Toxicity and biosorption of metals by *Saccharomyces cerevisiae*, *Amorphotheca resinae* and *Azolla filiculoides*.

M.Sc. Research Thesis

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of M.Sc. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 22nd September 1998

Dedicated to my family.

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ABSTRACT

Toxicity and biosorption of metals by *Saccharomyces cerevisiae*, *Amorphotheca resinae* and *Azolla filiculoides*.

M.Sc. Research Thesis

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The value of H^+ efflux in assessing and understanding metal interactions with *Saccharomyces cerevisiae* was investigated for its potential use as a rapid means of toxicity assessment for a range of metals Toxicity decreased in the order $Cu^{2+} > Cd^{2+} > Pb^{2+} > Co^{2+} > Sr^{2+}$ Toxic effects can be alleviated by external Ca^{2+} The effect of Cu^{2+} and Co^{2+} on *S cerevisiae* growth, and the intracellular localisation of Cu^{2+}, were studied in order to gain a better understanding of their toxicity

S cerevisiae, *Amorphotheca resinae* and *Azolla filiculoides* biosorbed various metals, with concomitant K^+ and Mg^{2+} release and pH change in certain situations *S cerevisiae* adsorbed Cu^{2+} and Cd^{2+} maximally to *ca* 150µmol g^{-1} and Sr^{2+} to *ca* 90µmol g^{-1} Cu^{2+}, Cd^{2+} and Sr^{2+} were adsorbed maximally to *ca* 70, 40 and 45µmol g^{-1} respectively by *A resinae* The data were also fitted to the Langmuir and Scatchard adsorption models

Both Cu^{2+} and Cd^{2+} were adsorbed to maximum uptake levels of 350 and 245 µmol g^{-1} respectively by native *A filiculoides* (*Azolla*) Native milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* exhibited Cu^{2+} uptake levels of *ca* 363 and 320µmol g^{-1} respectively from solutions with an initial Cu^{2+} concentration of 100mg l^{-1}

Milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla*, along with the native *Azolla*, successfully biosorbed influent Cu^{2+} during column studies Packed bed biosorption columns containing 1 0g and 2 5g of epichlorhydrin-immobilised *Azolla* were capable of complete removal of metal from *ca* 4 and 12 litres, respectively, of influent 5mg l^{-1} Cu^{2+} Even after 22 litres of influent solution had passed through, the 2 5g column was still at less than 75% saturation These columns were more efficient than those containing the same corresponding quantities of milled-sieved *Azolla* (*ca* 3 and 10 5 litres for 1 0 and 2 5g columns respectively), which in turn were more efficient than native *Azolla* (*ca* 3-4 litres for a 2 5g column) Selected results were compared to a computer model used to predict column profile The biosorbed Cu^{2+} was successfully recovered using Chelaton 3

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CURRENT PUBLICATIONS / PRESENTATIONS

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

Metals: pollution, biosorption and toxicity towards microbes.

1.1 Metal Pollution.

Developed and developing countries are struggling with burdens of heavy metal pollution in water and soil, resulting from expanding industrial activities These polluted natural resources pose health hazards to the population and deteriorate environmental quality of both aquatic and terrestrial ecosystems The term "heavy metal" refers to most metals with an atomic number greater than 20 and excludes alkali metals, alkaline earths, lanthanides and actinides (Mason, 1996) Heavy metals can enter the aquatic environment either from (a) point sources such as industrial premises and sewage-treatment plants or (b) diffuse sources such as road and agricultural runoff and aerial deposition Electroplating and metal finishing operations, electronic circuit production, mining and smeltering operations, paint and plastics production, steel and aluminium processes all produce large quantities of waste containing metals (Eccles, 1995, Mason, 1996, Volesky, 1990 *b*) A significant proportion of the heavy metals present in sewage effluents also originate from diffuse sources including domestic plumbing systems and road runoff (Zabel, 1993) Heavy metal pollution is well known to have a detrimental effect on soil processes and plant growth (Colpaert & Van Tichelen, 1996, Ernst *et al*, 1992, Gadd, 1993)

Cadmium, copper, zinc, nickel, mercury, thallium, tin, lead and arsenic are common examples of toxic elements, with cadmium being one of the most serious environmental pollutants (Hughes & Poole, 1989, Mason, 1996) and silver being probably

the most toxic element to microorganisms (Beveridge *et al*, 1997, Trevors, 1987) Strontium is a trace element that has no known essential biological role (Avery & Tobin, 1992) Concern has arisen over the fate of this radionuclide in the environment due to its continued discharge as a constituent of radioactive wastewaters from nuclear reactors and also because of the long half-life (*ca* 29 years) of the radioisotope ^{90}Sr (Macaskie, 1991, Watson *et al*, 1989) Furthermore, Sr^{2+} displays physicochemical properties similar to those of the biologically essential cations Ca^{2+}, Mg^{2+} and Na^{+} and competes directly for the same sites on biological cell surfaces (Urrutia, 1997) This may lead to interference with cellular transport systems, enzyme catalysis, structure stabilisation and the control of osmotic pressure

The European Union (EU) has compiled a "Black List" of pollutants (Mason, 1996) in Council Directive (76/464/EEC) on pollution caused by certain dangerous substances

(i) Organohalogen compounds and substances which may form such compounds in the aquatic environment,

(ii) Organophoshorous compounds,

(iii) Organotin compounds,

(iv) Substances having carcinogenic activity which is exhibited in or by the aquatic environment,

