set up with 50 ml half-strength Malt Extract broth (APPENDIX F) and a loopful of fungal mycelium. These cultures were incubated (30°C) until a fungal mat had developed and then aliquots of a concentrated copper solution were added to three of the flasks to obtain initial Cu^{2+} concentrations of 50, 75 and 100 mg l^{-1}. The fourth flask received no metal addition and served as a control. Samples were taken immediately after metal addition and at 15, 30, 60 and 120 minutes, 24 and 48 hours, and on day 5 thereafter, to assess the metal-uptake potential of this fungus by AAS (see section 2.2.4.2). Corresponding uninoculated controls containing only medium and appropriate concentrations of metal were also prepared, to determine the initial Cu^{2+} concentrations in the media.

7.2.2.4 Comparison of Cu^{2+}-uptake by killed and living fungus

Four flasks containing 49 ml half-strength Malt Extract Broth (APPENDIX F) were inoculated with a loopful of purified fungal mycelium obtained from the initial uptake experiment. These were incubated for 12 days at 30°C. Three of the cultures were then autoclaved (121°C/10 psi for 15 minutes) to kill the fungus. The volume was adjusted to 49 ml once more and then 1 ml aliquots of a concentrated Cu^{2+} solution (APPENDIX A) were added to these three dead cultures and to the remaining living culture to obtain a concentration of 100 mg l^{-1} Cu^{2+} in each case. Liquid samples (1.5 ml) for metal analysis by AAS (see section 2.2.4.2) were taken immediately after metal addition and at 15, 30, and 60 minutes and 24 hours thereafter. A corresponding uninoculated control containing only medium and 100 mg l^{-1} Cu^{2+} was established to determine the initial copper concentration in the medium. The semi-quantitative EDX technique was used to investigate Cu^{2+} uptake by the fungal biomass after 24 hours (see section 5.2.1.1).

7.2.2.5 Fungal growth on plates supplemented with metals

Mycelium of the fungus was inoculated onto half-strength Malt Extract Agar (APPENDIX F) plates supplemented with 100 mg l^{-1} or 200 mg l^{-1} Cu^{2+}, 100 mg l^{-1} Cd^{2+} or 100 mg l^{-1} Pb^{2+}, to determine the toxic effect of the metals on fungal growth.

7.2.2.6 Metal uptake by fungal mycelial mats cultured in Currie's Liquid Medium

Metal uptake by the fungus was also determined using mycelium cultured in Currie's liquid medium (APPENDIX G). Spores were inoculated onto the surface of the medium, allowed to germinate and grown for five days at 30°C to form a mycelial mat. The mat was washed in sterile distilled water and then cut up into 6 portions weighing approximately 0.5 g each and 6 portions weighing approximately 0.25 g each. One portion was then transferred to each of twelve 100 ml Erlenmeyer flasks.

Various copper ion solutions (50 ml) (Cu^{2+} concentrations indicated in Table 7.1) with no nutrients present were then added to each of the flasks. These flasks were incubated on a rotary shaker (200 rpm) at 30°C. The pH's of the metal ion solutions were initially decreased with 1 M HCl to between 4 and 5. Liquid samples (1.5 ml) for metal analysis by AAS (see section 2.2.4.2) were taken before exposure of the fungus to the metal, immediately after exposure to the metal solutions and 30, 60, 120 and 180 minutes and 24 hours thereafter.

Table 7.1   Wet mass of mycelial mats prepared for exposure to Cu^{2+} ion solutions.

Culture	Wet mass of mycelial mats (grams)			
	Sample 1	Sample 2	Sample 3	Sample 4
50 mg l^{-1} Cu^{2+}	0.547	0.535	0.209	0.221
100 mg l^{-1} Cu^{2+}	0.540	0.545	0.220	0.206
200 mg l^{-1} Cu^{2+}	0.537	0.558	0.240	0.245

Fungal mycelium was similarly washed, cut up and weighed for exposure to Pb^{2+} and Cd^{2+} ion solutions (concentrations shown in Table 7.2) with no nutrients present, and sampled as described above. The following wet weights of mycelium were used:

Table 7.2   Wet mass of mycelial mats prepared for exposure to Pb^{2+} and Cd^{2+} ion solutions.

Culture	Wet mass of mycelial mats (grams)	
	Sample 1	Sample 2
50 mg l^1 Cd^{2+}	0.328	0.589
100 mg l^1 Cd^{2+}	0.233	0.524
200 mg l^1 Cd^{2+}	0.203	0.650
50 mg l^1 Pb^{2+}	0.251	0.663
100 mg l^1 Pb^{2+}	0.256	0.613
200 mg l^1 Pb^{2+}	0.235	0.553

7.2.2.7 Metal uptake by fungal pellets cultured in Currie's liquid medium

Currie's liquid medium (APPENDIX G) was used to culture fungal pellets in submerged culture. The medium in the flasks was inoculated with spores of the fungus and incubated for 5 days at 30°C on a rotary shaker (200 rpm). The fungal pellets were washed with sterile distilled water and weighed (Table 7.3) into flasks as before, and then 50 ml of the Cu^{2+}, Cd^{2+} and Pb^{2+} solutions with no nutrients present added. Liquid samples for metal analysis by AAS (see section 2.2.4.2) or HPLC (see section 2.2.4.1) were taken before metal contact with the fungus, immediately after metal addition and 30, 60, 120, 180 minutes and 24 hours thereafter. Some of these pellets were analyzed by the energy dispersive microanalysis technique (EDX) (see section 3.3.3).

Table 7.3 Wet mass of fungal pellets prepared for exposure to Cu^{2+}, Cd^{2+} and Pb^{2+} solutions.

Culture	Wet mass of fungal pellets (grams)	
	Sample 1	Sample 2
50 mg l^{-1} Cu^{2+}	0.261	0.572
100 mg l^{-1} Cu^{2+}	0.199	0.553
200 mg l^{-1} Cu^{2+}	0.217	0.506
50 mg l^{-1} Cd^{2+}	0.248	0.500
100 mg l^{-1} Cd^{2+}	0.254	0.536
200 mg l^{-1} Cd^{2+}	0.232	0.552
50 mg l^{-1} Pb^{2+}	0.207	0.537
100 mg l^{-1} Pb^{2+}	0.235	0.563
200 mg l^{-1} Pb^{2+}	0.222	0.560

7.2.2.8 **Lead uptake by dried mycelial pellets and comparison with uptake by undried mycelial pellets**

Currie's liquid medium (APPENDIX G) was used to grow up fungal pellets in submerged culture, as described above. The resultant fungal pellets were washed twice with sterile distilled water, and weighed into 100 ml Erlenmeyer flasks. A proportion of the pellets was dried in an oven at 60°C under vacuum overnight before weighing so that exact weights of mycelium (i.e. 50, 100, 200 and 500 mg (dry wt)) could be obtained, to investigate the effect of biomass concentration on metal uptake. Aliquots (50 ml) of a lead nitrate solution (Pb^{2+} concentrations of 50, 100, 200 and 500 mg l^{-1}) adjusted initially to approximately pH 4 with 1 M HCl and with no nutrients present, were added to each of the flasks containing the dried and undried mycelial pellets. These experiments were made in triplicate. The flasks were incubated on a rotary shaker (200 rpm) at 30°C, and liquid samples (1.5 ml) for metal analysis were taken immediately following exposure to the metal solutions and 30, 60, 120 and 180 minutes, 24 and 48 hours thereafter. Samples for metal analysis were also taken from the various Pb^{2+}-containing solutions before mixing with the mycelial biomass. EDX investigations were also carried out.

7.3 RESULTS AND DISCUSSION

7.3.1 Use of Dead Microbial Biomass

7.3.1.1 Dead microbial biomass in Nutrient Broth

Samples of the supernatant were analyzed by HPLC to determine the concentration of metal remaining in solution and hence by subtraction the amount taken up by the dead biomass. There appeared to be efficient uptake of all the metals over the duration of the experiment (Fig. 7.1). However, organics in the Nutrient Broth may have complexed with some of the metals leading to their precipitation and thus giving an overestimate of the amount of metal taken up by the dead microbial biomass. No results were obtained for the Pb^{2+}-containing samples due to the precipitation problems encountered.
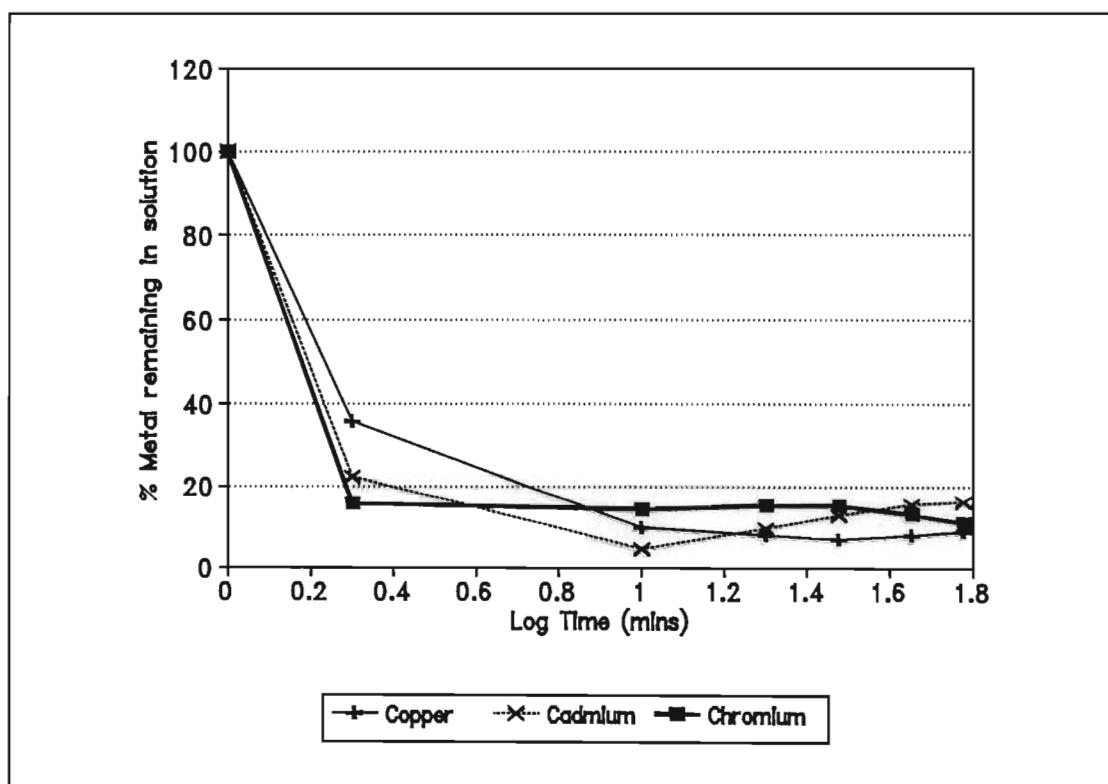
Figure 7.1 Percentage Cu^{2+}, Cr^{3+} and Cd^{2+} remaining in solution after metal uptake by dead microbial biomass in half-strength Nutrient Broth.

The accumulation of both lead and uranium by killed *Streptomyces* cells was found to be comparable with that of living cells and in some cases was slightly greater for both metals. Similar results have also been obtained for fungi, algae and bacteria (Golab *et al.* 1991). In general, the uptake of metals by the killed, unwashed cells was good in comparison with the living cultures used in the other experiments in this investigation. It must be kept in mind that dead cells may only act as ion-exchangers and are not able to use any intracellular uptake mechanisms which the living cells may make use of. Many workers have found little difference between living and killed biomass (see Siegel *et al.* 1990 for examples). However, environmental factors and the nature and composition of the microbial association play a major role in the metal uptake potential of microbial biomasses. It is important to note that when using killed cells the method employed to inactivate the biomass (heat or solvent) is likely to modify the surface properties of the biomass and, thus, affect its metal-binding properties (Brown 1991).

7.3.1.2 Dead microbial biomass in Ringers solution

The killed biomass took up no Cd^{2+} and only about 20% of the Cr^{3+} present, while approximately 40% of the copper was removed from solution (Fig. 7.2). Again no results were obtained for the Pb^{2+} samples due to the precipitation problems encountered. It is possible that the Ringers solution altered the chemistry of the environment thereby affecting uptake of the metals and leading to the low metal-uptake values obtained.
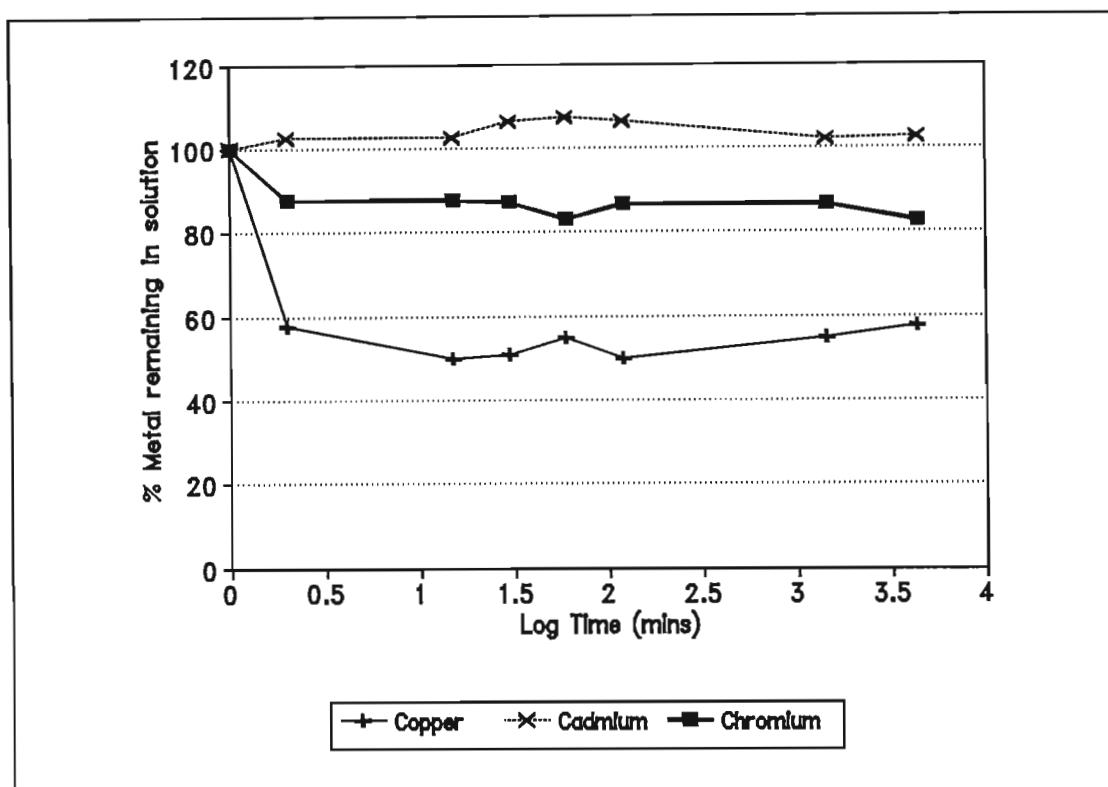
Figure 7.2 Percentage Cu^{2+}, Cr^{3+} and Cd^{2+} remaining in solution after uptake by dead microbial biomass in Ringers solution.

7.3.2 Use of Living Fungal Biomass

7.3.2.1 Isolation and identification of the fungus

On initial plating of the fungus the mycelium was found to be closely associated with a single bacterial species or several bacterial species. Streak plating was thus carried out to obtain monocultures of the fungus. The purified isolated fungus was found to grow in the presence of 100 mg l^{1} Cu^{2+} and not at 200 mg l^{1} Cu^{2+}, as it was originally able to do in the mixed microbial association.

Using light microscopy the fungus was identified as an *Aspergillus* species (Plate 7.1), as flask-shaped phialides were observed (Plate 7.1), as well as a swollen vesicle on the end of the conidiophore (Plate 7.1B). Using the dissecting and light microscopes it was further

determined that this species belonged to the *Aspergillus niger* group and most probably *Aspergillus niger*; the conidia were dark brown to black, 4-5μm in diameter and irregularly roughened; the conidial heads split into divergent columns as is characteristic of *Aspergillus niger* (Raper and Fennell 1965). For the purpose of this study the fungus will be known as *Aspergillus niger* strain 4. Other workers have previously used *Aspergillus niger* for metal uptake (Luef *et al.* 1991). It was also reported by Campbell and Martin (1990) that the most successful fungi in initial tests for metal uptake were species of *Penicillium* and *Aspergillus*.
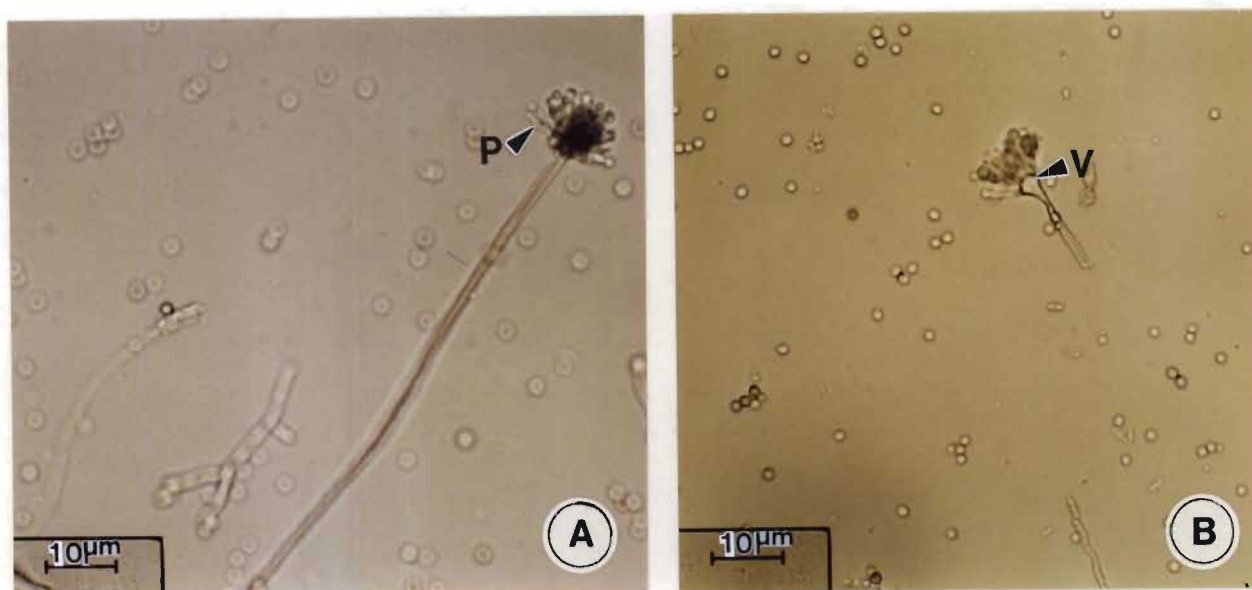
Plate 7.1   Light micrographs of *Aspergillus* species; A. phialide (P); B. vesicle on conidiophore (V).

7.3.2.2 Initial metal uptake by the fungus

After 48 hours fungal growth was observed in the control flasks. Some growth had taken place at 50 mg l^{-1} Cu^{2+}, while only very slight growth had occurred at 100 mg l^{-1} Cu^{2+} and no growth at 150 mg l^{-1} Cu^{2+}. After 5 days there was still no growth at 150 mg l^{-1} Cu^{2+}, but growth at 100 mg l^{-1} Cu^{2+} had increased. Supernatant samples taken immediately after inoculation (day 0) and on day 5 were analyzed by HPLC (see section 2.2.4.1), together with samples of the corresponding uninoculated controls.

In the control flasks, a mat of mycelium which covered the entire surface of the medium had developed after 48 hours. Growth in the 50 mg l^1 Cu^{2+}-containing culture was found to be more submerged with surface growth only starting after 7 days.

Copper uptake was low in the 50 mg l^1 and 150 mg l^1 Cu^{2+} cultures, possibly due to the small amount of fungal mycelium present, while uptake in the 100 mg l^1 Cu^{2+} culture was better at approximately 28% Cu^{2+} removal (Table 7.4).

Table 7.4 Percentage Cu^{2+} uptake by living fungal cultures (initial experiment) after 5 days.

Culture	Initial metal ion conc * (mg l^1)	Metal ion conc on day 0 (mg l^1)	Metal ion conc on day 5 (mg l^1)	% Metal uptake after 5 days
50 mg l^1 Cu^{2+}	53	47.5	45	15.1
100 mg l^1 Cu^{2+}	102.4	88	74	27.7
150 mg l^1 Cu^{2+}	150.4	126.8	130.5	13.2

* = Cu^{2+} concentration of corresponding uninoculated controls.

7.3.2.3 Cu^{2+} uptake by pregrown fungal mycelium

After 8 days incubation the fungus had grown sufficiently and the copper solution was added. It should be noted, however, that the amount of fungal mat in each flask varied and that the rates of uptake, if any, were thus not comparable.

There was little Cu^{2+} uptake from the 75 and 100 mg l^1 Cu^{2+} solutions, while about 40% of the Cu^{2+} from the 50 mg l^1 sample had been taken up after 48 hours (Fig. 7.3). The apparent increase in Cu^{2+} concentration in the 50 mg l^1 sample on day 5 could have been due to evaporation of the liquid leading to concentration of the metal ions (Fig. 7.3). The percentage copper taken up varied depending on the initial concentrations present as well as the amount of biomass present.
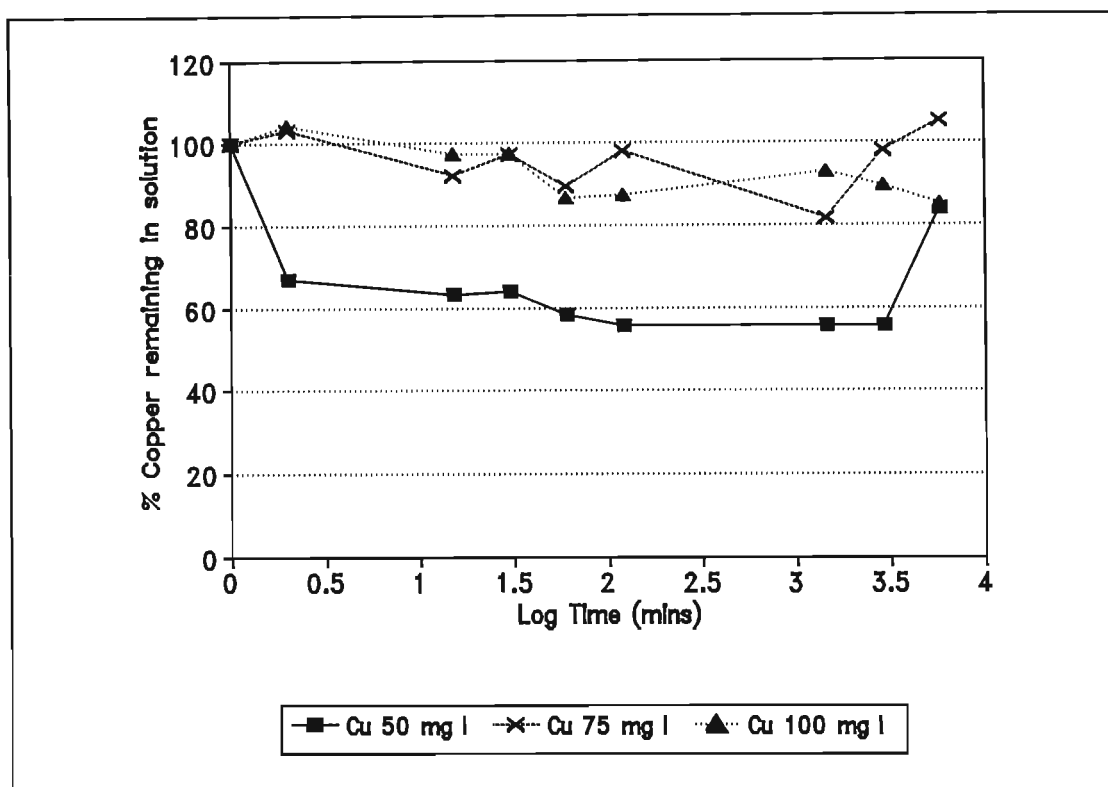
Figure 7.3 Percentage Cu^{2+} remaining in solution after uptake by pregrown fungal mycelium.

7.3.2.4 Comparison of Cu^{2+}-uptake by killed and living fungus

Using the semi-quantitative EDX technique, copper was found in different amounts in the following cultures (Table 7.5 and Fig. 7.4).

Table 7.5 Element % of copper associated with fungal mycelial cultures.

Culture	Element % on/in fungus - copper
F1 (dead)	48.3
F2 (dead)	55.2
F3 (dead)	24.8
F4 (living)	36.9

In the EDX spectra (Fig. 7.4), the phosphorus and sulphur originated from the proteins and other materials found within the fungal mycelium. Note that carbon, hydrogen, nitrogen and oxygen were not detected by this technique. In general the killed fungal mycelium appeared to take up more Cu^{2+} than the living culture.
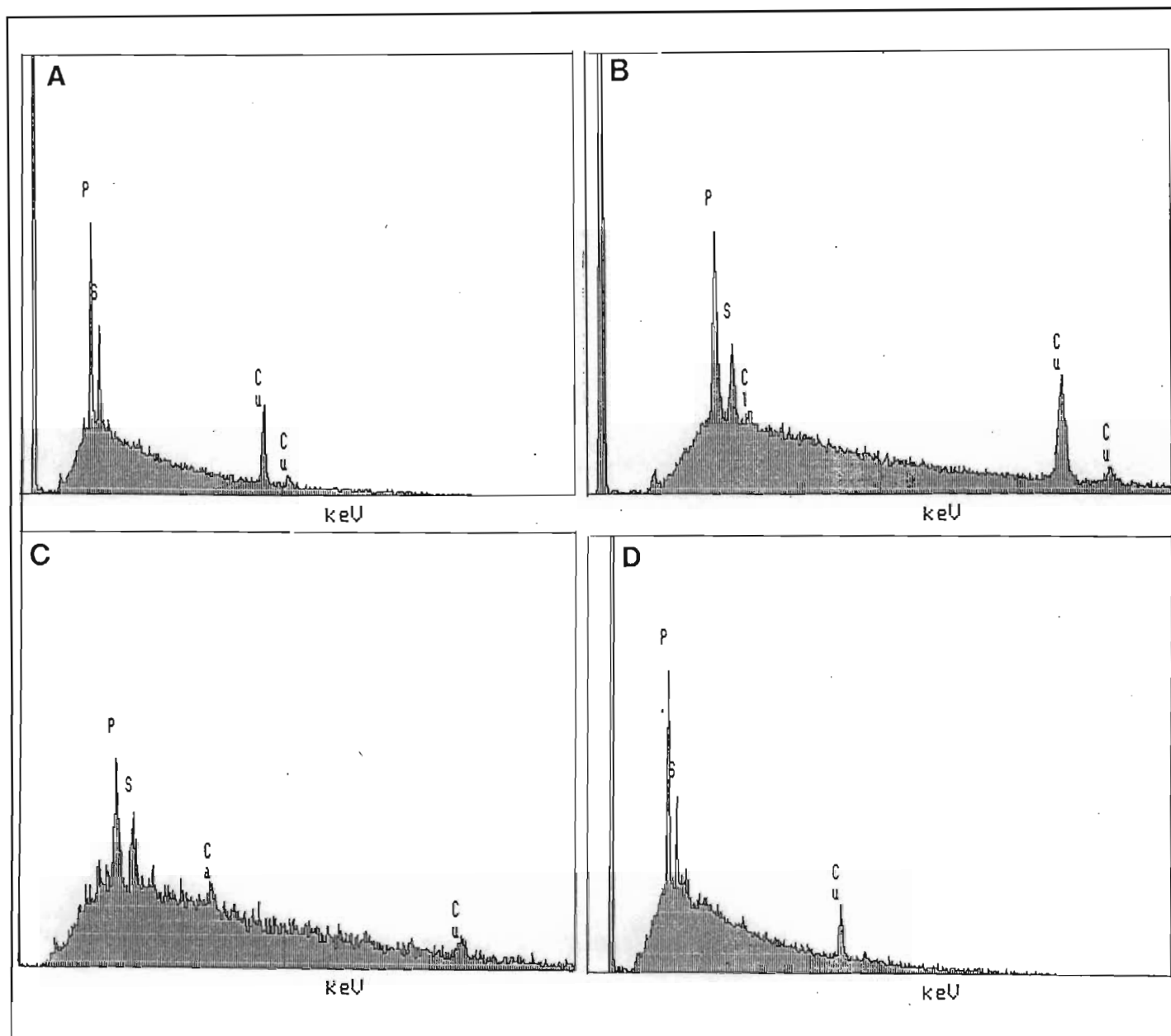
Figure 7.4 EDX spectrums of fungus cultures exposed to 100 mg l^{-1} Cu^{2+}; A. F1 (dead); B. F2 (dead); C. F3 (dead); D. F4 (living).

AAS analysis of the supernatant samples showed that most of the Cu^{2+} was still present in solution after 24 hours (Fig. 7.5). Culture F2 showed the greatest uptake (25%) which corresponded to the large copper peak observed on the EDX spectrum (Fig. 7.4B). The supernatant from the living fungal culture F4 showed the least uptake of Cu^{2+} from solution, when measured by AAS (Fig. 7.5) but not the lowest element % copper when analyzed by EDX (Table 7.5). This could have been due to an error in the semi-quantitative EDX analysis of culture F4.
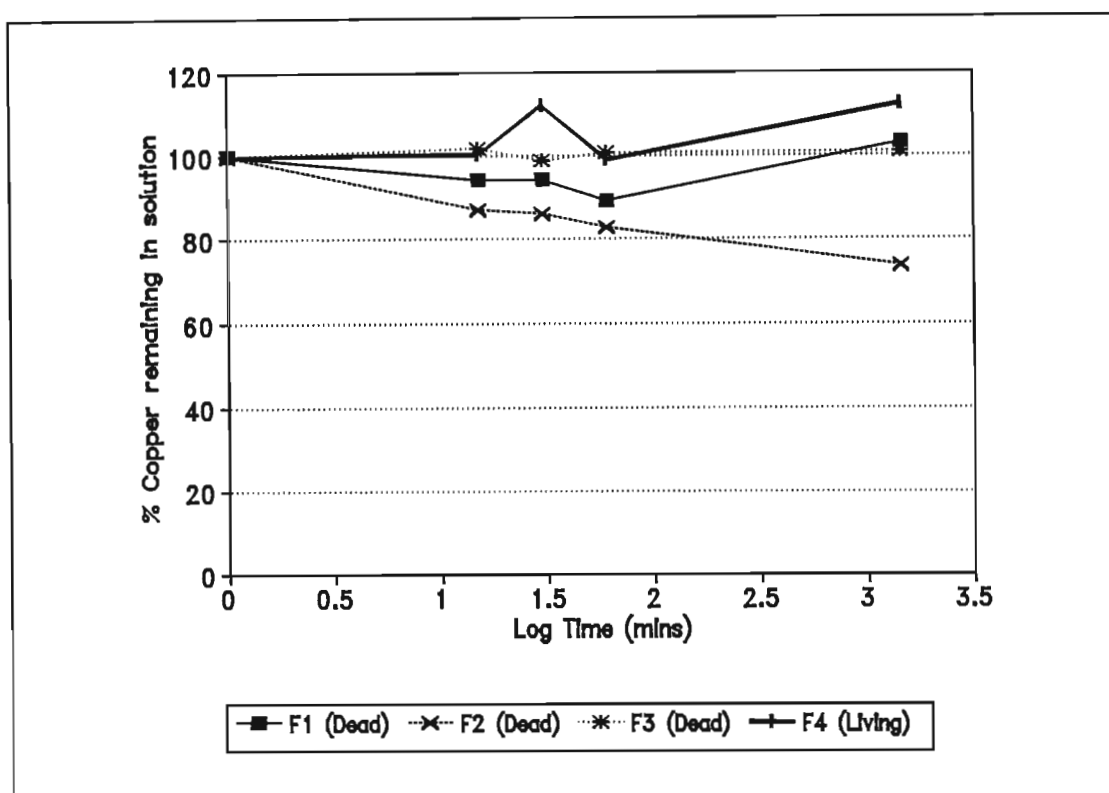
Figure 7.5 Percentage Cu^{2+} remaining in solution after 24 hours exposure to the killed and living fungal mycelium.

The accumulation of heavy metals by both living and heat-killed mycelium is largely an adsorptive process which is thought to involve inorganic ligands on the microbial surface (Duddridge and Wainwright 1980). The results obtained in this study suggested that Cu^{2+} uptake was more efficient by the killed fungus although efficient metal uptake by living fungal samples was also observed in other experiments (Fig. 7.3). Duddridge and Wainwright (1980) reported that less metal was accumulated by heat-killed mycelia, as it

was thought that heating altered or destroyed some metal-binding ligands present in the fungal wall. This was not observed in the present study.

7.3.2.5 Fungal growth on plates supplemented with metals

The fungus was able to grow in the presence of 100 mg l^{-1} Cu^{2+}, while growth at 100 mg l^{-1} Cd^{2+} and Pb^{2+} was limited, and bacterial contamination occurred in some of the cultures (Table 7.6).

Table 7.6 Visual assessment of fungal growth on plates.

Culture	Amount of growth after 24 hours	Amount of growth after 48 hours
Malt extract Agar + 100 mg l^{-1} Cu^{2+}	Very little growth	White mycelial growth
Malt extract Agar + 200 mg l^{-1} Cu^{2+}	Very little growth	No growth
Malt extract Agar + 100 mg l^{-1} Cd^{2+}	Slight growth	Fungal and bacterial growth
Malt extract Agar + 100 mg l^{-1} Pb^{2+}	Good growth, possibly bacterial	Fungal and bacterial growth
Malt extract Agar (Control)	Good mycelial growth	Good mycelial growth

7.3.2.6 Metal uptake by fungal mycelial mats cultured in Currie's Liquid Medium

The mycelial mats exposed to the various Cu^{2+} concentrations showed little metal uptake over 24 hours (Fig. 7.6A, B and C); perhaps a longer exposure time was required. The mycelium which took up 40% of the Cu^{2+} after 24 hours (culture Cu 200 (2); Fig. 7.6C) became blue in colour, possibly as a result of copper phosphate formation. This coloration of the mycelium was taken as evidence of Cu^{2+} uptake. The wet mass of mycelium used (Table 7.1) did not seem to significantly affect the amount of metal removed from solution with the larger biomass samples (samples 1 and 2) generally removing only slightly more metal than the lower biomass samples (samples 3 and 4) (see Fig. 7.6). Precise weights

could not be obtained using wet mass as the volume of water present in each sample varied.
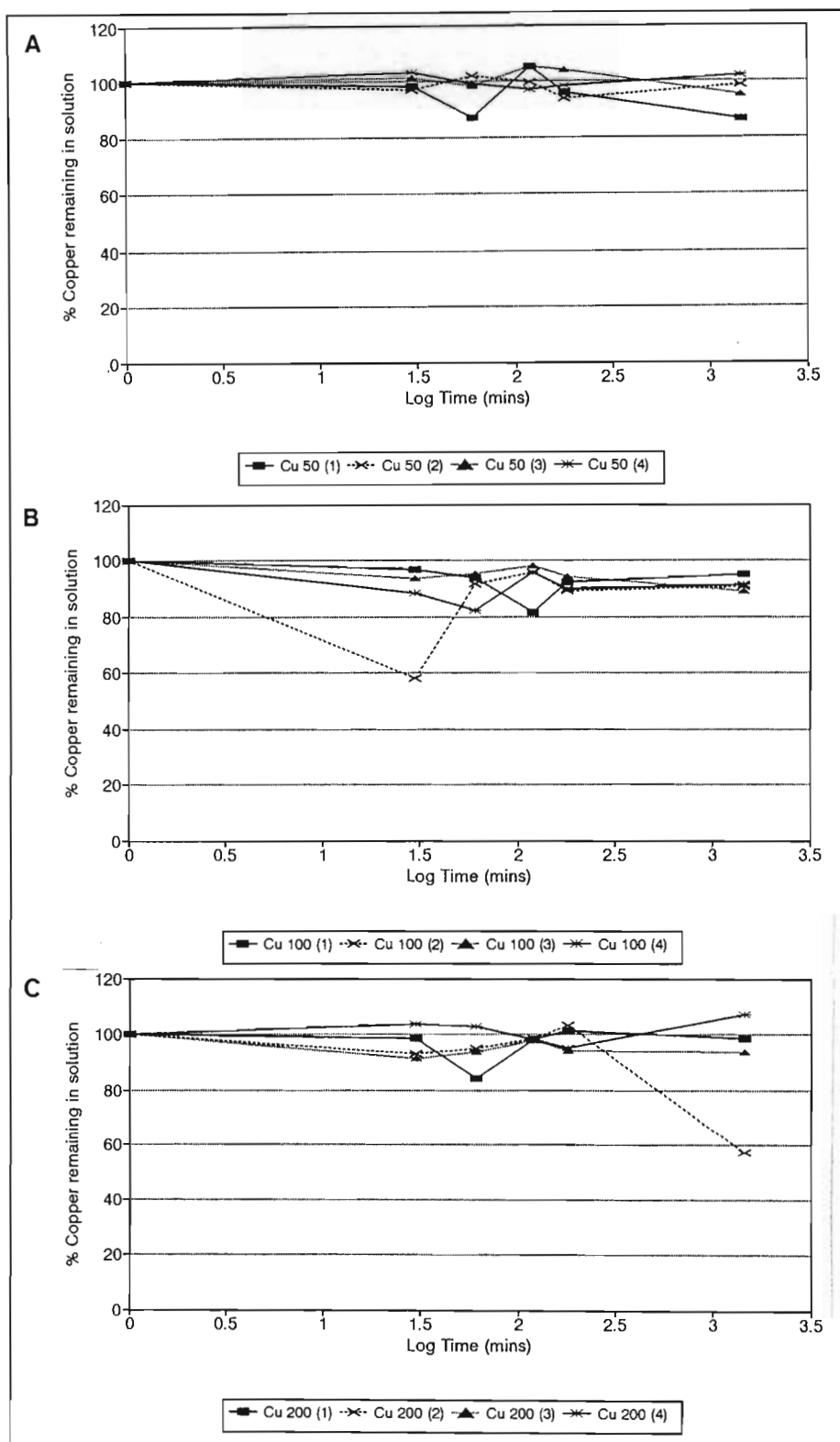
Figure 7.6 Percentage Cu^{2+} remaining in solution after uptake by mycelial mats; A. Cu^{2+} (50 mg l^{-1}); B. Cu^{2+} (100 mg l^{-1}); C. Cu^{2+} (200 mg l^{-1}). The numbers in parentheses indicate the sample numbers corresponding to those in Table 7.1.