(v) Mercury and its compounds,

(vi) Cadmium and its compounds,

(vii) Persistent mineral oils and hydrocarbons of petroleum,

(viii) Persistent synthetic substances

Less dangerous pollutants are placed on the EU "Grey List" (Mason, 1996) in Council Directive (76/464/EEC) and include the following metals/metalloids and their compounds

1 Zinc	2 Copper	3 Nickel	4 Chromium	5 Lead
6 Selenium	7 Arsenic	8 Antimony	9 Molybdenum	10 Titanium
11 Tin	12 Barium	13 Beryllium	14 Boron	15 Uranium
16 Vanadium	17 Cobalt	18 Thallium	19 Tellerium	20 Silver

In recent years there has been considerable interest in the area of metal accumulation from aqueous solutions by yeasts, fungi and plants (Brady & Tobin, 1994, 1995, Leusch *et al* , 1995, Tel-Or *et al*, 1996, Volesky & Holan, 1995) Due to their non-biodegradability, metals cannot be treated biologically "in-situ" and must instead be extracted from contaminated streams (Sahoo *et al*, 1992) Concerns over the significant and long-term environmental hazards posed by heavy metals has prompted research into developing novel processes for removal/recovery of such elements (Blackwell *et al* , 1995, Dostalek *et al* , 1995, Leusch *et al* , 1995, McHale & McHale, 1994) To decontaminate small amounts of concentrated wastewater, the use of precipitation followed by filtration or ultrafiltration is an option Reverse osmosis or ion-exchange materials can also be used It may be uneconomical, however, to apply these techniques to purify large volumes of waste water containing a low concentration of contaminants

1.2 The role of Microorganisms.

Microbe-based adsorption has recently attracted much attention as a potential alternative to existing methods (Blackwell *et al*, 1995, Norris & Kelly, 1979, de Rome & Gadd, 1991, Strandberg *et al*, 1981) Metal uptake by microbial biomass consists of an initial non-metabolic step followed, in certain circumstances, by a second metabolism-dependent step, depending on the viability of the biomass, and is influenced by a number of environmental and experimental factors (Blackwell *et al*, 1995, Junghans & Straube, 1991) The nonmetabolic physico-chemical interactions between metals and microbial biomass are commonly referred to as "biosorption" (Gadd, 1993, Veglio & Beolchini, 1997) This initial biosorption step is often rapid (Avery & Tobin, 1992, Brady & Duncan, 1994 *a*, White & Gadd, 1987 *a*) although rates will depend on factors such as the type and concentration of both metal ion and biomass (Gadd, 1993) Biosorption is, however, independent of temperature (Mowll & Gadd, 1984), metabolic energy (de Rome & Gadd, 1987) and the presence of metabolic inhibitors (White & Gadd, 1987 *a*) Binding is almost always thought to involve the microbial cell wall, the exception being biosorption by extracellular polymers, and is attributed to ion-exchange, adsorption, complexation, precipitation and crystallisation within the multilaminate, microfibrillar cell wall structure (Mullen *et al*, 1992, Remacle, 1990, Tobin *et al*, 1984) Metabolism-dependent intracellular accumulation, often termed "bioaccumulation", is slower, but may lead to higher levels of uptake (Avery & Tobin, 1992), and is influenced by factors such as temperature, pH and the presence of metabolic inhibitors (Mowll & Gadd, 1984, Okorokov *et al*, 1977, de Rome & Gadd, 1987, White & Gadd, 1995)

The cell wall, considered to be the primary site involved in metal binding, contains a variety of functional groups including carboxylate, phosphate, thiolate and amine groups These ligands serve as potential binding sites, with varying specificities, for the sequestration of metal ions (Tobin *et al*, 1984) and each metal can exhibit a different affinity for individual wall biomolecules (Avery & Tobin, 1993) Pearson (1963) classified metallic ions according to a "hardness scale" defined by the strength of their binding with nonpolarisable (hard) or polarisable (soft) ligands A refinement of this classification was described by Nieboer and Richardson (1980) by using the covalent index, Xm^2r, as an index to class B ("soft") behaviour, where Xm is the metal-ion electronegativity (Allred, 1961) and r its ionic radius (Shannon & Prewitt, 1969, Shannon & Prewitt, 1970) The principle predicts that hard metals, which are generally nontoxic and often essential macronutrients for microbial growth, bind preferentially to oxygen-containing (hard) ligands, whereas soft metals, which often display greater toxicity, form more stable bonds with nitrogen- or sulphur-containing (soft) ligands The hard-and-soft principle also predicts that bonds formed between hard metals and hard ligands are predominantly ionic, whereas those of soft metal-ligand complexes are more covalent in character (Pearson, 1963) Accordingly, the covalent index of Cu^{2+} is greater than that of Cd^{2+} and the Cu^{2+} ion is expected to possess a greater degree of class B character and enhanced potential to form covalent bonds with biological ligands Similarly, Sr^{2+} is a class A ("hard") ion with a lower covalent index than either Cu^{2+} or Cd^{2+} and is expected to form bonds that are principally ionic

A range of microbes has proved successful for the extraction of metal ions from solution (Gadd, 1990), but these microbial-based biosorbents must be technologically and economically competitive with existing processes if they are to be used on an

industrial scale (Brierly *et al*, 1986 *a*, Holan *et al*, 1993) Most proposed metal ion-sequestering processes using microbial or plant biomass involve either a batch or column configuration incorporated into standard effluent treatment configurations (Bedell & Darnall, 1990) Column reactors are more readily adapted to continuous flow and automation than batch reactors and may offer greater metal-binding capacity and higher efficiency (*i e* higher purity effluents) (Bedell & Darnall, 1990)

The use of these microbial-based biosorbents for industrial applications has been hindered by problems associated with the physical characteristics of the material (McHale & McHale, 1994) Low mechanical strength of the biomass can cause difficulties associated with separation of the biomass from effluents which, in turn, contribute to limitations in process design A further problem is associated with fragmentation of the biomass causing flow restrictions in continuous-flow contact vessels Cross-linking or immobilisation technologies, stiffening of the biomass into a desirable particulate or granular form, may overcome many of these problems (McHale & McHale, 1994, Volesky, 1990) by reinforcing the biomass leading to improved physical characteristics of the biomass for process applications This may also enhance the sorption performance (Leusch *et al*, 1995, Ting & Teo, 1994), assist its use in column reactors and the reusability should improve the process economics (Volesky, 1990 *b*)

The development of a process which generates significant quantities of secondary waste, or smaller quantities of waste which is difficult to handle/dispose of or more toxic, does not solve the problem (Eccles, 1995) Any novel effluent clean-up process must comply with several criteria and those that contribute directly to cost effectiveness and environmental considerations will have by far the most significant

influence on the selection of the treatment process Eccles (1995) lists the major criteria for any clean-up process as

- **Compatibility** with existing operations, in particular with up-stream processes,
- **Cost Effectiveness** - environmental processes add to overall production costs,
- **Flexibility** - to handle fluctuations in quality and quantity of effluent feed,
- **Reliability** - as most effluent processes will operate continuously,
- **Robustness** - to minimise supervision and maintenance,
- **Simplicity** - to minimise the need for skilled operators and reduce costs,
- **Selectivity** - to remove only the contaminants (metals) under consideration

Yeasts possess an acknowledged potential for accumulating a range of metal cations (Blackwell *et al* , 1995) and large amounts of this metal can remain associated with the yeast cell wall (Murray & Kidby, 1975, Strandberg *et al* , 1981) Protein and carbohydrate fractions of yeast cell walls have previously been shown to be involved in binding Cu^{2+}, Cd^{2+} and Co^{2+} ions (Brady & Duncan, 1994 *b*) Isolated components of yeast cell walls (mannans, glucans and chitin) were also observed to accumulate greater quantities of metal than intact cell walls (Brady *et al* , 1994)

Recently there has been considerable interest in the potential of magnetic immobilisation systems for biotechnology applications ranging from protein recovery to enzyme and DNA purification (O'Brian *et al* , 1996) and improved fermentation performances with magnetically immobilised biocatalysts (Brady *et al* , 1996, Ivanova *et al* , 1996) Processes for metal recovery from waste streams using both metabolising and non-metabolising cultures in magnetic biosorbent systems have been

described (Elwood *et al* , 1992, Lloyd & Macaskie, 1996, Sly *et al* , 1993, Thomas & Macaskie, 1996, Wong & Fung, 1997) In metabolising cell systems metal precipitation by reaction with metabolically liberated phosphate or sulphide ions has been observed to result in magnetically responsive biosorbent This approach has been shown to be applicable to a wide range of metals and removal levels of up to 200μmol Hg^{2+} g^{-1} cells have been achieved in large scale tests (Ellwood *et al* , 1992) although the application of this system is limited to non-toxic effluents Alternative strategies have involved adsorption of cells to various oxide surfaces with relatively low cell loadings of the order of 10% w/w (Shabtai & Fleminger, 1994, Sly *et al* , 1993, Wong & Fung, 1997) Thus, while good metal binding to the cells has been achieved, overall biosorbent uptake levels were decreased and the durability of the biosorbent in large scale applications remains largely untested

Nonmagnetic particulates can be magnetised through a process known as seeding (Dauer & Dunlop, 1991) This facilitates rapid and simple removal of particulates, in this context the biosorbent, from solution using a magnetic separator or a permanent magnet For example in high gradient magnetic separation (HGMS) (Kolm *et al* , 1975) magnetic traction forces have been employed to capture magnetic particles on a magnetised fibre filtration matrix (Dauer & Dunlop, 1991)

Two types of magnetic biosorbent were recently prepared by novel protocols from epichlorhydrin-cross-linked *S cerevisiae* cell walls and their biosorption characteristics were compared to those of non-magnetic cell walls (Patzak *et al* , 1997) The magnetic biosorbents I and II were capable of binding Cu^{2+} maximally to 215 and 50 μmol g^{-1}, Cd^{2+} to 90 and 25μmol g^{-1} and Ag^{+} to 80 and 45μmol g^{-1} respectively These values compare with 400, 125 and 75 μmol g^{-1}, respectively, for

non-magnetic cell walls The advantages of using magnetic supports include both facile, selective recovery and recycling of magnetically immobilised adsorbents (Halling & Dunnill, 1980, Wong & Fung, 1997)

Rather than being viewed as an alternative to conventional chemical methods for treating metal-polluted aqueous systems, microbe-based adsorption may be used in tandem with these techniques, possibly as an additional final purification step It may also prove economical to direct research towards the utilisation of waste fungal biomass from industrial sources, for example from the fermentation industry (Unz & Shuttleworth, 1996)

Metal uptake processes are complex and dependent not only on specific surface properties of the organisms, but also on the cell physiology, chemistry of the metal ions and the physico-chemical influence of the cell's environment, for example metal concentration, pH and temperature (Gadd & de Rome, 1988, Gadd, 1993, Sag *et al*, 1995) A given metal ion may also be accumulated by different mechanisms in different microorganisms (Sag *et al*, 1995)