The fungal mycelium exposed to Pb^{2+} showed efficient uptake of the metal, except for the mycelium exposed to Pb^{2+} at 200 mg l^1 (Fig. 7.7) where there appeared to be complete uptake of the metal ions in the initial 30 minutes but then the concentration of lead in solution increased to over 100% before decreasing again in 24 hours. There was obviously an error in the analysis of some of the samples.
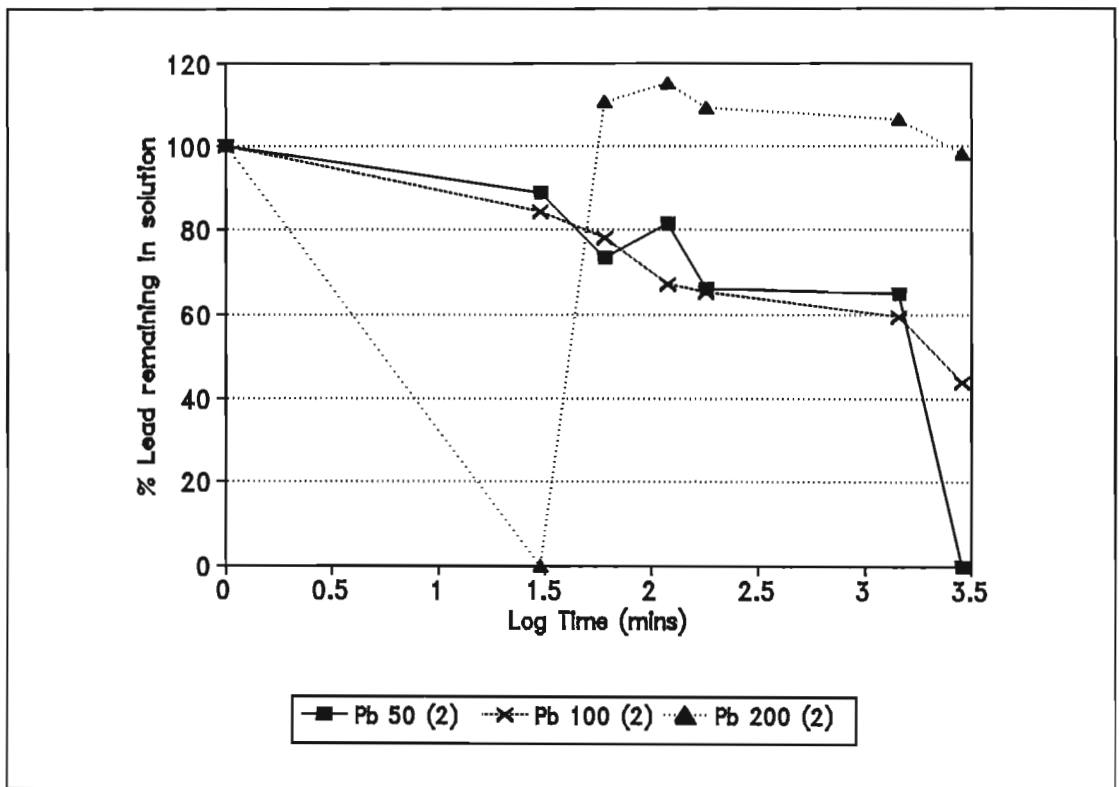
Figure 7.7 Percentage Pb^{2+} remaining in solution after uptake by mycelial mats. The numbers in parentheses indicate the sample number corresponding to those in Table 7.2.

The uptake of Cd^{2+} was generally poor, with little or no uptake by the fungus exposed to 50 and 200 mg l^1 Cd^{2+}. Sample Cd^{2+} concentrations of over 100% were obviously erroneous, possibly due to problems with the analysis. Cd^{2+} uptake by the mycelium exposed to 100 mg l^1 Cd^{2+} was erratic (Fig. 7.8), indicating possible cycles of metal adsorption and desorption by/from the mycelium.
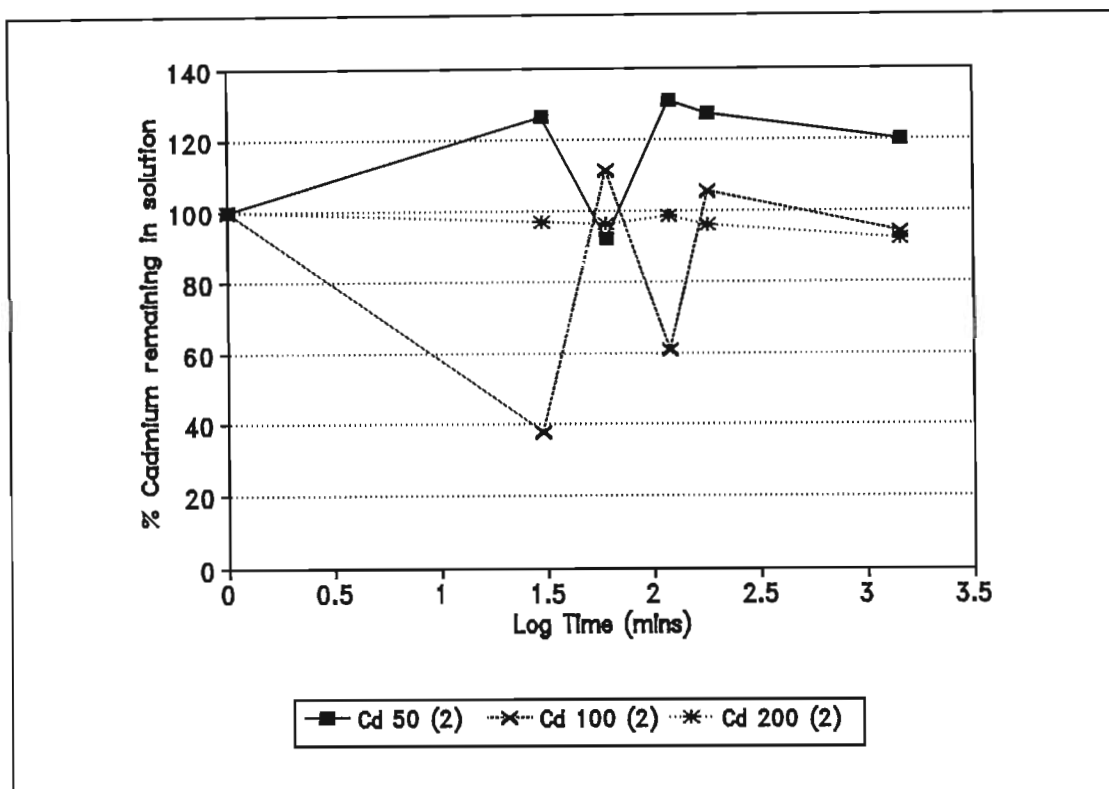
Figure 7.8 Percentage Cd^{2+} remaining in solution after uptake by mycelial mats. The numbers in parentheses indicate the sample numbers corresponding to those in Table 7.2.

7.3.2.7 Metal uptake by fungal pellets cultured in Currie's liquid medium

The pellets exposed to the copper solutions were left in contact with the metal for an extended time period and the mycelial mass took on a blue colour after 60 days. Samples of the culture supernatant were analyzed by HPLC (Fig. 7.9) (see section 2.2.4.1), and the fungal pellet samples by EDX (Fig. 7.10).

Figure 7.9 shows the uptake of Cu^{2+} from solution by the fungal pellets. There appeared to be little uptake of copper in the 100 mg l^{-1} culture after 24 hours, while the copper uptake in the 50 mg l^{-1} culture was 65% after 60 days. There was an initial fairly active uptake of copper in the 200 mg l^{-1} culture after 120 minutes which then decreased dramatically in 60 days; this could have been due to evaporation from the flasks, or

alternatively Cu^{2+} was desorbed from the mycelium after the initial uptake. The EDX results, however, did not substantiate the latter explanation.
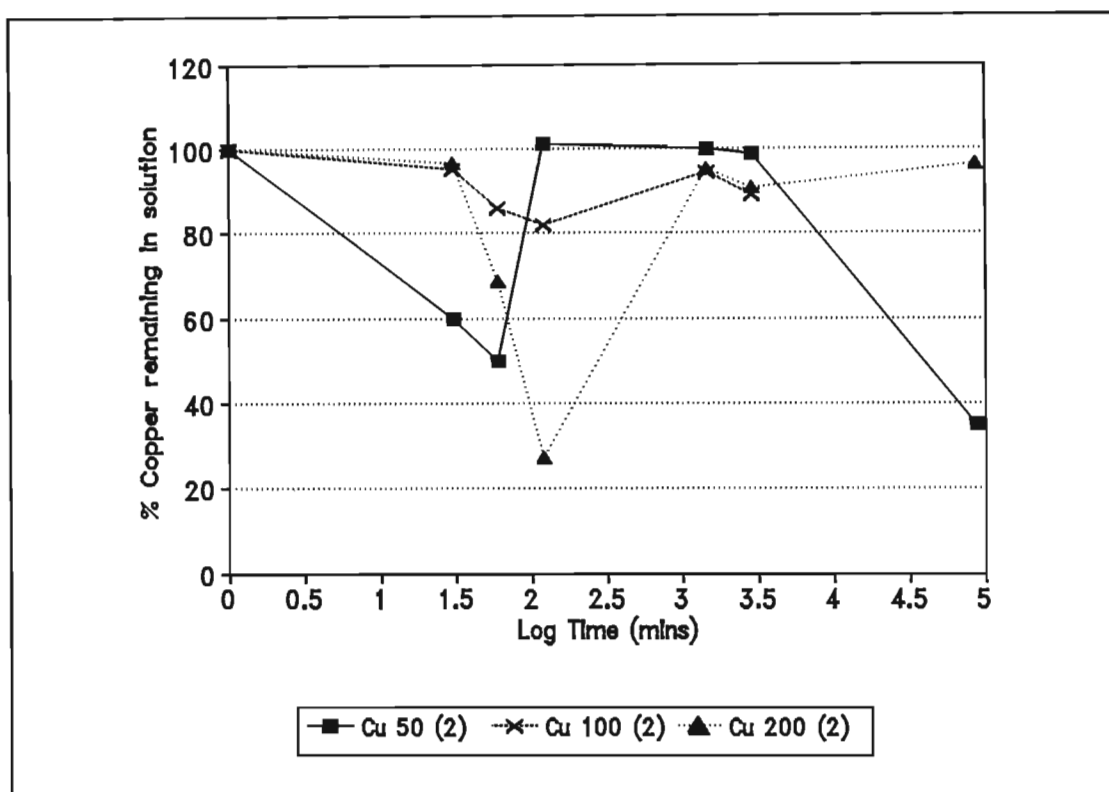
Figure 7.9   Percentage Cu^{2+} remaining in solution after uptake by fungal pellets. Numbers in parentheses indicate the sample numbers corresponding to those in Table 7.3.

Figure 7.10 shows the EDX spectrum of pellets exposed to the 200 mg l^{1} Cu^{2+} solution. There was some Cl^{-} present but its concentration was considerably lower than that of the Cu^{2+}, i.e. 13.4% Cl^{-} and 86.6% Cu^{2+}, indicating that the Cu^{2+} was not all complexed with chloride to form $CuCl_2$. The same trend was observed with the fungal sample exposed to 50 mg l^{1} Cu^{2+} (results not shown).
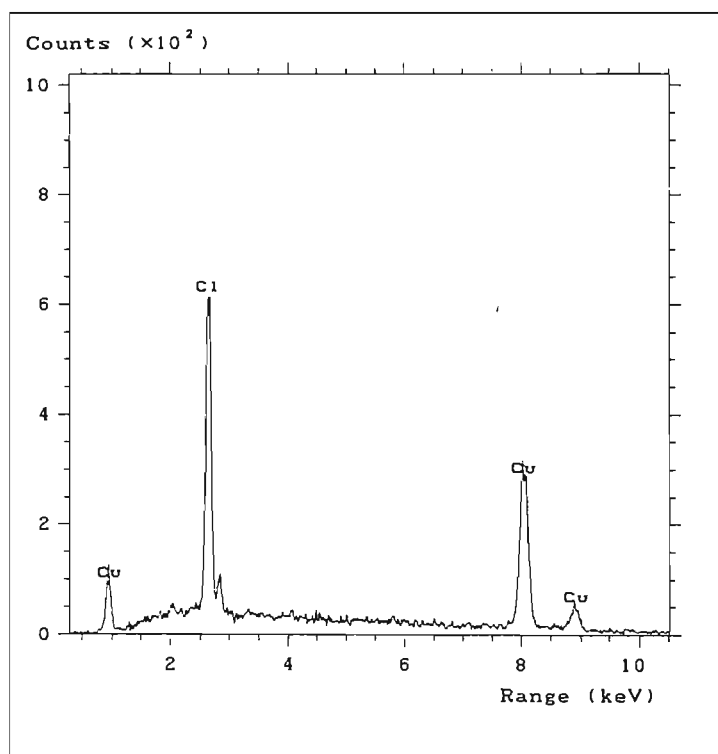
Figure 7.10 EDX spectrum of a fungal pellet exposed to 200 mg l^{-1} Cu^{2+}.

The pellets suspended in the Pb^{2+} solutions turned a brown colour after 60 days contact with the metal. Large amounts of Pb^{2+} were visible on/in the pellets exposed to both the 100 mg l^{-1} and 200 mg l^{-1} Pb^{2+} solutions. The EDX spectrum of pellets exposed to the 100 mg l^{-1} Pb^{2+} solution for 60 days is shown in Fig. 7.11.
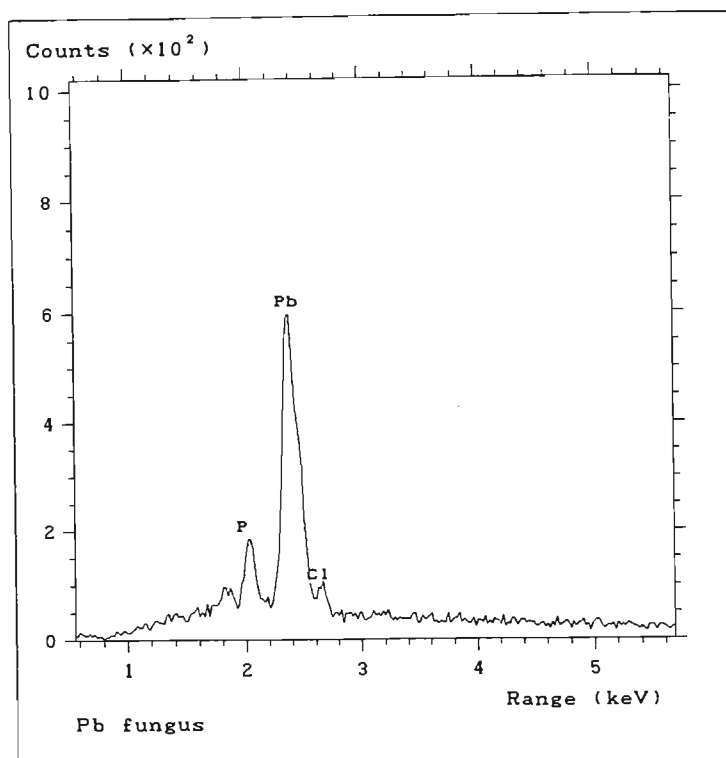
Figure 7.11 EDX spectrum of fungal pellets exposed for 60 days to 100 mg l^{-1} Pb^{2+}.

The liquid media from which these pellets were removed were analyzed by HPLC (see section 2.2.4.1) and it can be seen from Fig. 7.12 that Pb^{2+} was indeed removed from solution during the time the pellets were exposed to the metal. Sample concentrations above 100% were due to errors which occurred during analysis. No results were obtained for the 50 mg l^{-1} Pb^{2+} culture. Note that the concentrations of Pb^{2+} in the solutions before exposure to the fungus were very low but this Pb^{2+} appeared to be removed over the 60 days period of the experiment. Complete removal occurred with the 100 mg l^{-1} sample while with the 200 mg l^{-1} sample, 60% removal was achieved.
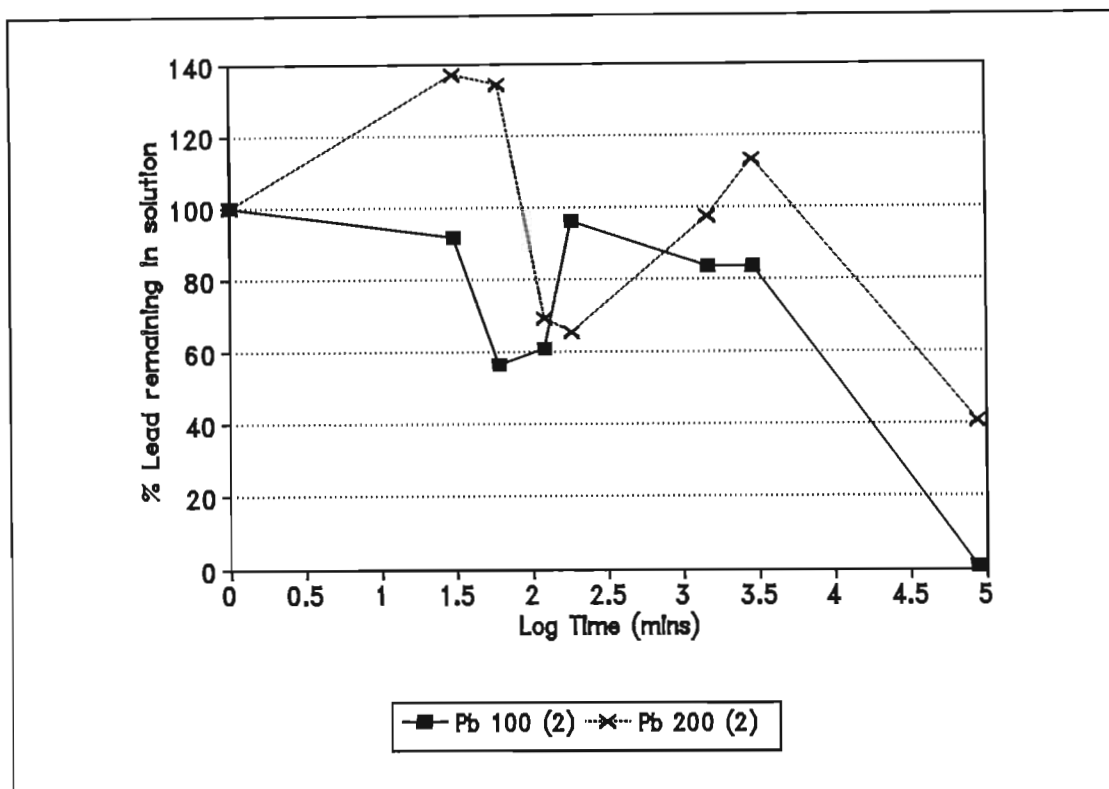
Figure 7.12 Percentage Pb^{2+} remaining in the solution after uptake by fungal pellets exposed to 100 and 200 mg l^{1} Pb^{2+}. Numbers in parentheses indicate sample numbers corresponding to Table 7.3.