The source of the raw biomass and the cost of an immobilisation process play a major role in determining the overall manufacturing cost of the biosorbent material (see Table 1 1) while the versatility, ease of use and other technology characteristics will also contribute to the overall process costs, both capital and operational (Eccles, 1995, Kuyucak, 1990) It is difficult to obtain comparable cost data for different technologies in a liquid effluent clean-up system, and Eccles (1995) believes that any costs will be dependent on parameters such as

- plant process capability (m^3 day^{-1}),
- concentration of metals in solution,

- operational mode of the equipment,
- secondary treatments needed, such as regeneration of ion exchange resins,
- selectivity of ion exchange resins, coupled with their respective capacities for given metal(s),
- disposal of secondary wastes such as sludges

Source	Price of sorbent
Byproduct of fermentations	No cost or nominal plus drying and transportation
Activated sludge	No cost or nominal plus drying and transportation
Specifically cultured biomass e g fungi, yeast[a]	$1-$5/kg
Marine algae[a]	$1-$2/kg
Specifically cultured algae[a]	$2-$16/kg
Ion-exchange resins	$14-$28/kg
Activated carbon	$2-$4/kg

a Dry weight

Table 1.1: Sorbent types and their corresponding prices (from Kuyucak, 1990)

The microbial cell wall represents a key part of the microbe and offers protection against environmental toxicity The wall is in direct contact with the environment external to the cell and interacts with substances soluble in the liquid medium This interaction may be particularly pronounced in the case of metallic ions

due to the anionic characteristics of the cell wall (Remacle, 1990) The composition of the cell wall can be characteristic of the fungal genera (Table 1 2) and effectively determines metal binding capacity

Metal uptake behaviour by biosorbents may be profoundly affected by the growth conditions In a study of propagation and characterisation of *Rhizopus* biosorbents, the medium composition and growth condition were shown to affect the uptake of copper, manganese and chromium by *R javanicus* (Yerushalmi, 1990) When K_2HPO_4 was the only source of phosphate in the grown medium, both biomass production and copper uptake increased as the salt concentration was increased from 0 5 to 2 g l^{-1} (Yerushalmi, 1990)

Varying culture conditions and stages of the life cycle of the organism result in significant variations in fungal cell wall structures (Remacle, 1990) thereby influencing passive adsorption of test ions (Meikle *et al* , 1990) Increasing biomass concentration has also been reported to reduce metal uptake per gram of biomass in a number of systems (Brady and Duncan, 1994 *a*, Fourest & Roux, 1992, Singleton and Simmons, 1996) This may be due to a number of factors including electrostatic interactions, interference between binding sites, limited availability of solute and reduced mixing at high biomass densities (Fourest & Roux, 1992, Meikle *et al*, 1990, Singleton and Simmons, 1996)

Fourest and Roux (1992) report that zinc uptake decreases when *R arrhizus* biomass concentration is increased They also report that reduction in biomass concentration in the suspension at a given metal concentration enhances the metal/biosorbent ratio, and thus increases metal uptake per gram of biosorbent, as long as the latter is not saturated

Cell wall category	Taxonomic group	Representative genera
I Cellulose-glycogen	Acrasiales	*Polysphondylium, Dictyostalium*
II Cellulose -β-glucan	Oomycetes	*Phytopthora, Pythium, Saprolegnia*
III Cellulose-chitin	Hyphochytridiomyces	*Rhizidiomyces*
IV Chitin-chitosan	Zygomycetes	*Mucor, Phycomyces, Zygorhynchus*
	Chytridiomycetes	*Allomyces, Blastocladiella*
V Chitin -β- glucan	Euascomycetes	*Aspergillus, Neurospora*
	Homobasidiomycetes	*Schizophilum, Fomes*
VI Mannan-β-glucan	Hemiascomycetes	*Saccharomyces, Candida*
VII Chitin-mannan	Heterobasidiomycetes	*Sporobolomyces, Rhodotorula*
VIII Galactosamine - galactose polymers	Trichomycetes	*Amoebidium*

Table 1.2: Cell Wall Composition and Taxonomy of Fungi (from Bartnicki-Garcia, 1970)

The process of metal recovery using microbial-based biosorbent materials is basically a solid-liquid contact process consisting of a metal uptake (sequestering) cycle and the metal desorption (elution) cycle In its technological configuration, it would be very similar to that used in the ion-exchange process or activated carbon applications The metal-laden solution is contacted with the solid sorbent phase in a batch, semicontinuous, or continuous-flow arrangement Appropriate contact between the solution and the solid phase can be accomplished routinely in any modification of the following apparati batch-stirred tank contactor, continuous-flow stirred-tank contactor, fixed packed-bed contactor, pulsating-bed contactor, fluidised-bed contactor or multiple-bed contact arrangements

The metal-sequestering cycle which results in the metal being immobilised onto the solid biosorbent material has to be followed by a cycle which would result in the release of the metal in a concentrated form This part of the process, similar to that of ion-exchange, is based on eluting the metal by a small volume of an appropriate solution which should ideally regenerate the biosorbent for subsequent reuse Ashing of the metal-laden biosorbent material presents a possible process where the biosorbent is not considered for reuse (Volesky 1990 *b*)

1.3 Biosorption by Waste Biomass from the Fermentation and Pharmaceutical Industries.

Recovering or recycling metals following wastewater treatment can decrease the cost of the overall process Waste biomass from fermentation industries represents an important potential resource and suggested uses include ergosterol / ribonucleic acid isolation and the preparation of a biosorbent for heavy metal uptake