The concentrations of Cd^{2+} remaining in the solutions after contact with the fungal pellets are shown in Figure 7.13. There was little uptake of Cd^{2+} by the fungal pellets in any of the cultures. The 50 and 100 mg l^{1} Cd^{2+} cultures seemed to demonstrate a cycle of adsorption and desorption (Fig. 7.13) as seen in other cadmium removing fungal cultures (Fig. 7.8). The uptake of Cd^{2+} by the fungal pellets was slightly better than uptake by the surface grown fungal mycelial mats (Fig. 7.8) with approximately 20% of the Cd^{2+} removed from the 200 mg l^{1} Cd^{2+} solution in about 36 hours. However, uptake of Cd^{2+} by this fungus was generally poor.
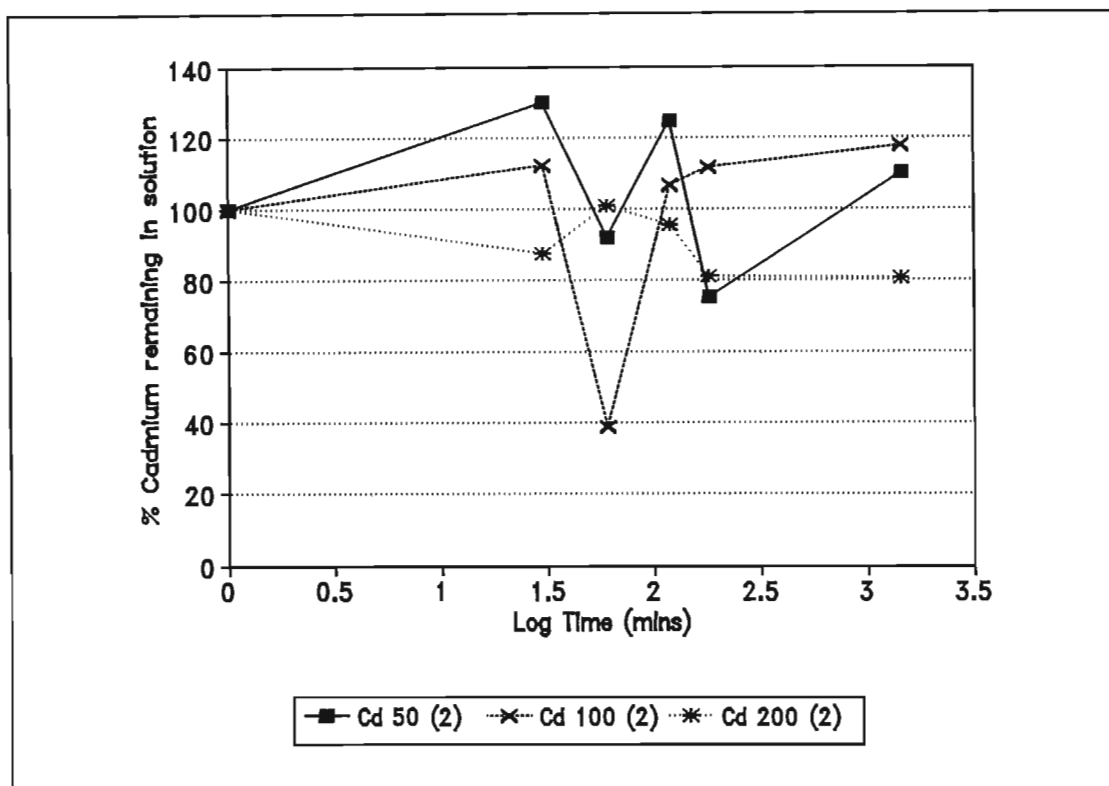
Figure 7.13 Percentage Cd^{2+} remaining in solution after uptake by fungal pellets. Numbers in parentheses indicate sample numbers corresponding to Table 7.3.

7.2.3.8 Lead uptake by dried mycelial pellets and comparison with uptake by undried mycelial pellets

Lead uptake by the pregrown fungus varied depending on the initial metal concentration and the amount of biomass present. The energy dispersive X-ray microanalysis spectrum obtained from the dried mycelial pellet after exposure to the lead solution, showed the presence of lead on and/or in the mycelial pellet. The various other elements (Na, Cl, K and Ca) also detected are commonly found in fungal cells (Fig. 7.14). Figure 7.14 is an example of one of the EDX spectra obtained for a 500 mg biomass sample exposed to 500 mg l^{-1} Pb^{2+} for 48 hours, and clearly shows the presence of lead associated with the fungal pellets.
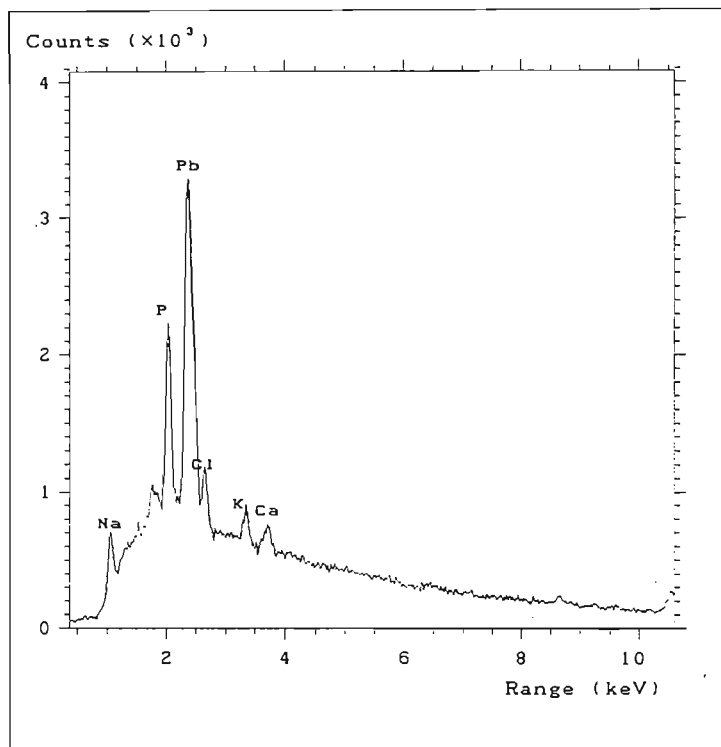
Figure 7.14 EDX spectrum of dried fungal pellets exposed for 48 hours to 500 mg l^1 Pb^{2+}.

Dried mycelial pellets were used to assess the affect of biomass concentration on Pb^{2+} uptake as exact dry weights could be obtained. With different dry weights of mycelium it was determined that the greater the mass of mycelium present, the greater was the amount of Pb^{2+} removed from solution, since the percentage Pb^{2+} remaining in solution decreased as the biomass increased. This applied to all the Pb^{2+} concentrations tested (Fig. 7.15). In each case, however, the percentage Pb^{2+} removed from solution decreased at the 500 mg l^1 Pb^{2+} concentration compared to the 200 mg l^1 Pb^{2+} concentration, indicating that the adsorptive capacity of the biomass was finite since the system seemed to become overloaded above a threshold concentration/biomass ratio (Fig. 7.15).
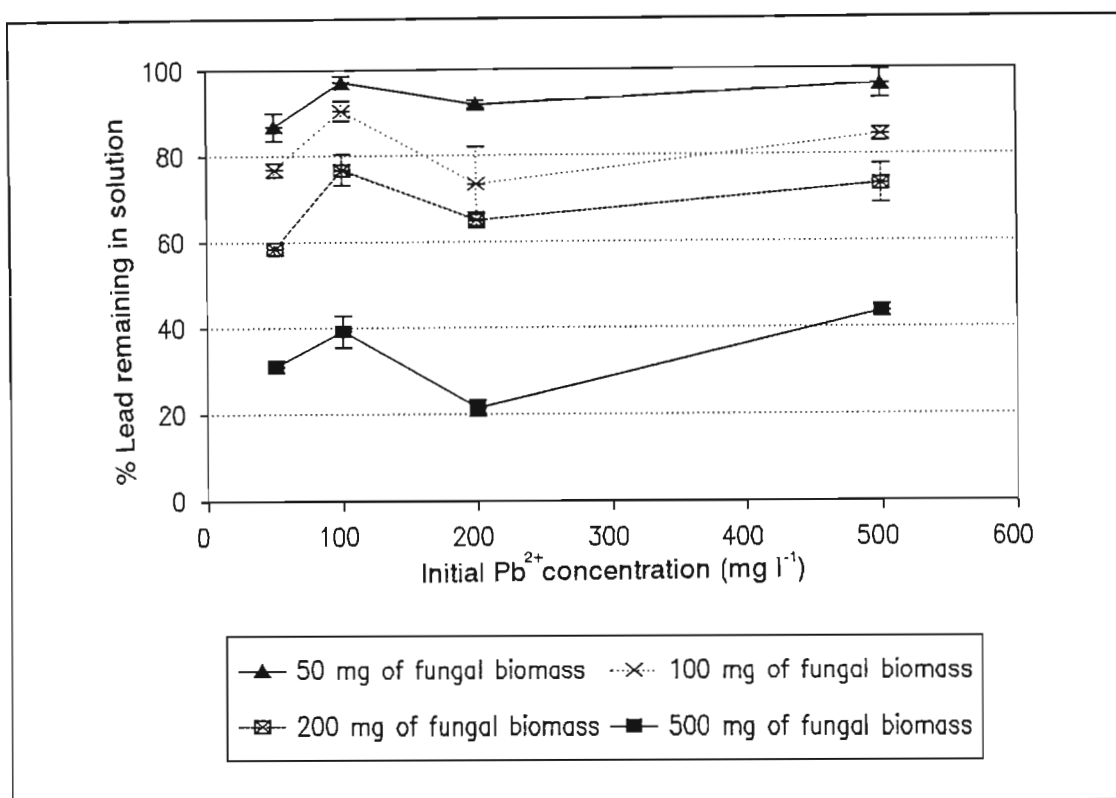
Figure 7.15 Percentage Pb^{2+} remaining in solution after 24 hours contact with the various masses of dried mycelium. Initial Pb^{2+} concentrations were 50, 100, 200 and 500 mg l^{-1}.

However, when the amount of Pb^{2+} removed per g of dried biomass was considered, there appeared to be an opposite effect (Fig. 7.16), especially at the higher initial Pb^{2+} concentrations. More Pb^{2+} appeared to be taken up per unit mass by the 100 mg (biomass concentration of 2 mg ml^{-1}) samples than by the 200 (4 mg ml^{-1}), 500 (10 mg ml^{-1}) and 50 mg (1 mg ml^{-1}) samples. A similar effect was observed by Gadd and White (1989b) with thorium uptake by a *Penicillium* sp., *Aspergillus niger* and *Saccharomyces cerevisiae*. They concluded that this decrease in uptake at higher biomass concentration was probably due to interference between binding sites and reduced mixing during exposure to the metal ion solutions (Gadd & White 1989b). Fourest and Roux (1992) also observed that zinc uptake decreased as the *Rhizopus arrhizus* biomass concentration increased. They concluded that at a given metal concentration, reducing the biomass concentration enhanced the metal/biosorbent ratio and, therefore, as long as the biosorbent is not

saturated, the metal uptake per g of biosorbent increases. The 50 mg sample was too small to demonstrate significant uptake of Pb^{2+} ions and did not follow this trend. In these experiments this effect was more significant with the higher initial Pb^{2+} concentrations of 200 and 500 mg l^1 (Fig. 7.16). It appeared that at these higher lead concentrations blocking of the active uptake sites in the presence of greater biomass was more pronounced than with the lower lead concentrations. It seems that although a greater percentage of lead was removed by the greater masses of mycelium, not all the active metal-uptake sites were used, i.e. with high biomass concentrations (4-10 mg ml^{-1}) the efficiency of uptake was reduced. This suggested that an optimum biomass concentration needs to be determined for each system to ensure maximum metal uptake efficiency. This theory regarding non-utilization of all potential metal-uptake sites was possibly supported by the uneven distribution of metal ions in the surface layers of hyphae exposed to Cu^{2+} (see Plate 8.3A and related text in Chapter 8).

It has also been reported that the biosorptive process by *Rhizopus arrhizus* can only deal with low concentrations of metallic effluents if a high percentage removal is required (Zhou and Kiff 1991). Metal removal is dependent upon the binding sites available on the fungal surface and a reasonably high ratio of fungal biomass to pollutant strength should always be maintained for satisfactory metal ion removal (Zhou and Kiff 1991).
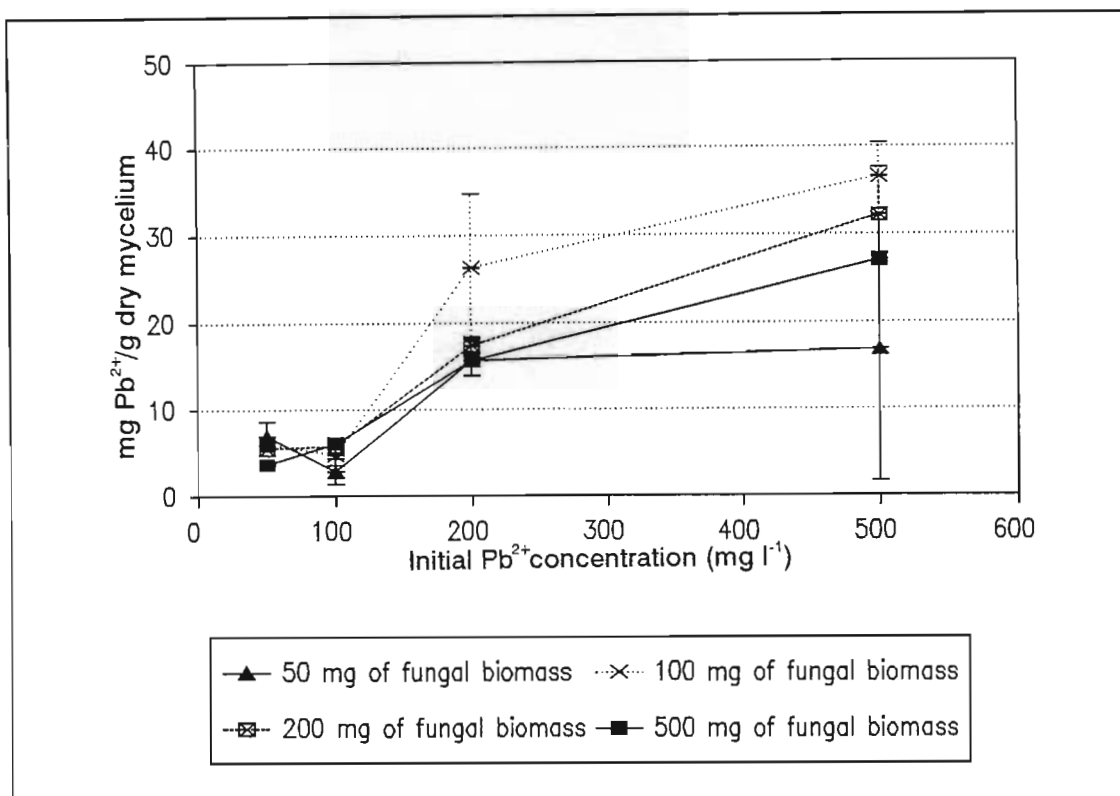
Figure 7.16 Amount of Pb^{2+} per unit mass of mycelium taken up after 24 hours by the various masses of dried mycelium at initial Pb^{2+} concentrations of 50, 100, 200 and 500 mg l^{-1}.

When comparing the uptake of lead by the dried and undried pellets, very little difference in their metal uptake capabilities was found (Fig. 7.17). Drying at 60°C apparently had no effect on the metal uptake sites on the surface of the fungus. The Pb^{2+} uptake capacity of *Penicillium digitatum* was also found to be unaffected by heat (100°C for 5 minutes) or any other pretreatments, while the uptake of other metals was affected by the various pretreatments used (Galun *et al.* 1987). When using killed cells it is important to remember that the method employed to inactivate the biomass (heat or solvent) may modify the surface properties of the biomass and thus affect its metal-binding properties (Brown 1991). Dead or killed biomass is not subject to metal toxicity, needs no nutrient supply and merely acts as an ion-exchanger (Gadd 1990).
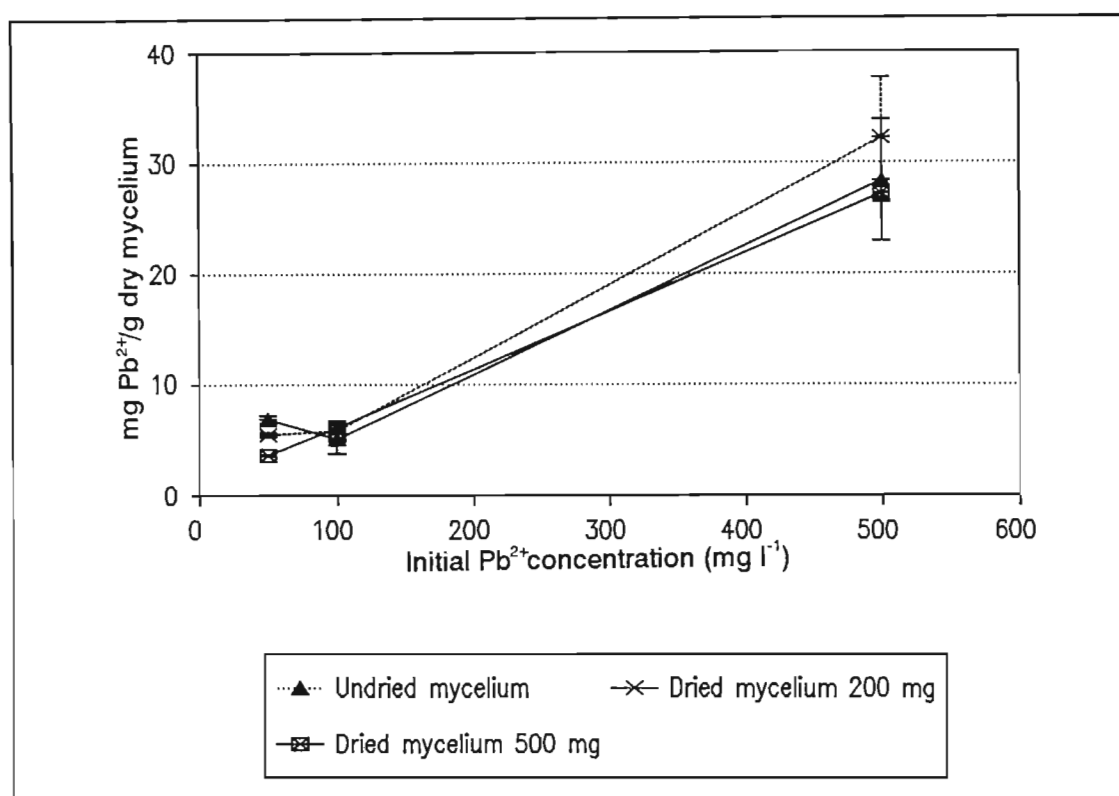
Figure 7.17 Comparison of Pb^{2+} uptake by dried mycelium (200 and 500 mg) and undried mycelium (280 mg) after 24 hours at initial Pb^{2+} concentrations of 50, 100 and 500 mg l^{-1}.

A pH close to 4 was used as it is known that metal ions bind to fungi best at pH values between 4 and 5, with a reduction in binding at pH 3 and no binding at pH 2 (Wales & Sagar 1990), due to competition with hydrogen ions in a stoichiometric ratio of 1:1 (Nui *et al.* 1993). *Penicillium chrysogenum* was also found to have maximum Pb^{2+} adsorptive capacity in the pH range of 4 to 5 (Nui *et al.* 1993). A pH of 3.6 has also been used to avoid precipitation of metal oxides (Tobin *et al.* 1984). There was negligible change in the pH of the Pb^{2+} solutions over the 48 hour period of contact with the fungal mycelium.

Living, but non-growing (i.e. pregrown) fungal material might be better to use for metal uptake since, in addition to the non-requirement for nutrients, the surface sites for metal adsorption and uptake are not destroyed by heat, solvents or other methods used to kill the fungus. Instead, the unmodified mycelium is merely transferred to a medium which does

not support its growth. Some authors have reported that environmental factors and the nature and composition of the microbial association play a major role in the metal uptake potential of microbial biomasses (Siegel *et al.* 1990).

7.4 CONCLUSIONS

Metal uptake by the killed microbial biomass varied with good uptake by the unwashed cells in half-strength Nutrient Broth (7.2.1.1) and poor uptake by the cells which were washed and resuspended in Ringers solution (7.2.1.2). Metal uptake by living and dead biomass was found to be comparable but each microbial association and metal solution must be tested to obtain the most efficient metal uptake system.