Conventional methods for treatment of metal-loaded waters include evaporation, neutralisation/precipitation, carbon adsorption, ion exchange, dialysis, electrodialysis and reverse osmosis Effluent clean-up processes must be able to effectively deal with a feed stream which may vary in both quality and quantity Domestic and industrial wastes often exhibit variability in chemical and physical parameters such as concentration of toxic component, pH ranging from strongly acidic to basic, simultaneous presence of inorganic and organic components, dissolved and volatile species, colloids and emulsions (Eccles, 1995, Zhao & Duncan, 1998) Processes incapable of dealing with such variables require upstream buffering/treatment facilities which adds significantly to the overall treatment cost It is also important to consider recycling the recovered metals due to increasing costs of solid waste disposal, with selective removal of metals subsequently being required (Eccles, 1995)

A biomass composed of *Bacillus* spp derived from a commercial fermentation process has been used for production of a granular biosorption agent (Brierley, 1990, Brierley *et al* , 1986 *a, b*) The system, called AMT BIOCLAIM^{TM}, has proved very effective in the treatment of metal-bearing effluents both in fixed-bed

canisters and fluid-bed reactor systems Several properties of this biosorbent make this a practical and an economically feasible treatment process (see Table 1 3)

	Ability of system to respond to variation in					
	Metal ion conc	pH	Suspended solids	Other contaminants (oils)	Other metal ions	Expected effluent conc (mg l^{-1})
Hydroxide precipitation	no	some	yes	yes	yes	2-5
Sulfide precipitation	no	no	yes	yes	yes	2-5
Ion Exchange	some	some	no	no	some	<1
Reverse osmosis	yes	some	no	no	some	<1-5
Ion transfer	yes	some	no	no	some	<1
Evaporation	yes	yes	yes	some	yes	1-5
Electro-dialysis	yes	some	no	no	some	2-5
AMT BIOCLAIM™	yes	yes	yes	yes	yes	<1

Table 1.3: Performance characteristics of heavy metal wastewater treatment technologies and comparison with the AMT BIOCLAIM™ process derived from *Bacillus* spp (derived from Brierley *et al* , 1986 *a* and Eccles, 1995)

As a waste by-product from alcohol production, brewing strains of *Saccharomyces* are a readily available and cost effective biosorbent with the potential for high metal uptake capacity and metal recovery (Omar *et al* , 1996, Wilhelmi and Duncan, 1996) Previously, the majority of biosorption studies have involved the use of laboratory cultured strains, but these may exhibit considerable differences from waste fermentation yeast due mainly to variations in culture conditions (Omar *et al*, 1996) Biosorption of Sr^{2+} by an industrial strain of *Saccharomyces cerevisiae* has previously been shown to exceed that of a laboratory strain (Avery & Tobin, 1992)

The raw material for the production of biosorbents, the microbial biomass, can be produced by fermentation processes and is preferably obtained as a waste material from the fermentation industries Biosorbents have been examined which originate from

-the food and beverage industry which produces beer and wine using the yeast *Saccharomyces cerevisiae* (Morley *et al* , 1996, Yerushalmi, 1990),

-the pharmaceutical industry, the products of which include citric acid from the fungus *Aspergillus niger* (Morley *et al* , 1996, Yerushalmi, 1990),

-the enzyme industry, using the fungi *A niger*, *Trichoderma reesii* and *Rhizopus arrhizus* and the *Bacillus* spp of bacteria (Brierley *et al* , 1986 *a*, *b*, Brierley, 1990, Yerushalmi, 1990)

1.4 Metal toxicity towards microorganisms.

Many metals are essential for cell growth and metabolism and consequently mechanisms exist for physiological uptake of metals including Cu, K, Mg, Ca, Na, Mn, Fe, Zn, Ni, and Co (Ehrlich, 1997, Morley *et al*, 1996) Toxicity results when a metal with no known biological function (cadmium or strontium for example) competes with or replaces a functional metal (Hughes & Poole, 1989) Effects of toxic metals include the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, the blocking of functional groups of biologically important molecules (*e g* enzymes and transport systems for essential nutrients and ions), conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Hughes & Poole, 1989, Ochiai, 1987)

The chemical state of metals is an important factor contributing to toxicity The toxicity of organometals, where the metal is bound to an organo group through a metal-carbon bond, is often much greater than that of the inorganic form (Gadd, 1992, Hughes & Poole, 1989, Zabel, 1993) as increased liposolubility results in greater uptake into the cell (Gadd, 1993) Toxicity varies both with the number and the identity of the organic groups (Cooney & Weurtz, 1989)

Cation release may be an indication of an ionic interaction between metal and biomass (Avery & Tobin, 1992) but is also frequently the result of membrane damage and resulting ion leakage from the cell interior (Belde *et al*, 1988, Brady & Duncan, 1994 *c*, Gadd & Mowll, 1983, Joho *et al*, 1995, Passow & Rothstein, 1960) *S cerevisiae* cellular K^+ levels have been observed to decrease after incubation in $CdSO_4$ and $CuSO_4$ (Norris and Kelly, 1977) while Sr^{2+} uptake has been observed previously to cause little or

no ion release for *S cerevisiae* (Avery & Tobin, 1992), but Sr^{2+}-induced ion release has been observed for denatured *R arrhizus* (Brady & Tobin, 1994)