Initial experiments with *Aspergillus niger* strain 4 showed a poor uptake of Cu^{2+} with the killed mycelium displaying a slightly better uptake than its living counterpart.

Metal uptake by the pregrown fungus varied depending on the initial metal concentration and the type of fungal mycelium used. For example, uptake of Cu^{2+} by the fungal pellets was found to be better than by the mycelial mats. Pb^{2+} was taken up by both the fungal pellets and the mycelial mats, while Cd^{2+} uptake was also found to be better by the pellets than by the mycelial mats.

Pregrown living fungal material is better than dead/killed biomass for use in metal uptake as no nutrients are required and the surface sites for metal adsorption and uptake are not destroyed by heat or solvents as the fungus is not killed but merely transferred to a medium which does not support its growth.

Using various masses of dried mycelial pellets it was determined that the biomass concentration affects metal uptake. The greater the mass of biomass present, the greater the total percentage lead removed from solution. However, the most efficient removal of lead (mg g^{-1} of biomass) was obtained with the 100 mg 50 ml^{-1} sample, as this biomass concentration (2 mg ml^{-1}) was able to remove a greater amount of lead per gram of biomass. Simple experiments, such as determining the biomass concentration, can be used

to generate information regarding scale-up processes to optimise industrial effluent clean-up (Fourest and Roux, 1992).

Fungal biomass, especially filamentous forms, can act as adsorbents or ion exchangers for the removal of heavy metals, especially lead, from polluted waters and has good potential for use in metal clean-up systems as it is cheap and readily available as a waste material from several industries. Further research and development is needed to optimise these processes. Determination of the mechanisms involved in metal uptake is important for optimising such metal removal processes.

CHAPTER 8: MECHANISM OF METAL UPTAKE BY PELLETISED *ASPERGILLUS NIGER*

8.1 INTRODUCTION

Fungi are known to have good metal uptake systems (Gadd 1986). A variety of mechanisms exist for the removal of heavy metals from solution, ranging from purely physico-chemical interactions, such as adsorption to cell walls, to mechanisms dependent on metabolism, such as intracellular compartmentalisation and extracellular precipitation by excreted metabolites (Gadd 1988). Metabolism-independent biosorption appears to be the most efficient mechanism of uptake (Tobin *et al.* 1994). This involves several chemical processes such as ion exchange, adsorption, co-ordination and covalent bonding, with the cell walls playing an important role, due to the presence of various uptake sites containing electronegative, anionic and N-containing groups (Tobin *et al.* 1994). A full understanding of the mechanisms controlling metal sorption will be highly worthwhile for the optimisation of metal recovery processes (Fourest and Roux 1992).

The biomass of filamentous fungi contains a relatively high percentage of cell wall material (Luef *et al.* 1991). The mechanical strength, morphological features and biological activity of fungal walls is dependent on their particular composition (Farkas 1979). Polysaccharides represent 80 to 90% of the dry matter of fungal cell walls and are composed of amino sugars, hexoses, hexuronic acids, methylpentoses and pentoses (Farkas 1979). The fibrous and non-fibrous wall polysaccharides are not the exclusive or unique components of the biosorptive system (Farkas 1979). Wall polymers consisting of cellulose, glycan, chitosan, polyuronides and chitin, also provide the chemical basis for localisation of many ionic species onto the cell surface (Siegel *et al.* 1990). Different fungal cell envelopes vary in detailed chemical composition (Siegel *et al.* 1990). There is a certain degree of stratification in cell walls of fungi and, in general, the outer surfaces of the wall are smooth or slightly granular in texture and composed of amorphous material (often glycoprotein in nature) (Farkas 1979).

The biosorbent properties of *Penicillium* species reside in the hyphal cell wall. These walls can be characterised as a system of biopolymer fibres interwoven to form an ion-exchange and coordination surface, which is patterned with fixed ionogenic groups and fixed neutral ligand sites derived from basic, neutral and acidic glucans, phosphoglucans and proteins (Galun *et al.* 1987). The varying suitability of different fungal mycelia for zinc biosorption was related to the presence of different numbers of functional groups within the mycelial material (Luef *et al.* 1991).

Pregrown pellets of *Aspergillus niger* strain 4 which had been exposed to metal solutions were investigated using electron microscopy and EDX techniques to determine their mechanism of metal uptake.

8.2 MATERIALS AND METHODS

8.2.1 Metal Uptake by Fungal Pellets

Pregrown fungal pellets were exposed to separate heavy metal solutions of lead and copper (Section 7.2.2.7) and their mechanism of metal uptake investigated using combinations of electron microscopic and EDX techniques.

8.2.2 Scanning Electron Microscopy and EDX Mapping

Initial EDX digital mapping (section 3.2.3.2) and EDX dot mapping (section 3.2.3.3) experiments were carried out on critical point dried pellets to determine whether metals were indeed associated with the fungal biomass.

8.2.3 Transmission Electron Microscopy Investigations

Transmission Electron Microscopy was carried out on thin sections of fungal pellets to investigate the mechanism of metal uptake. Mycelial pellets exposed to separate solutions of copper and lead (7.2.2.7), and corresponding control pellets (not exposed to the metals) were fixed in 3% (v/v) glutaraldehyde overnight, washed twice in 0.05 M cacodylate

buffer (30 minutes), post fixed in 2% (v/v) osmium tetroxide for 2 to 4 hours, washed again with 0.05 M cacodylate buffer, dehydrated in an ethanol series (10 minutes in 30, 40, 50, 60, 70, 80, 90% and 3 times in 100%), embedded in Spurr's resin [2 hours in 25% Spurr's: 75% ethanol (caps on), 2 hours in 50% Spurr's: 50% ethanol (caps on), 2 hours in 75% Spurr's: 25% ethanol (caps off), overnight in 100% Spurr's (caps on)], and finally polymerised in a second change of 100% Spurr's resin at 70°C for 16 hours. The embedded pellets were mounted on perspex blocks and sectioned on a LKB Ultrotome III Ultramicrotome (sections were 100-150 nm thick) before viewing in a Jeol 100CX TEM equipped with a Link eXLII EDX system. The sections were not stained. Preliminary EDX investigations were carried out at an accelerating voltage of 80 kV.

Similar thin sections cut from the same fungal pellets were sent to The Netherlands to be analyzed on a Philips CM120/STEM with a BioTWIN objective lens, equipped with an EDAX Energy dispersive X-ray detector.

8.3 RESULTS AND DISCUSSION

8.3.1 Metal Uptake by Fungal Pellets

Both Cu^{2+} and Pb^{2+} were taken up by the mycelial pellets from solution. The results, expressed as the percentage metal remaining in solution after fungal uptake, are shown in section 7.3.2.7. Although the microscopic and EDX techniques used here were carried out on pellets exposed to Cu^{2+} and Pb^{2+} solutions for 60 days, similar results were obtained, using these same techniques, on pellets exposed to these metal solutions for only 24 hours (results not shown).

8.3.2 Scanning Electron Microscopy and EDX Mapping

Fungal pellets exposed to copper solutions were initially investigated using Scanning Electron Microscopy and EDX mapping techniques. Figure 8.1 shows the digital map of pellets exposed to a 200 mg l^{-1} Cu^{2+} solution for 60 days. It is evident that the Cu^{2+} ions were associated with the fungal mycelium. The EDX spectrum obtained from this sample is shown in Fig. 7.10 and the metal ion analysis of the supernatant samples is shown in Fig. 7.9.
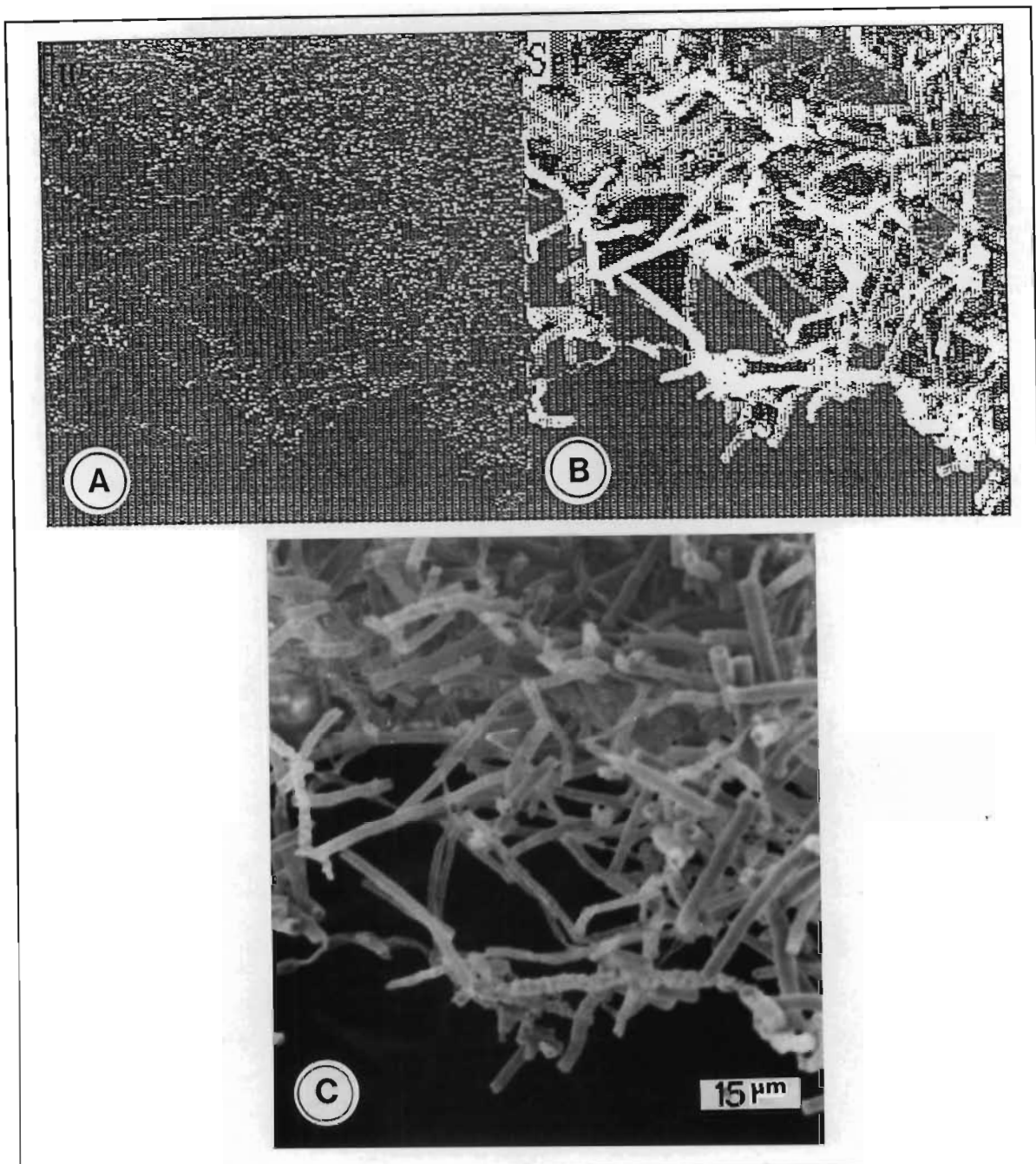
Figure 8.1 Digital map of part of a fungal pellet exposed for 60 days to 200 mg l^{-1} Cu^{2+}; A. copper map; B. secondary image; C. electron micrograph corresponding to secondary image.

Pellets exposed to 100 and 200 mg l^{-1} Pb^{2+} were also analyzed by the EDX digital mapping technique and this analytical technique clearly showed the presence of Pb^{2+} associated with the mycelium of the pellet (Figs 8.2 and 8.3). The presence of lead is

indicated by the area shown as white on the map which corresponds to the area on the pellet depicted in the secondary image and electron micrograph (Fig. 8.2). The corresponding EDX spectrum and the results of the supernatant analyses are shown in Figs 7.11 and 7.12 respectively. This technique does not show the actual location of the lead in the pellets as X-rays are able to penetrate into the mycelial strands. However, it is most probable that the lead was adsorbed to the external hyphal surfaces, i.e. metabolism-independent biosorption had taken place, as the fungus was not metabolising since no nutrients were present in the medium. Under these conditions intracellular uptake was unlikely to occur.
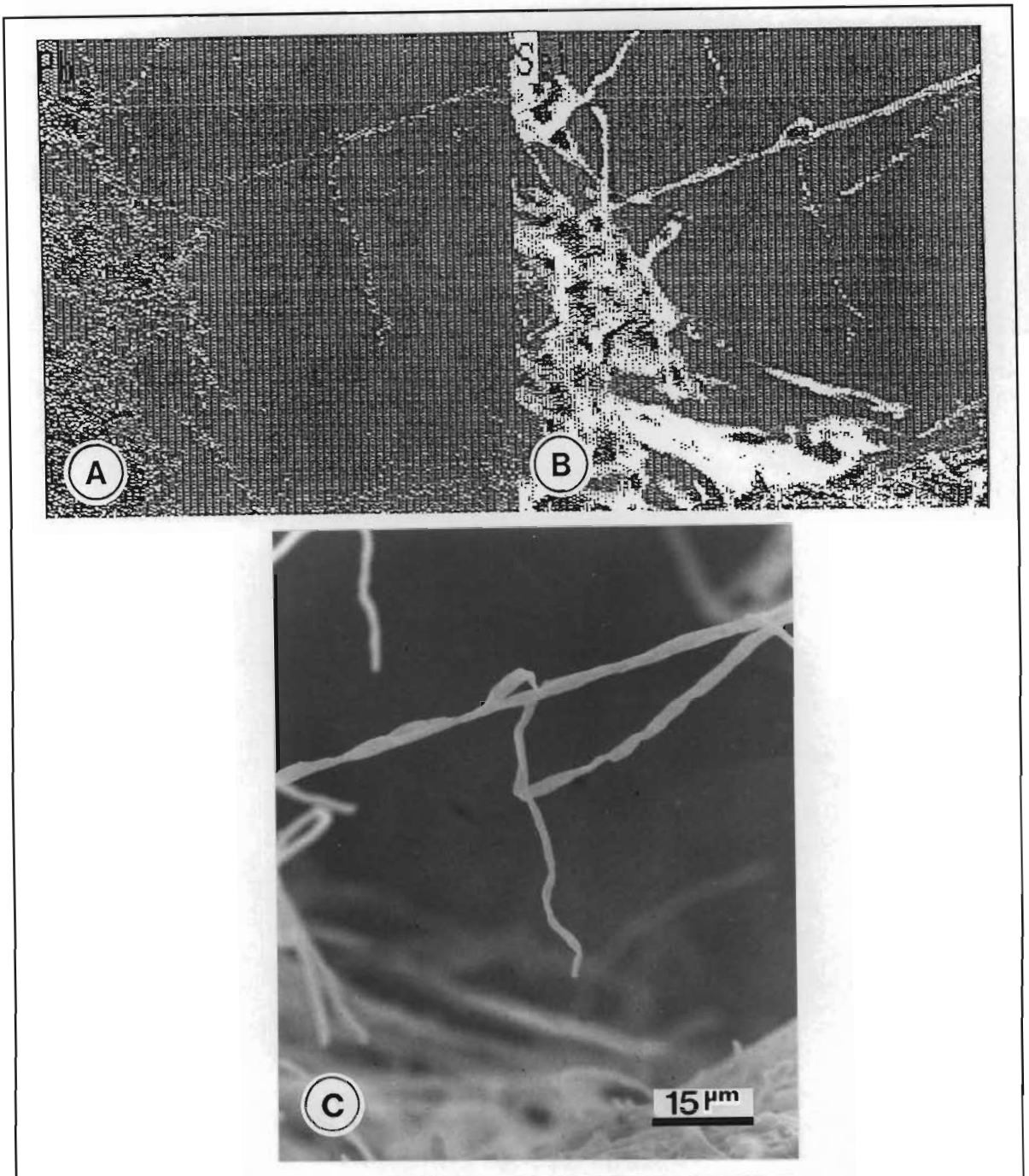
Figure 8.2   Digital map of part of a fungal pellet exposed for 60 days to 100 mg l^{-1} Pb^{2+}; A. lead map; B. secondary image; C. electron micrograph corresponding to secondary image.
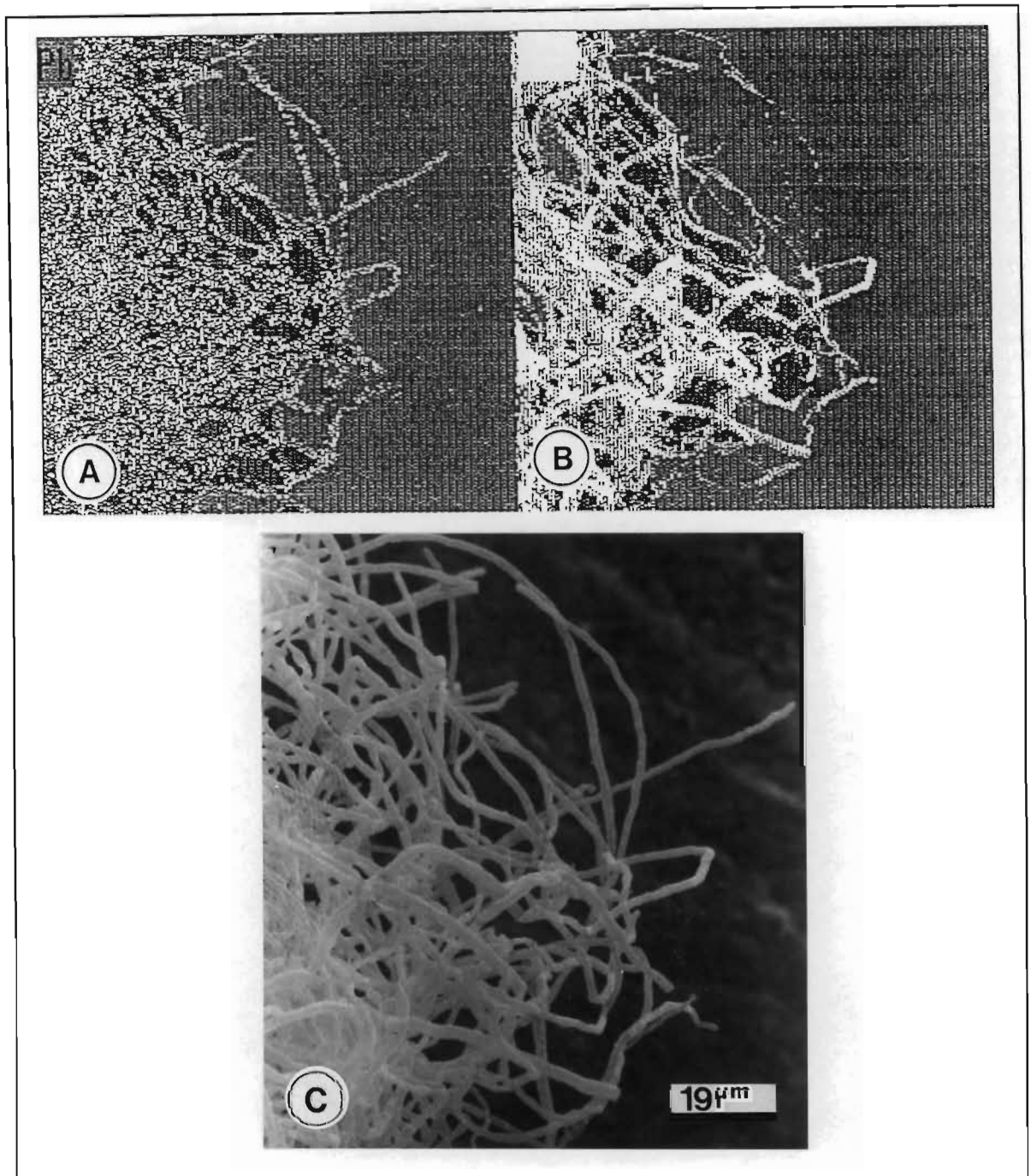
Figure 8.3 Digital map of part of a fungal pellet exposed for 60 days to 200 mg l^{-1} Pb^{2+}; A. lead map; B. secondary image; C. electron micrograph corresponding to secondary image.

Pellets exposed to both copper and lead solutions were also analyzed by the dot mapping technique (3.2.3.3). Plates 8.1 and 8.2 show the dot maps obtained from pellets exposed

to 200 mg l^{-1} copper and 100 mg l^{-1} lead, respectively, and indicate that both metals were associated with the respective pellets. The presence and location of the metals can be distinguished as the white dots on the maps. This mapping technique is considerably easier to carry out than the digital mapping technique. When using the digital mapping technique problems such as image drifting and charging occurred due to the instability of these samples, and the clarity of the digital map and secondary image was adversely affected. Dot mapping results can also be obtained more quickly and this technique is especially useful for investigating the location of only one element in a system, as was the case here.
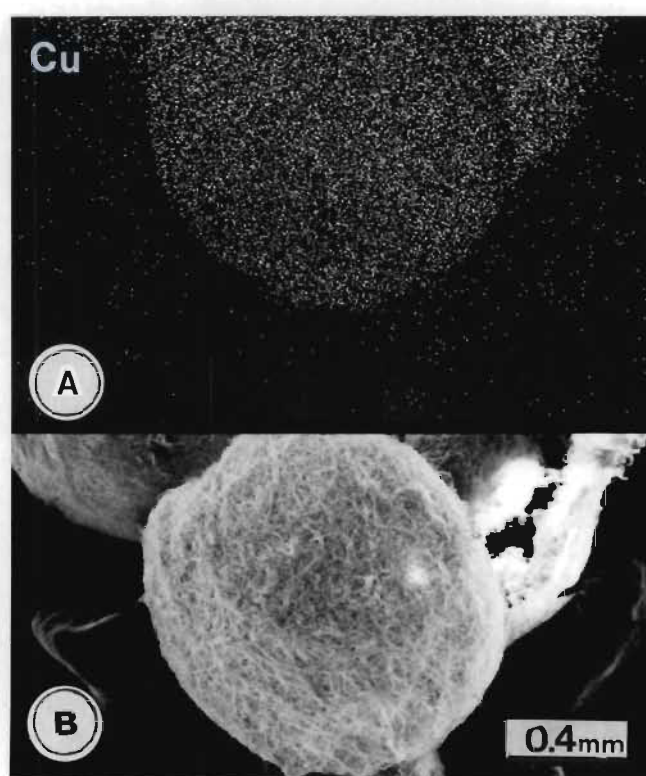
Plate 8.1   EDX dot map of a fungal pellet exposed for 60 days to 200 mg l^{-1} Cu^{2+}; A. dot map indicating the location of copper; B. corresponding electron micrograph. The corresponding EDX spectrum is shown in Fig. 7.10.
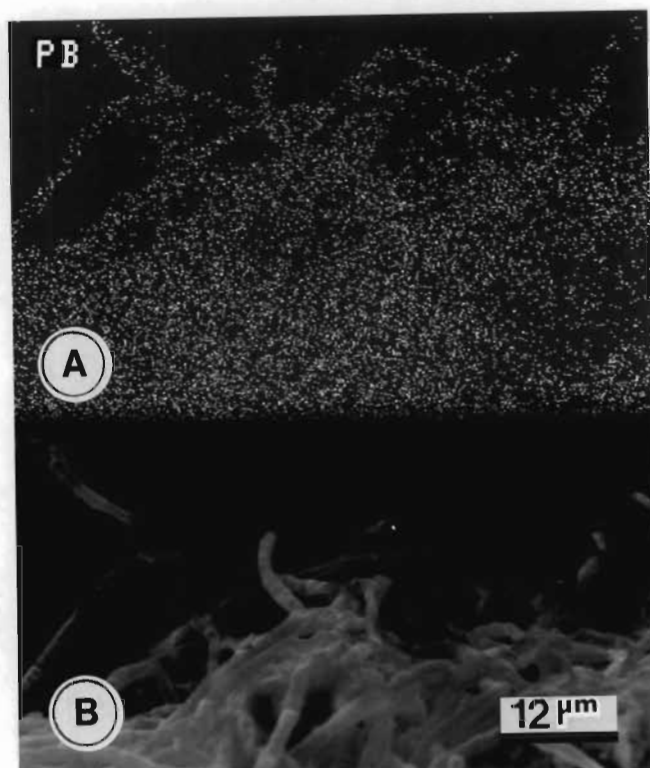
Plate 8.2   EDX dot map of hyphae of a fungal pellet exposed for 60 days to 100 mg l^{-1} Pb^{2+}; A. dot map indicating the location of lead; B. corresponding electron micrograph. The corresponding EDX spectrum is shown in Fig 7.12.

Since Scanning Electron Microscopy could not indicate the precise location of the metal within/on the mycelial strands, Transmission Electron Microscopy investigations were made.

8.3.3 Transmission Electron Microscopy Investigations

Plate 8.3 (A and B) show electron micrographs of thin, unstained sections through hyphae which had not been exposed to any metal solutions, i. e. control pellets. The cell wall (W) can be clearly seen and did not contain any electron dense areas. The EDX spectrum of these control pellets is shown in Fig. 8.4. No lead was present and the other elements detected originated from the microscope itself, i.e. molybdenum (Mo) from the apertures, iron (Fe) from the column and copper (Cu) from the column, sample holder and grid. Thus, the TEM used was not suitable for EDX analyses since metals in the microscope column were also detected and interfered with the metals of interest.

Plate 8.3   A. Cross section of a hyphal strand of a control pellet, which was not exposed to any metal solutions, showing the cell wall (W) and cell membrane (M) structure; B. longitudinal section of a hyphal strand of a control pellet showing the cell wall (W); C. cross section of a hyphal strand exposed to a 100 mg l^{-1} Pb^{2+} solution. The EDX spectrum presented in Fig. 8.5 corresponds to the part of the cell wall indicated by arrow 1, and the EDX spectrum shown in Fig. 8.6 to the central intracytoplasmic region of the hyphal strand indicated by arrow 2; D. cross section of a hyphal strand exposed to a 100 mg l^{-1} Pb^{2+} solution. Arrows indicate the electron dense cell wall (W) and electron dense material associated with the extracellular material (E); E. tangential section of a hyphal strand exposed to a 100 mg l^{-1} Pb^{2+} solution. The arrow indicates electron dense granular material associated with the cell wall.

A

W

M

0.5 μm

B

W

0.5 μm

C

1

2

0.5 μm

D

E

W

0.2 μm

E

0.5 μm
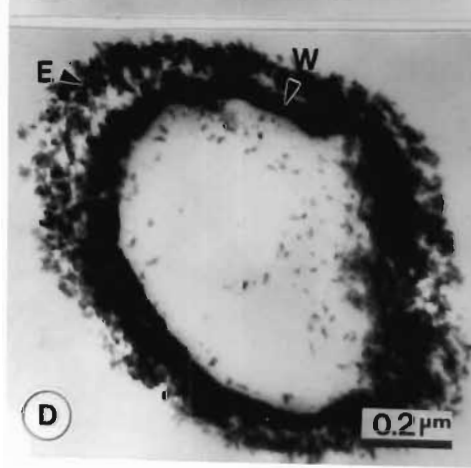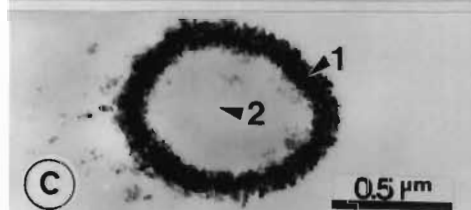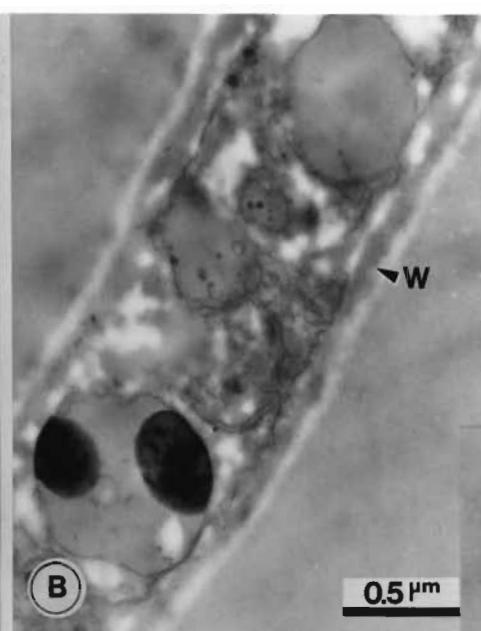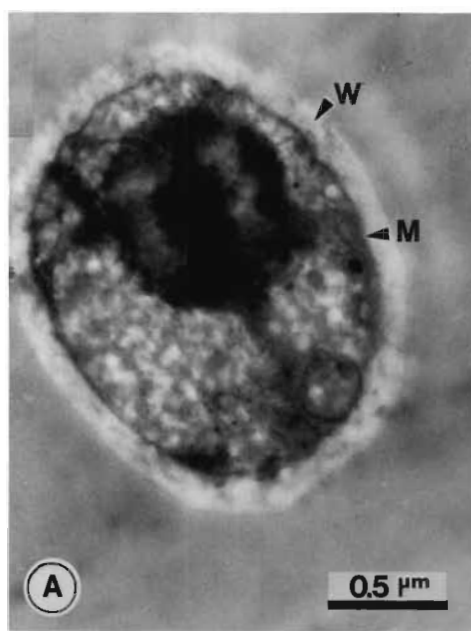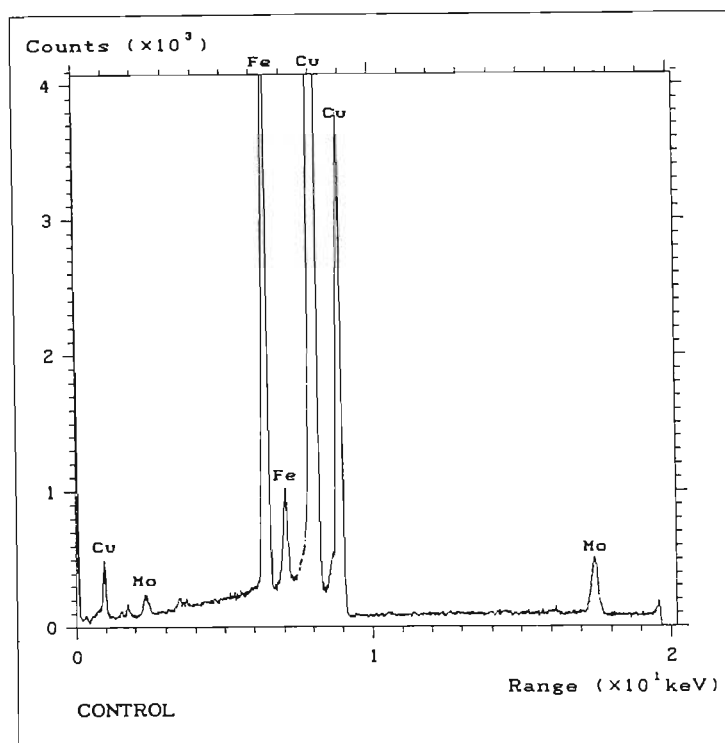
Figure 8.4 EDX spectrum of a fungal pellet which was not exposed to any metal solution.

Preliminary Jeol TEM investigations showed the presence of dark electron dense areas in the hyphal cell walls. Since these electron dense granules were only observed in the pellets exposed to lead solutions (Plate 8.3C, D and E) and not in the control pellets (Plate 8.3A and B) it can be assumed that they were comprised of lead.

Figure 8.5 shows the EDX spectrum obtained from the spot on the cell wall indicated by arrow 1 in Plate 8.3C. A small amount of lead was detected but the peaks were insignificant (Fig. 8.5) compared to the contaminating metal elements originating from within the TEM itself. This spectrum (Fig 8.5) was very similar in many respects to the EDX spectrum of the control fungal pellet (Fig 8.4). Figure 8.6 shows the EDX spectrum obtained from the spot within the centrally located cytoplasmic area of a hyphal strand indicated by arrow 2 in Plate 8.3C. Considerably less Pb^{2+} was detected here than in the cell wall. The spot size was too large and could not be reduced and, therefore, the possibility that the small amount of lead detected was associated with the cell wall could

not be discounted. EDX analyses of spots on the cell wall and in the central region of the other hyphal strands shown in Plate 8.3D and E were similar, and are not shown.
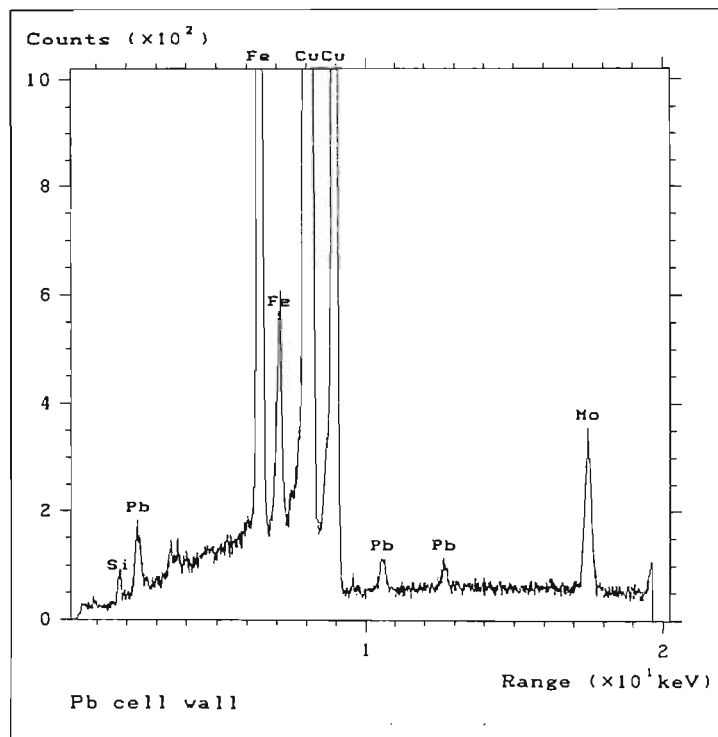
Figure 8.5   EDX spectrum of a section of the cell wall (arrow 1 in Plate 8.3C) of a fungal hypha which had been exposed to 100 mg l^{-1} Pb^{2+}.
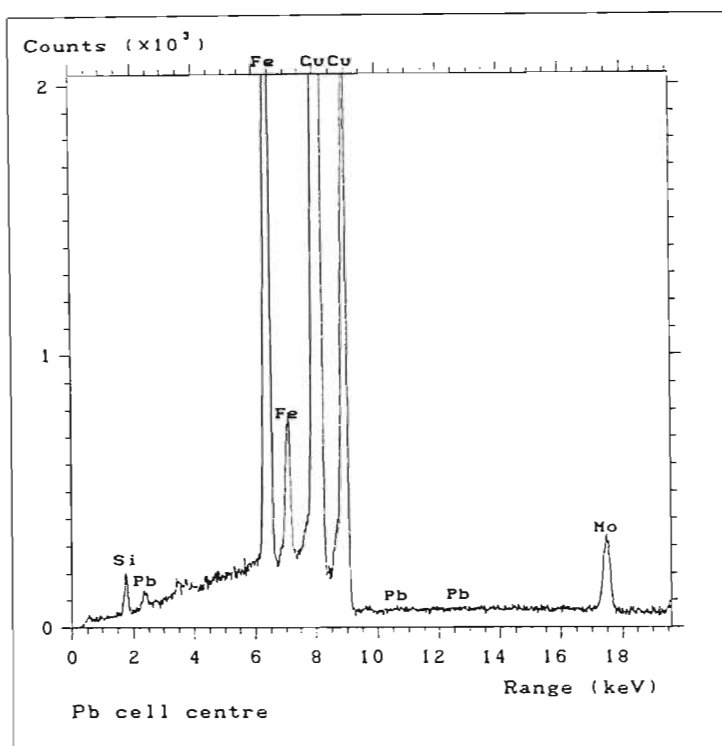
Figure 8.6 EDX spectrum of an area towards the centre of a hyphal strand (arrow 2 in Plate 8.3C) which had been exposed to 100 mg l^{-1} Pb^{2+}.

Plate 8.3D shows an electron dense area in the vicinity of the plasmalemma closely associated with the cell wall. The outer electron dense area may have been due to lead associated with extracellular material, such as excreted melanins. Melanins are fungal pigments which are located in and around the cell walls and in the external medium, and enhance survival under conditions of environmental stress (Bell and Wheeler 1986). Fungal melanins contain phenolic units, peptides, carbohydrates, aliphatic hydrocarbons and fatty acids (Gadd and De Rome 1988). From these preliminary observations it appeared that lead was taken up onto the cell walls of the hyphal strands of *Aspergillus niger* strain 4.

Transmission electron microscopy of thin sections of *Rhizopus arrhizus* non-living cells exposed to uranium solutions also revealed electron-dense layers throughout the fungal cell wall. Electron microscopy of the cell interior did not indicate any concentration of electron dense material anywhere within the cell. The electron dense material deposited

in the cell wall was identified as uranium using X-ray Energy Dispersive Analysis (Tsezos and Volesky 1982). *Penicillium chrysogenum* biomass was likewise shown to develop a higher electron density in the cell walls. This was due to the concentration of lead as confirmed by EDAX (Nui *et al.* 1993).

Transmission electron micrographs of thin sections of *Streptomyces* cells exposed to Pb^{2+} solutions also showed electron dense areas in the cell wall structure, indicating that Pb^{2+} uptake was primarily connected with metal ion binding to cell surface structures (Golab *et al.* 1991). The importance of functional adsorption sites and biomass surface area was shown by the substantial increase in metal ion abstraction when prepared cell wall material was used instead of whole killed cells (Golab *et al.* 1991).

Thin sections of pellets exposed to lead were also investigated with the Philips STEM equipped with EDX. The thin section through a hyphal strand exposed to 100 mg l^1 lead contained electron dense areas in the cell wall (Plate 8.4A, B). It appeared that the Pb^{2+} accumulated in certain areas, almost as a crystalline precipitate. The majority of the metal was associated with the cell wall system, extending towards the cell membrane (indicated by the arrow on plate 8.2B). The EDX spectrum obtained from the point indicated by the arrow (Plate 8.4B) is shown in Plate 8.4C and provided incontrovertible evidence that the electron dense precipitate, concentrated in the cell wall region, was lead. Osmium was also detected as it was used in the preparation of these pellets for viewing in the TEM.

Plate 8.4   A. Philips STEM micrograph of a longitudinal section of a hyphal strand exposed to 100 mg l^1 Pb^{2+}; B. enlargement of the boxed area in 8.4A showing the precipitate-like crystals (arrows) formed in the cell wall structure; C. EDX spectrum of area indicated by the lower arrow in B, showing that the electron dense areas contained significant amounts of lead.