Brady and Tobin (1994) observed a H^+ release of the order $Cu^{2+}>Cd^{2+}>Sr^{2+}$ for *R arrhizus* and H^+ displacement has been reported to be characteristic of covalent bonding (Avery & Tobin, 1992) This trend is in keeping with the hard and soft principle whereby softer ions will tend to interact with biological ligands predominantly through covalent bonding (Brady & Tobin, 1995, Shuman *et al*, 1983) leading to H^+ release and a reduction in pH

The presence or absence of a metal may exert a profound influence on the effects of other metals due to competition between them (Hughes & Poole, 1989) For example Mg^{2+} can prevent harmful effects of Cu^{2+} and Co^{2+} towards *S cerevisiae* (Karamushka & Gadd, 1994) and magnesium and calcium are reported to reduce the toxicity of a range of metals to microbes (Ainsworth *et al*, 1980, Babich & Stotzky, 1981, 1982, 1983, Karamushka & Gadd, 1994, Karamushka *et al*, 1996)

The plasma membrane is an important initial site of biological interaction between cells and their environment and is a selective barrier that can control both the influx and efflux of metal ions (Gadd, 1993, Karamushka & Gadd, 1994) The transport of a variety of solutes across yeast and fungal plasma membranes is dependent on the plasma membrane H^+-ATPase activity (Gadd, 1993, Jones & Gadd, 1990) Three main classes of H^+-pumping ATPases have been identified in fungi and yeasts (Serrano, 1984, 1985) in mitochondrial, vacuolar and plasma membranes, the latter two being associated with ion transport, regulation of intracellular pH and intracellular compartmentation (Jones & Gadd, 1990)

The addition of a carbon source to an *S cerevesiae* suspension leads to acidification of the external medium This is thought to be mainly due to H^+-efflux through a membrane bound H^+-ATPase (Ramos, 1985) rather than due to release of organic acids resulting from fermentation As this plasma membrane H^+-ATPase is responsive to external physico-chemical factors, it may be a useful indicator of metal toxicity (Karamushka & Gadd, 1994) and has many potential applications both in toxicity assessment and for investigations into metal-microbe interactions

1.5 Metal biosorption by plant biomass.

Plants have the ability to extract and concentrate a range of metals from the environment (Salt *et al* , 1995, Sela *et al* , 1988, Tel-Or *et al* , 1996) Metals essential for the growth and development of plants include Cu, Mg, Mo, Zn, Fe, Mn and possibly Ni and these are accumulated from soil and water (Raskin *et al* , 1994, Salt *et al* , 1995) Some metals with no known biological function, such as Cd, Co, Pb, Cr, Ag, and Se, can also be accumulated by certain plants although excessive accumulation may lead to toxicity (Raskin *et al* , 1994)

Plants have recently gained attention as potential metal biosorbents for use in the treatment of metal-bearing effluents (Baker *et al* , 1988, Salt *et al* , 1995, Sela *et al* , 1989, Tel-Or *et al* , 1996) *Azolla filiculoides* is a floating water fern common in many parts of the world (Sela *et al* , 1990) and may prove to be the most useful plant-based metal biosorbent The use of plants for environmental bioremediation, termed phytoremediation (Raskin *et al* , 1997), may be divided into (i) phytoextraction - the transport and concentration of metals from the soil into the

harvestable parts of roots and above-ground shoots by metal accumulating plants (Kumar *et al*, 1995 Raskin *et al*, 1997), (ii) rhizofiltration - the adsorption, precipitation and concentration of toxic metals from polluted effluents by plant roots (Dushenkov *et al*, 1995), (iii) phytostabilization - the reduction of heavy metal mobility by metal tolerant plants, thereby reducing the risk of further environmental degradation by leaching into the ground water or airborne spread (Salt *et al*, 1995), (iv) plant assisted bioremediation - plant roots in conjunction with their rhizospheric microorganisms used to remediate soils contaminated with organics (Walton & Anderson, 1992, Anderson *et al*, 1993)

1.6 Fungal Melanins and their Interactions with Metals

1.6.1 *Introduction to fungal melanins*

Melanins are high molecular mass dark brown or black pigments (Vidal-Cross *et al*, 1994, Wheeler & Bell, 1988) and are formed by the oxidative polymerization of phenolic compounds They are found in animals, plants, fungi and bacteria (Wheeler & Bell, 1988, Bell & Wheeler, 1986) and in fungi they are located either in cell walls or exist as extracellular polymers On electron micrographs they appear as electron dense granules (Gadd, 1993) Extracellular melanin arises from a number of different sources Wall-bound melanin may be released into the external environment, although this is generally identical to that located in the cell wall Melanins are more correctly defined as "extracellular" if they are synthesised externally from the cell This may occur through secretion of either phenol compounds which are later oxidised or through secretion of the phenol oxidases to oxidise phenolics in the medium external to the fungus (Bell & Wheeler, 1988)

Melanins are often poorly soluble in alkali and generally insoluble in water, aqueous acids and common organic solvents Conjugation with carbohydrates or proteins sometimes occurs, in which case the melanins become water soluble (Wheeler & Bell, 1988)

Melanins may exist as free radicals (Longuet-Higgins, 1960, Mason *et al*, 1960) which are easily formed under various conditions such as incubation at increased temperatures (Zhdanova *et al*, 1980) irradiation with ultraviolet (UV) rays or γ-rays (Slawinska *et al*, 1975, Zhdanova *et al*, 1978) or reaction with chemical reductants They can act as proton receivers or donors (Lillie, 1969) and may be reduced by silver ions and oxidised by H_2O_2 (Wheeler & Bell, 1988)

Purification of melanins involves their dissolution in alkali and reprecipitation in acid Prolonged hydrolysis in aqueous acid removes associated proteins, carbohydrates and lipids Alternating cycles of organic solvents and hot acids provides additional purification (Wheeler & Bell, 1988, Ellis & Griffiths, 1974)

Melanins are not essential for fungal growth as both pigmented and albino strains of the same fungi exist Rather, the pigments bestow upon the fungus an added advantage in certain environments Numerous pathogenic fungi synthesize melanin to increase virulence (Jacobsen & Emery, 1991, Jacobsen & Tinnel, 1993, Kubo *et al*, 1985, Woloshuk *et al*, 1980) Mutants of *Verticillium dahliae*, *Cochliobolus miyabeanus* and *Magnaporthe grisea*, which lack the ability to synthesize melanin, are unable to penetrate the host leaf and therefore lose their pathogenicity (Kubo *et al*, 1985, Woloshuk *et al*, 1980, Lundqvist *et al*, 1993)

Melanins also enhance the survival of fungi under environmental stress The melanin present in fungal conidia reduces damage due to UV light (Durrell, 1964, Wang

& Casadevall, 1994), solar radiation (Carlile & Watkinson, 1994), γ-irradiation (Durrell, 1964) and x-rays (Lukiewicz & Ablewicz, 1974) The degree of protection towards UV light is proportional to the melanin concentration in conidial walls (Durrell, 1964) Melanins may also play a role in fungal resistance to dessication and high temperatures, although no definitive work has been carried out in this area The inhibition of cellulase by melanin is time-dependent and results in the formation of an irreversible enzyme-melanin complex Results point to a primary involvement of electrostatic bonding in formation of these complexes (Bull, 1970)

1.6.2. *Interaction of Melanins with Metals*

1.6.2. (a) *Functional groups*

Melanins contain carboxyl, phenolic, hydroxyl and amine groups, which provide many potential binding or biosorption sites for metal ions (Haider et al , 1975, Meuzelaar *et al* , 1977, Schnitzer *et al* , 1973, Schnitzer & Neyroud, 1975) Fungal melanins appear to be similar to soil humic acids with respect to volatile compounds released upon pyrolysis, amino acids released upon acid hydrolysis, IR and ^{13}C NMR spectra and ion exchange capacity (Saiz-Jiminez, 1983, Saiz-Jiminez *et al* , 1975) Humic acid carboxylic groups are known to bind metal ions by ion exchange, with release of H^+ ions Reaction with Cu(II) involves chelation by *o*-hydroxycarboxylic acid and *o*-dicarboxylic acid ligands An alternative reaction may be that of metal ions complexing the same types of oxygen-containing functional groups without forming a chelate ring (Boyd *et al* , 1981) An electron spin resonance (ESR) study of copper binding to humic acids showed that the direct Cu^{2+}-organic interactions are largely ionic and involve surface oxygen atoms (McBride, 1978)

In melanins, it appears likely that, as in other biomass types (Tobin *et al*, 1990), various functional groups may contribute in differing degrees to metal binding resulting in an array of multiple non-equivalent binding sites It has been reported that copper binds principally to carboxyl groups in catechol melanin at pH<5 but at pH>6 it is mainly phenolic hydroxyl groups which bind the copper (Froncisz *et al*, 1980) In contrast, ESR studies of iron and copper uptake by fungal phenolic polymers have suggested that nitrogen-containing groups of the proteinaceous moiety are involved in binding (Saiz-Jiminez & Shafizadeh, 1984) whereas multiple quinone groups have been proposed as the coordination sites for uranium uptake (Sakaguchi & Nakajima, 1987)

The maximum binding capacity of fungal melanins has been reported to be in the order Cu>Ca>Mg>Zn (Saiz-Jiminez & Shafizadeh, 1984, Zunino & Martin, 1977) The authors suggest that the preferential binding of Cu^{2+} may be due to its borderline status between classes A and B which allows it to receive electrons from all functional groups present in fungal melanins (Saiz-Jiminez & Shafizadeh, 1984)

1.6.2. (**b**) *Metal binding and desorption*

Melanins from a variety of fungal sources exhibit biosorption behaviour (see Table 1 4) In a comprehensive study of Cu^{2+} binding by extracellular melanin and intact biomass of albino and melanized strains of *Cladosporium resinae* (now *Amorphotheca resinae*) and *Aureobasidium pullulans*, the amount of copper adsorbed per unit weight of adsorbate was observed to increase with increasing copper concentration in the solution (Gadd & de Rome, 1988) Extracellular melanin of *A pullulans* was found to bind significantly more Cu^{2+} than either the pigmented or albino biomass Also, the pigmented biomass had a significantly higher Cu^{2+} uptake capacity than the albino biomass indicating