A

B

0.5 µm

0.1 µm

C

PbMa

NiKa

PbLa
OsLb

PbLb

OsLb

AlK

CuL

CuKa

CuKb

Os

PbLg

2.00   4.00   6.00   8.00   10.00   12.00   14.00   16.00   18.00   20.00

FS : 836   CPS : 218   Cnts : 0   KeV : 0.08
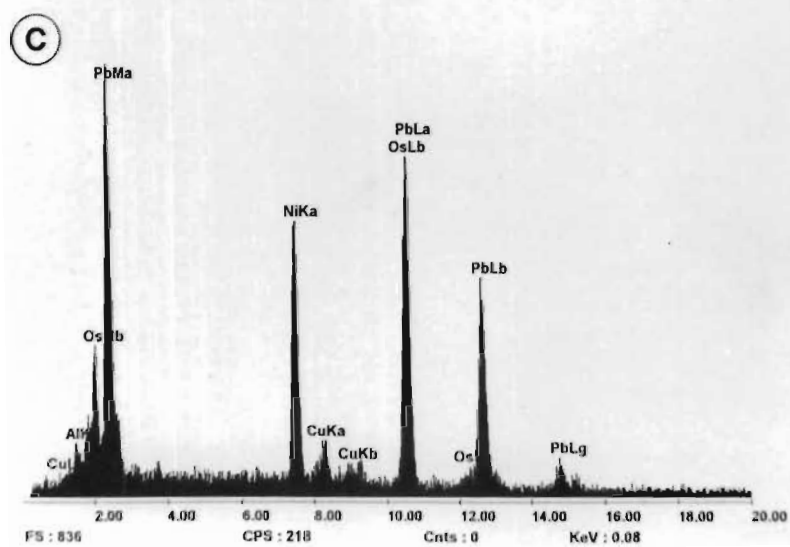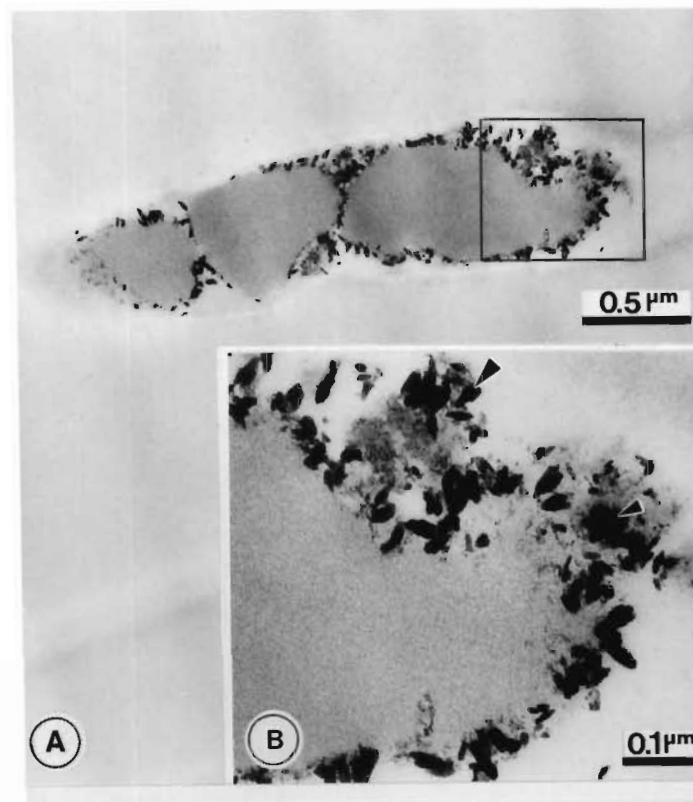
Copper also appeared to accumulate in the cell surface layers where electron dense areas were observed, but could not be detected due to the presence of copper in the fabric of the Jeol TEM (results not shown). These sections were subsequently also analyzed with the Philips STEM.

Plate 8.5A is an electron micrograph taken with the Philips STEM and shows a cross section of a hyphal strand exposed to 200 mg l^1 copper. Little copper was detected at location 1 (Plate 8.5A) an area of low electron density between two regions of much greater electron density (Fig. 8.7A). The nickel which was detected was from the specimen grid (Fig. 8.7A). Location 2 (Plate 8.5A), an electron dense area towards the outside surface of the hyphal wall contained higher levels of copper than location 1 (Fig. 8.7B). A chlorine peak was also present (Fig. 8.7B), possibly because the pellet was exposed to copper as a $CuCl_2.2H_2O$ solution (APPENDIX A). At location 3 (Plate 8.5A), an electron dense area towards the inner surface of the cell wall, very large copper peaks and a much smaller chlorine peak were observed, (Fig. 8.7C), indicating that copper ions can penetrate the entire fabric of the cell wall and that very little of this copper was associated with chlorine.

Plate 8.5B is also an electron micrograph of a cross section of a hyphal strand exposed to 200 mg l^1 copper. A relatively thick electron dense layer is apparent in the outer regions of the cell wall (Plate 8.5B) and EDX analysis at location 1 (Fig 8.8A) showed that large amounts of copper were associated with this layer. The electron dense material inside the cell (location 2; Plate 8.5B) also had copper associated with it, although at a much lower concentration (Fig. 8.8B). Osmium (from the fixation process) and a very small amount of chlorine were also detected in this area (Fig. 8.8B). As this was the only example of intracellularly detected Cu^{2+} it is possible that it might be an artifact, i. e. possibly some wall material was picked up on the specimen grid. Alternatively, some copper may have been taken up intracellularly by this *Aspergillus niger* strain but, in general, most of the copper accumulated within the cell wall layers.

Plate 8.5   A. TEM micrograph of a cross section of a hyphal strand exposed to a 200 mg l^{-1} Cu^{2+} solution. The EDX spectra (Fig. 8.7A, B and C) correspond to the locations indicated by the numbered arrows 1, 2 and 3, respectively, and indicate the elements present at these locations on/in the hyphal strand; B. TEM micrograph of a cross section of a hyphal strand exposed to a 200 mg l^{-1} Cu^{2+} solution.   The EDX spectra shown in Fig. 8.9A and B correspond to the locations indicated by the arrows 1 and 2, respectively.

A

0.5 μm

B

0.5 μm
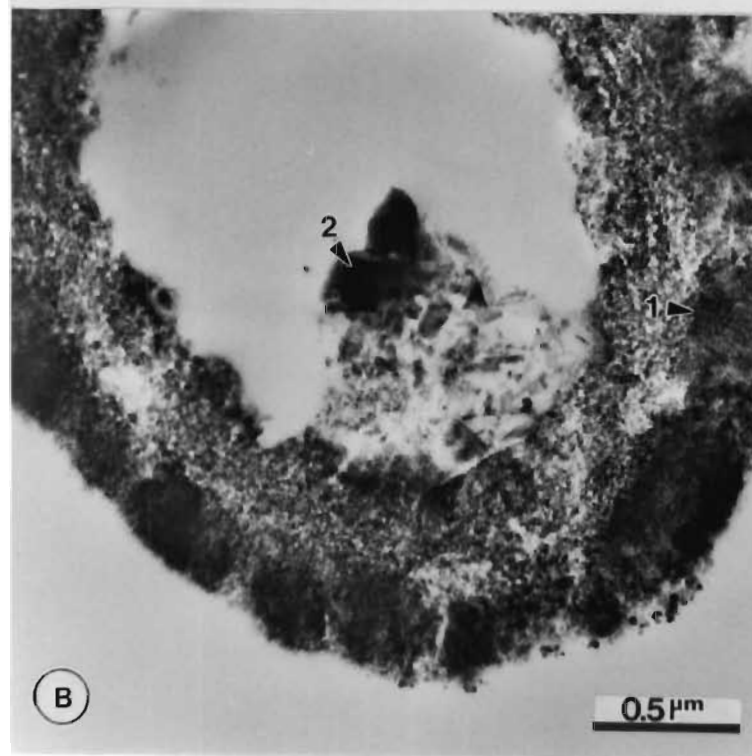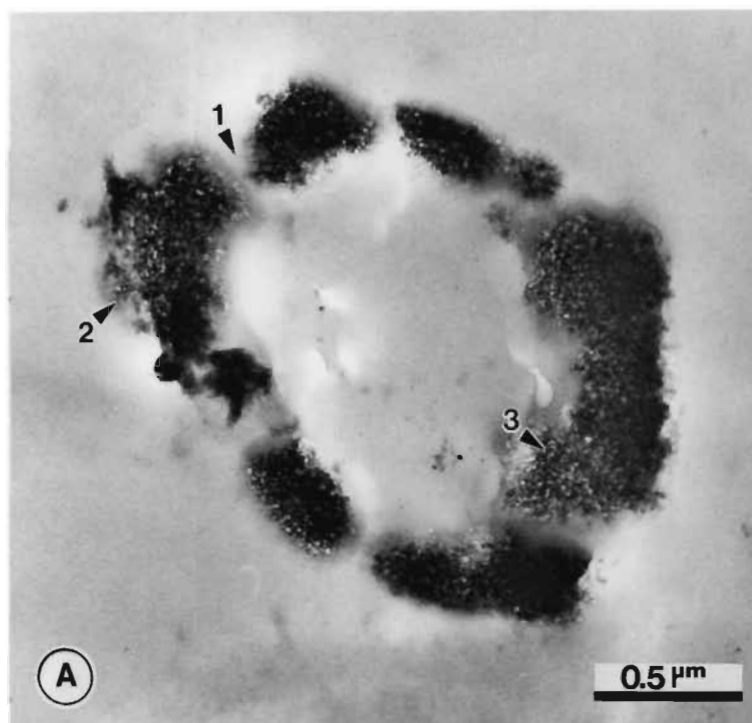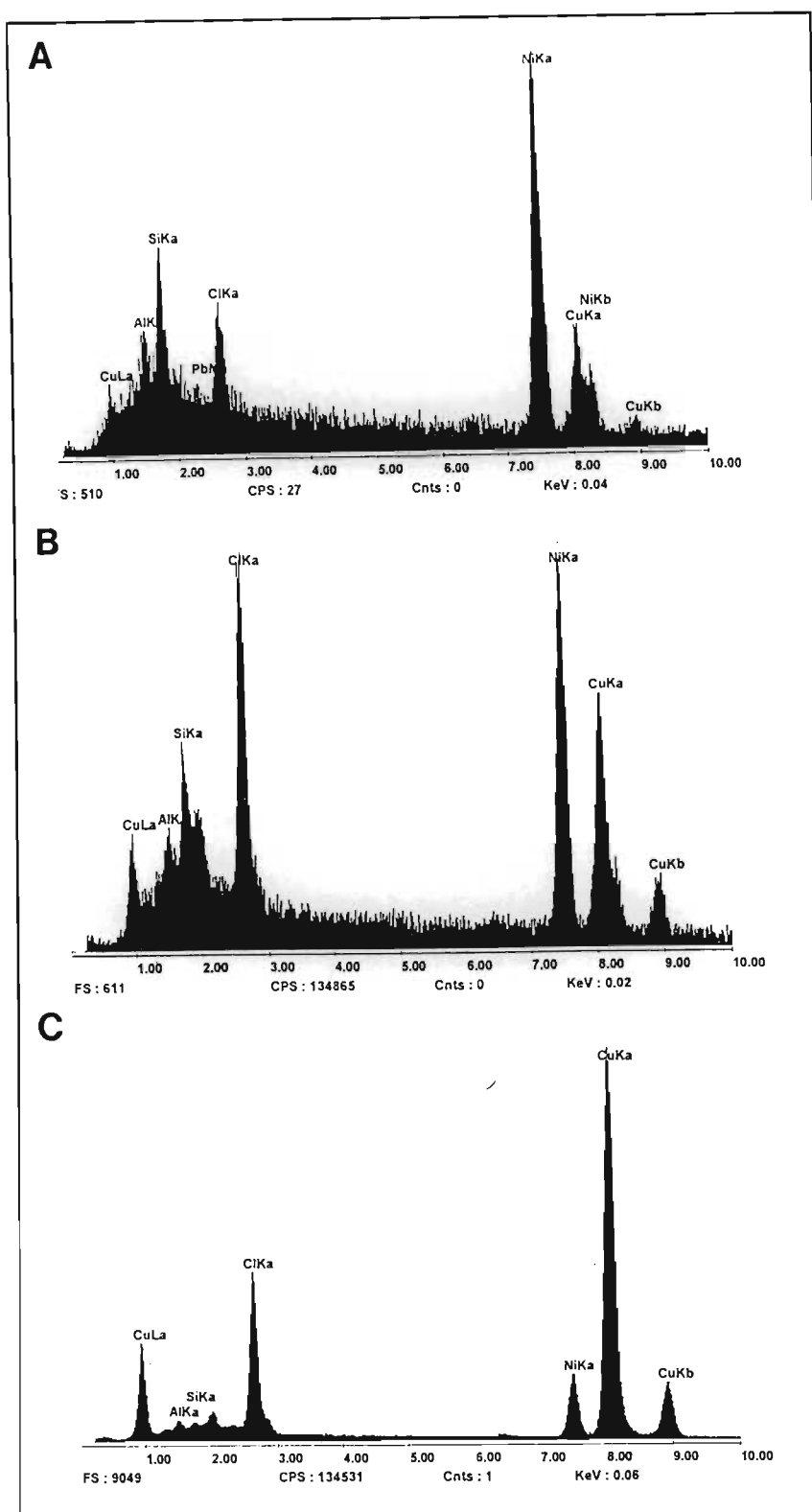
Figure 8.7   A. EDX spectrum of location indicated by arrow 1 in Plate 8.5A, showing a relatively small amount of copper; B. EDX spectrum of location indicated by arrow 2 in Plate 8.5A showing a larger copper peak; C. EDX spectrum of location indicated by arrow 3 in Plate 8.5A showing a very large copper peak. Note the nickel peak from the supporting grid.
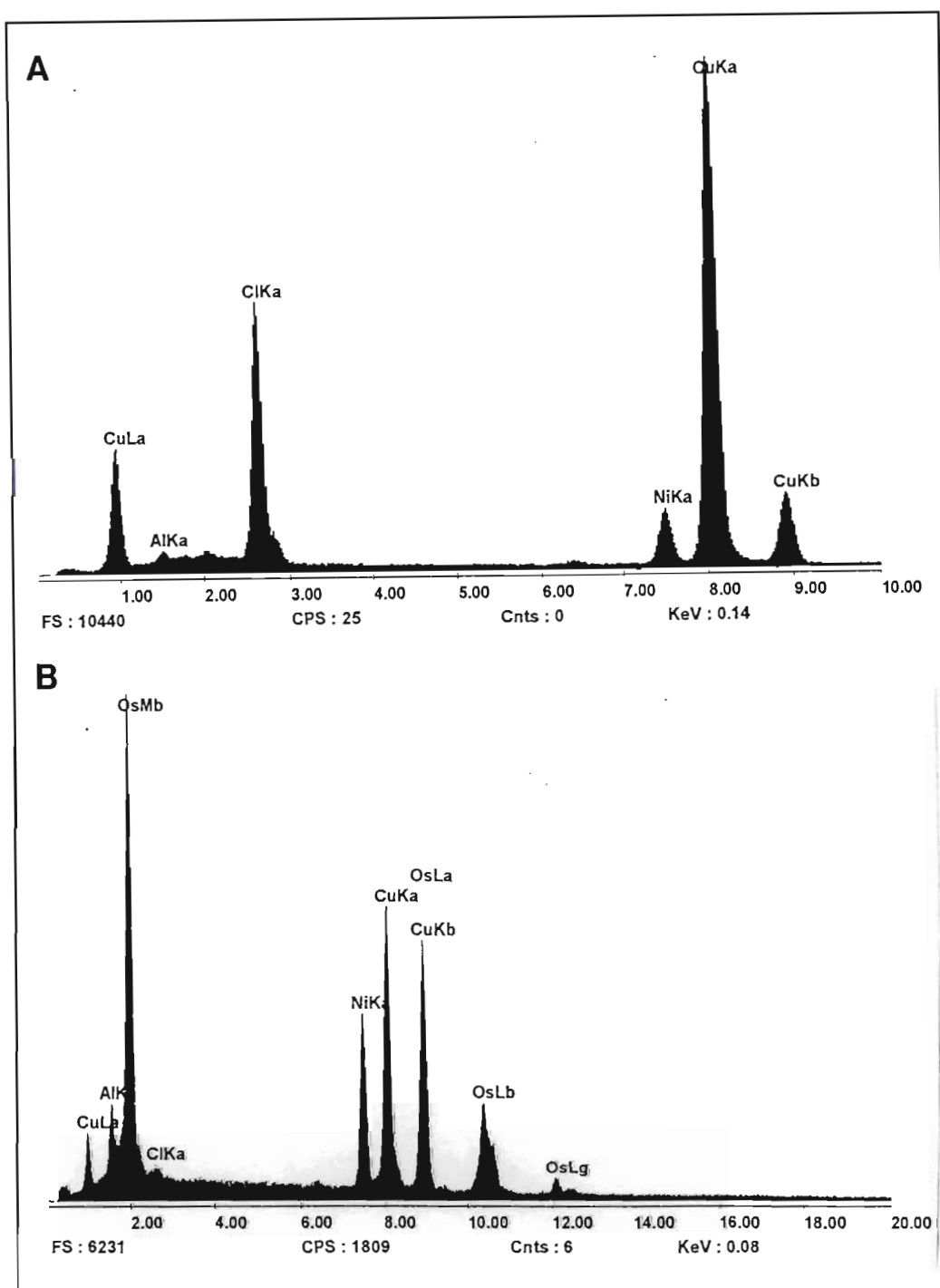
Figure 8.8 A. EDX spectrum of location indicated by arrow 1 in Plate 8.5B showing a large copper peak. Note the presence of chlorine at this site; B. EDX spectrum of location indicated by arrow 2 in Plate 8.5B showing a relatively much smaller copper peak than that observed at location 1. Osmium peaks (from the fixative) and a small chlorine peak (from $CuCl_2$) are also evident.

Subramanyam *et al.* (1983) found that *Neurospora crassa* accumulated copper to such an extent that the mycelium acquired a blue coloration. They determined that 90% of the accumulated copper was associated with the cell wall, which contained more chitosan, phosphate and amino groups but less chitin than mycelia grown in the absence of Cu^{2+}. Elevated concentrations of Cu^{2+} altered the metabolism of *N. crassa* so that more cell wall components capable of binding Cu^{2+} were synthesised (Subramanyam *et al.* 1983). The *Aspergillus niger* pellets exposed to copper solutions in the present study also displayed a blue coloration and copper was determined by TEM and EDX to be associated mostly with the cell wall structure.

Granules formed by a *Penicillium ochro-chloron* strain were found to sometimes contain copper, although the greater proportion of copper taken up by this fungus was accumulated in the cell surface system and little copper was taken up into the cell (Fukami *et al.* 1983). A similar Cu^{2+} distribution was observed in the present investigation, indicating that the *Aspergillus niger* strain studied had an analogous copper uptake mechanism.

Zhou and Kiff (1991) determined the main mechanism of metal uptake by *Rhizopus arrhizus* to be adsorption onto the fungal cell wall surface, i. e. onto negatively charged ligands, such as amines, on the cell surface. These authors suggested that the total sorptive surface area of the fungus was proportional to the amount of biomass present and determines how many Cu^{2+} ions will be bound by the cells (Zhou and Kiff 1991). Thus, the mechanism of Cu^{2+} uptake by *R. arrhizus* was through binding of metallic ions to the cell surface. The electron microscopic and EDX techniques used in the present investigation indicated that a similar Cu^{2+} ion binding mechanism operated in the *Aspergillus niger* strain studied.

In the present study it was observed that the Cu^{2+} ions were not adsorbed evenly on the surface of the hyphal strands. There appeared to be specific locations on the cell wall where metal binding sites were concentrated (Plate 8.5A).

8.4 CONCLUSIONS

The EDX mapping technique employed here clearly showed the metal ion uptake capacity of the fungal biomass and illustrated its potential for use in metal clean-up systems. Using a combination of AAS/HPLC and EDX the metal uptake potential of a specific microorganism, as well as the amount of biomass necessary to adsorb a known concentration of solubilized metal ions can be determined.

The preliminary TEM and EDX results indicated that lead was associated with the cell walls of the *Aspergillus niger* strain studied. The major proportion of copper taken up by this fungus was also found to be associated with the cell surface system, with little of the metal taken up into the cell. This is similar to the mechanism of metal uptake reported for *Penicillium* (Fukami *et al.* 1983) and *Rhizopus* species (Zhou and Kiff 1991). A combination of TEM and EDX techniques is a useful tool in ascertaining the main mechanism of metal uptake by any microbial process.

These results indicated that *Aspergillus* species (eg. *Aspergillus niger*) which are often waste products from fermentation industries, may be useful for the removal of heavy metals such as copper and lead, and possibly other metal ions, from industrial effluents. As the major mechanism of metal uptake appeared to be metabolism-independent biosorption onto the cell surface, dead/killed biomass can be used. This has several advantages in that no nutrient supplies are needed and toxicity effects are minimised. This fungus would, thus, be useful for the recovery of metal ions and the biomass could be used repeatedly following desorption processes.

CONCLUDING REMARKS

Enriched microbial associations were adapted, over a period of several months, to survive and grow in increasing concentrations of metals in a low-strength nutrient solution. This was necessary as they would need to survive in high metal concentrations if they were to be used in heavy metal polluted water clean-up processes.

Attached microorganisms were found to be more stable and also more efficient at metal uptake. Of the different materials examined, ground glass and polystyrene, rather than smooth glass, were found to offer the best surfaces for biofilm formation, indicating that surface roughness is important in biofilm development. Polystyrene would be the better surface to use in commercial clean-up processes as it is cheaper than glass and would, therefore, be more appropriate for use on an industrial scale. Electron microscopic observations of the development of the biofilm in the Cr^{3+}-containing medium over time showed that a progression of microorganisms was involved in biofilm formation. It would be interesting to see if the metal removing capacity of the biofilm could be enhanced by manipulating the biofilm population.

In this study some metals were found to be taken up more readily than others from solutions containing mixtures of metal ions. In general, it was found that Pb^{2+} ions were taken up more readily than Cd^{2+} ions which in turn were more actively removed from solution than Cu^{2+} ions by the microbial associations investigated. It could prove beneficial to expand this work to include other metal ions. Association of the metals with the various microorganisms was clearly demonstrated by the EDX techniques used. However, the actual mechanism involved, i.e. whether the metal ions were simply adsorbed onto the cell surfaces or whether they were taken up intracellularly, could not be resolved with these techniques.

Varying the proportions of pregrown inocula (adsorbents) generally did not affect the uptake of any of the specific metals tested to any great extent. In general, however, it was found that microbial associations which had previously been exposed to a metal took up that particular metal more readily. The metal-adapted microbial associations also showed

enhanced uptake of other metals to which they had not previously been exposed. Conceivably these microbial associations could efficiently adsorb other metals that were not tested in these investigations, thereby extending their usefulness as metal pollution control agents.

pH is another factor which affects metal uptake. Only Cu^{2+}-uptake was investigated in this regard as many problems were encountered with Pb^{2+} and Cd^{2+} ions due to precipitation. Using various buffer systems and acid/base adjustments, the optimum pH range for Cu^{2+} uptake by the Cu^{2+}-adapted microbial association was found to be 5.8-7.0. Other buffer systems should be used to determine the pH range over which Pb^{2+} and Cd^{2+} ions would be most effectively removed from solution by the Pb^{2+}- and Cd^{2+}-adapted microbial associations.

Many advantages and disadvantages have been cited for using either dead or living microbial biomass for metal uptake. The uptake of metals by heat-killed cells was investigated here and found to be efficient. However, as it is known that different killing methods have different affects on various surface components of microbial cells, other means of inactivating the microorganisms should be investigated. Such treatments may produce a biomass with improved metal-uptake ability by modifying the surface components/structures and exposing additional metal-binding sites.

Fungi are known to be more tolerant than other microorganisms to heavy metal stress and other unfavourable conditions. Furthermore, they can be readily and cheaply obtained because they constitute the waste products of many fermentation industries. Both pregrown, but non-metabolising, and heat-treated, non-living fungal mycelia were investigated for their metal-uptake capacity. In general, the pregrown *Aspergillus niger* mycelium offered the better metal-uptake potential, especially for Pb^{2+} and Cu^{2+} ions. Biomass concentration was found to be an important factor in establishing an efficient metal uptake process with pregrown fungal pellets and, therefore, had to be optimised. Scanning Electron Microscopy and EDX mapping techniques proved useful for identifying the association between the metal ions and the fungal mycelium. Additional information on the mechanism of metal uptake was obtained by examining thin sections of hyphae by

a combination of Transmission Electron microscopy and EDX techniques. It was determined that both copper and lead were adsorbed to the surface layers of the fungal hyphae, including wall and extracellular components, and that no, or very little, intracellular uptake occurred. Thus, pregrown or dead fungal biomass would be useful in a metal uptake process as the metal ions could be more easily recovered and the biomass reused. An understanding of the mechanism(s) governing metal uptake by microbial cells is essential for establishing an efficient biological metal uptake process.

When selecting a microbiologically-based clean-up process it is important to keep in mind the various factors which are known to affect metal uptake by microorganisms. This investigation showed that an attached, metal-adapted microbial association comprised of living or dead cells, or pelletised fungal mycelium, offered an efficient biologically-based system for removing heavy metal(s) from polluted waters. Factors such as pH, the presence or absence of organic and/or inorganic molecules and ions, and the nature of the support matrix should also be examined in more detail.

Microbial systems will probably never entirely replace existing processes for metal ion removal from effluents but if used in combination with these conventional processes the overall efficiency of the system might be improved, particularly in cases where low concentrations of heavy metals are present, as microorganisms are very efficient under such conditions. There is also much scope for the metal-removing potential of microorganisms to be further exploited. Genetic manipulations of microorganisms for increased metal tolerance and further research into metallothioneins should be undertaken to expand the applications of bioremediation of South Africa's limited water resources.

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APPENDIX A

Heavy Metal Solutions:

$CdCl_2$ (mw=183.28); Cd^{2+} mw=112.41 (61.3%)

A stock solution of 10000 mgl^{-1} Cd^{2+} was prepared as follows:

1.631g $CdCl_2$ were weighed out, dissolved in distilled water and diluted to 100 ml in a volumetric flask.

$CuCl_2.2H_2O$ (mw=170.45); Cu^{2+} mw=63.546 (37.3%)

A stock solution of 10000 mgl^{-1} Cu^{2+} was prepared as follows:

2.68g $CuCl_2.2H_2O$ were weighed out, dissolved in distilled water and diluted to 100 ml in a volumetric flask.

$Pb(NO_3)_2$ (mw=331.21); Pb^{2+} mw=207.2 (62.6%)

A stock solution of 10000 mgl^{-1} Pb^{2+} was prepared as follows:

1.5974g $Pb(NO_3)_2$ were weighed out, dissolved in distilled water and diluted to 100 ml in a volumetric flask.

$HgCl_2$ (mw=271.496); Hg^{2+} mw=200.59 (73.9%)

A stock solution of 100 mgl^{-1} Hg^{2+} was prepared as follows:

0.00677g $HgCl_2$ were weighed out, dissolved in distilled water and diluted to 100 ml in a volumetric flask.

$Cr(NO_3)_3.9H_2O$ (mw=400.016); Cr^{3+} mw=51.996 (13%)

A stock solution of 10000 mgl^{-1} Cr^{3+} was prepared as follows:

7.6923g $Cr(NO_3)_3.9H_2O$ were weighed out, dissolved in distilled water and diluted to 100 ml in a volumetric flask.

These were stored at 4°C after use.

APPENDIX B

Eluent for HPLC (Microsep)

Into a 1 litre volumetric flask were added:

    ~ 700 ml ultra-pure water

    0.433 g sodium octanesulfonate (Sigma)

    7.510 g tartaric acid (Sigma)

The flask was thoroughly shaken until the ingredients were dissolved and then 20 ml acetonitrile (HPLC grade) were added.

The flask was filled to the mark with ultra-pure water and the contents thoroughly mixed. The pH of the eluent was adjusted to 3.3 with NaOH pellets and then finally adjusted to pH 3.4 +/-0.01 with 1 M NaOH. It was critical to adjust the eluent pH correctly, otherwise significant differences in retention times were observed. The eluent was filtered and degassed using a 0.45 μm Millipore HA filter.

APPENDIX C

Post-column Reagent (Microsep)

NOTE: This was prepared in a well ventilated fume hood.

Into a 500 ml beaker with stirring bar were added:

~ 100 ml ultra-pure water

103 ml concentrated ammonium hydroxide (ACS grade)

0.026 g PAR (Sigma)

The contents were mixed until dissolved and then 29 ml concentrated acetic acid (ACS grade) were slowly added.

Care was taken as this is an exothermic reaction, giving off white fumes.

Once the ingredients had dissolved, the mixture was transferred to a 500 ml volumetric flask and diluted to the mark with ultra-pure water. The reagent was filtered and degassed using a 0.45 μm Millipore HA filter.

This reagent degraded in the presence of light and air, and was thus not used for longer than 24 hours.

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APPENDIX D

Examples of calibration curves used in HPLC ion Analysis:

Copper (Cu^{2+}):

Table 1   Concentrations of Cu^{2+} standards and peak heights for calibration curve

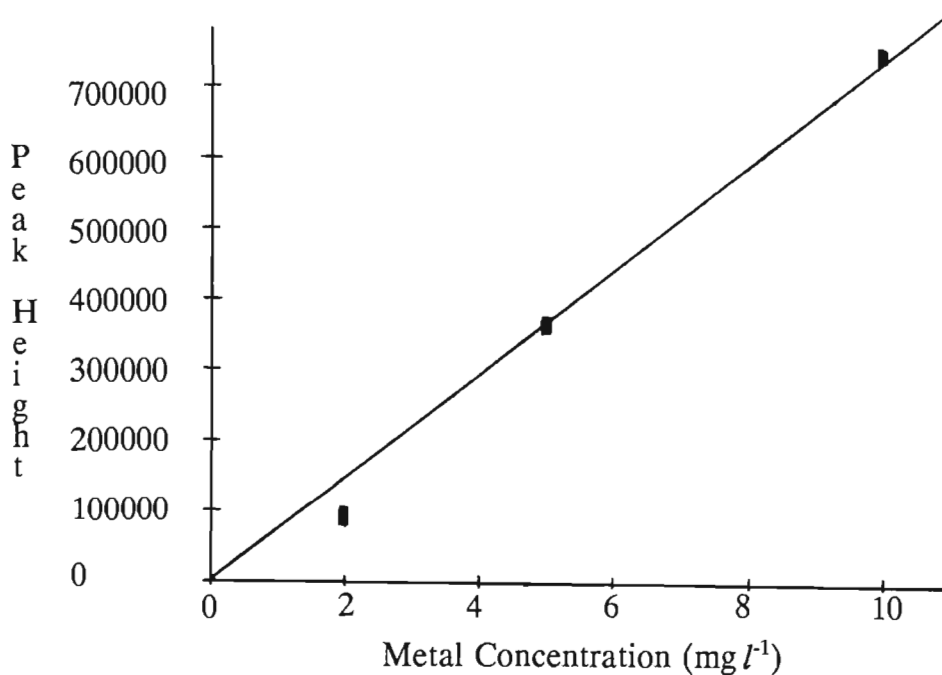| Standard Number	Metal Concentration (mg l^{-1})	Peak Height
1	2	88773.398
2	2	94570.757
3	5	363491.875
4	5	357035.562
5	10	730503.500
6	10	733614.500

Figure 1   Calibration curve used for determining concentrations of copper in solution.

Calibration Equation : 13679.179688x

Curve Type   : Linear forced through origin

Weighting   : Equal

Correlation Coefficient   : 0.9772705

Cadmium (Cd^{2+}):

Table 2   Concentration of cadmium standards and peak heights for calibration curve.

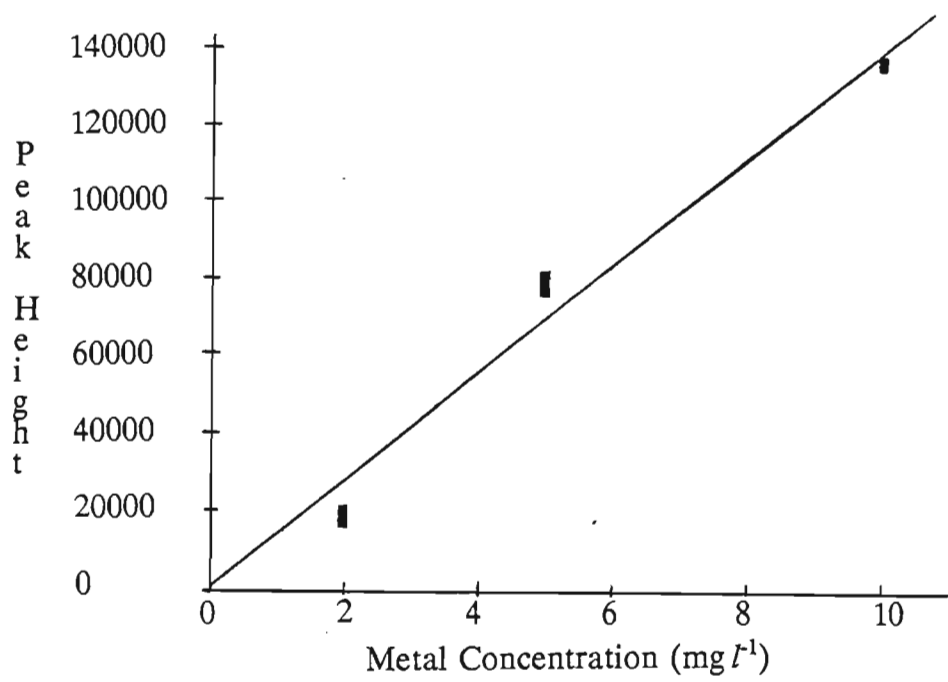| Standard Number	Metal Concentration (mgl^{-1})	Peak Height
1	2	16369.505
2	2	19296.228
3	5	75113.273
4	5	79615.367
5	10	134435.000
6	10	133990.375

Figure 2   Calibration curve used for determining concentrations of cadmium in solution.

Calibration Equation : 13679.179688x

Curve Type   : Linear forced through origin

Weighting   : Equal

Correlation Coefficient   : 0.9772705

Lead (Pb^{2+}):

Table 3 Concentration of lead standards and peak heights for calibration curve.

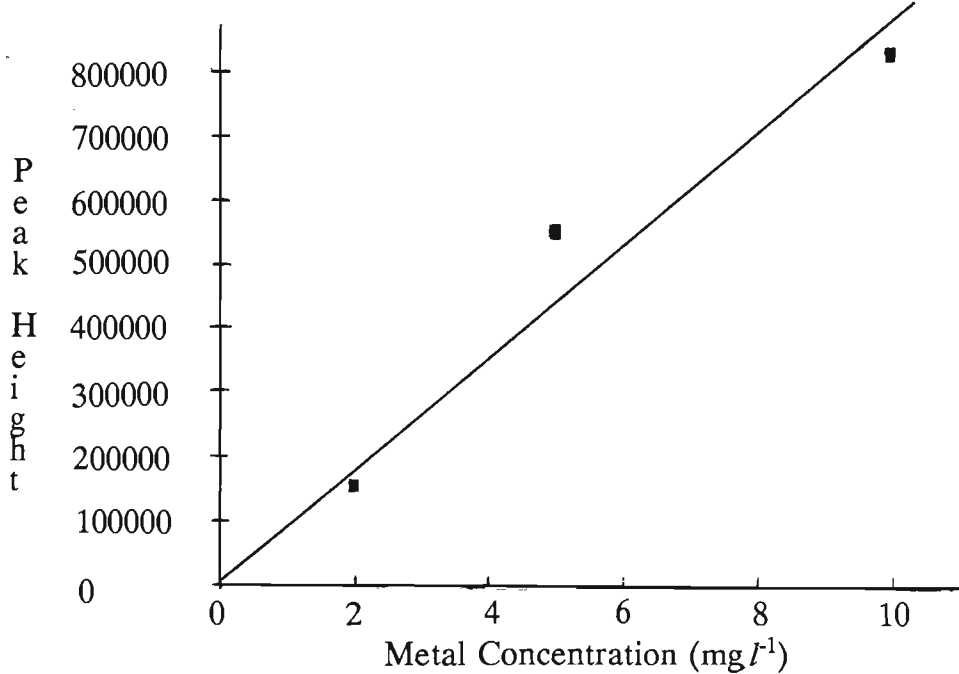| Standard Number	Metal Concentration (mg l^{-1})	Peak Height
1	2	148128.734
2	2	150223.796
3	5	540233.375
4	5	543624.062
5	10	818546.250
6	10	813651.937

Figure 3 Calibration curve used for determining concentrations of cadmium in solution.

Calibration Equation : 86581.296875x

Curve Type   : Linear forced through origin

Weighting   : Equal

Correlation Coefficient   : 0.933513

APPENDIX E

Sodium-phosphate buffer salts used:

Salts were weighed out, dissolved in distilled water and made up to 1 litre with distilled water:

$Na_2HPO_4.12H_2O$: 0.2 M solution = 71.64 g l^{-1}

$NaH_2PO_4.2H_2O$: 0.2 M solution = 31.21 g l^{-1}

Citric acid/Sodium-phosphate buffer salts used:

Salts were weighed out, dissolved in distilled water and made up to 1 litre with distilled water:

$C_6H_8O_7.H_2O$: 0.1 M solution = 21.01 g l^{-1}

$Na_2HPO_4.12H_2O$: 0.2 M solution = 71.64 g l^{-1}

Table 1 Volumes of sodium phosphate salt solutions (0.2M) used in making sodium phosphate buffer of different pH values.

pH	0.2 M $Na_2HPO_4.12H_2O$ (ml)	0.2 M $NaH_2PO_4.2H_2O$ (ml)
5.8	2	23
7.0	15.25	9.75
8.0	23.68	1.32

Table 2 Volumes of 0.1M citric acid and 0.2M sodium phosphate salt solutions used in making citric acid/$Na_2HPO_4.12H_2O$ buffer of different pH values.

pH	0.1 M Citric acid (ml)	0.2 M $Na_2HPO_4.12H_2O$ (ml)
2.6	22.275	2.725
3.0	19.863	5.137
4.0	15.363	9.637
5.0	12.125	12.875
5.8	9.888	15.112

Table 3 Volumes of sodium phosphate salt solutions (0.2 M) used in making sodium phosphate buffers with pH values close to neutrality.

pH	0.2 M $Na_2HPO_4.12H_2O$ (ml)	0.2 M $NaH_2PO_4.2H_2O$ (ml)
5.8	2	23
6.4	6.625	18.375
7.0	15.25	9.75

APPENDIX F

Malt Extract Broth

Malt Extract Broth was made up by dissolving the following ingredients in 1 litre of distilled water:

17 g malt extract

3 g peptone

This medium was then autoclaved at 115°C/10 psi for 10 minutes.

Half-strength Malt Extract Broth was made up by dissolving the following ingredients in 1 litre of distilled water:

8.5 g malt extract

1.5 g peptone

This medium was autoclaved at 115°C/10 psi for 10 minutes.

APPENDIX G

Currie's liquid medium (Currie, 1917)

The following were added to 1 litre of distilled water:

130 g sucrose

2.25 g ammonium nitrate

0.9 g potassium dihydrogen phosphate

0.21 g magnesium sulphate

The pH was adjusted to 3.4-3.5 with 1 M HCl or 1 M NaOH, and the medium was autoclaved at 115°C/10psi for 10 minutes.