Adsorbent	Metal	Initial / Equilibrium metal concentration[a]	Metal uptake (nmol mg^{-1} dry wt)	Source
Albino *C resinae*	Cu	C = 80μM	120	Gadd & de Rome, 1988
Pigmented *C resinae*			180	
C resinae melanin			350	
Albino *A pullulans*	Cu	C = 200μM	45	Gadd & de Rome, 1988
Pigmented *A pullulans*			80	
A pullulans melanin			115	
Commercially prepared melanin	U	I = 42μM	84	Sakaguchi & Nakajima, 1987
Pigmented *A pullulans*	TBTC[b]	I = 1 0mM	240	Gadd *et al*, 1990
Non-pigmented *A pullulans*		C = 2 5 mM	700	
Pigmented *A pullulans*		C = 2 5mM	900	
A Pullulans melanin		C = 0 4mM	22000	
Albino *P digitatum*	Cd	Not Available	7 4	Siegel *et al*, 1986, Siegel *et al*, 1990
Albino *C cladosporoides*			8 8	
Pigmented *C cladosporoides*			21 5	
Albino *P digitatum*	Cd	Not Available	4 9[e]	Siegel *et al*, 1986, Siegel *et al* 1990
Albino *C cladosporoides*			5 0[e]	
Pigmented *C cladosporoides*			17 0[e]	
C cladosporoides + 2 5mM PABA[c]			8 6[e]	
C cladosporoides + 2 5mM PTU[d]			9 1[e]	
Albino *P digitatum*	Ni	I = 150μM	10 5[e]	Siegel *et al*, 1986
	Cu	I = 150μM	10 6[e]	
	Zn	I = 150μM	9 4[e]	
	Pb	I = 90μM	8 5[e]	
Pigmented *C cladosporoides*	Ni	I = 150μM	52 0[e]	Siegel *et al* 1986
	Cu	I = 150μM	58 1[e]	
	Zn	I = 150μM	51 7[e]	
	Pb	I = 90μM	23 2[e]	
Albino *P digitatum*	Ni	I = 350μM	35 0	Siegel *et al*, 1986
	Cu		39 0	
	Zn		38 0	
	Cd		30 0	
	Pb		32 0	
Pigmented *C cladosporoides*	Ni	I = 330μM	155 0	Siegel *et al*, 1986
	Cu		140 0	
	Zn		90 0	
	Cd		110 0	
	Pb		165 0	

[a]I = Initial metal concentration, C = Equilibrium metal concentration (see text)
[b]TBTC = Tributyltin chloride
[c]PABA = *p*-Amino Benzoic Acid, inhibits melanin synthesis
[d]PTU = Phenylthiourea, inhibits melanin synthesis
[e]Rate of metal uptake (nmol mg^{-1} h^{-1})

Table 1.4: Biosorption by albino and pigmented biomass and purified melanin (From Fogarty & Tobin, 1996)

the significant role of melanin in biosorption In the case of *C resinae* there was a much greater difference in uptake between the melanin and intact biomass, with the melanin adsorbing almost double the amount of Cu^{2+} (*ca* 350 nmol mg^{-1}) as compared with the pigmented biomass (*ca* 180 nmol mg^{-1}) and almost three times that of the albino (*ca* 120 nmol mg^{-1}) at an equilibrium Cu^{2+} solution concentration of 80 µM

Metal desorption by dilute mineral acids was observed to increase with decreasing pH and the percentage desorption was lowest for extracellular melanin when compared with the intact biomass of both fungal types (Gadd & de Rome, 1988) At the lowest pH level (pH 2 3) only *ca* 20% of the total adsorbed Cu^{2+} was desorbed from the melanin of *C resinae* in comparison with *ca* 47% from melanin of *A pullulans* These values are significantly lower than those for intact biomass, where desorption from the pigmented strains was lower than from the albino strains Similarly, less than 20% of total Cu^{2+} was desorbed from Cu^{2+}-loaded extracellular melanin of *C resinae* by metal sulphates at cation concentrations up to 250µM while Cu^{2+} desorption from pigmented and albino cells ranged from 40-80% *A pullulans* cells were reported to exhibit similar behaviour

In the presence of copper, melanin production by both *C resinae* and *A pullulans* increased (Gadd & de Rome, 1988, Gadd & Griffiths, 1980) For pigmented *C resinae* extracellular melanin production increased from 22 to 25 µg mg^{-1} in the presence of 5 mM $CuSO_4$, although the final yield of biomass for both pigmented and albino strains was reduced by 30% The melanin was also produced earlier in the presence of copper (after 24 h compared to 36 h in the absence of copper) (Gadd & de Rome, 1988) This may be due to the fact that DHN melanin polymerization is thought to be controlled by a copper-containing laccase so copper deficiency blocks melanin synthesis (Bell & Wheeler, 1986)

A slight decrease in toxicity as evidenced by increased growth rate and biomass yield upon addition of melanin to the growth medium may be attributed to melanin metal binding and sequestration

Sakaguchi and Nakajima (1987) observed that commercially-prepared melanin absorbed uranium from seawater almost quantitatively and in batch experiments maximum uptake levels of 84 nmol mg^{-1} were observed from seawater supplemented to 10 mg uranium l^{-1} In similar batch studies, pigmented *C cladosporoides* biosorbed 2 5 to 4-fold more Ni, Cu, Zn, Cd and Pb and at 4 to 6-fold higher rates than albino *Penicillium digitatum* (Siegel *et al*, 1986, Siegel *et al*, 1990) Both *P digitatum* and *C cladosporoides* are non-pigmented after 4 and 2 days growth respectively and capable of removing *ca* 7 4 and 8 8 mM kg^{-1} of Cd^{2+} from solution After 4 days growth, however, *C cladosporoides* is green-black in colour typical of early melanization and is capable of removing *ca* 21 5 mM kg^{-1} of Cd^{2+}

Phenylthiourea (PTU) and p-amino benzoic acid (PABA) are inhibitors of melanin synthesis and provide additional evidence for the role of melanin in Cd^{2+} uptake *P digitatum* after 4 days and *C cladosporoides* after 2 and 4 days growth had relative melanin levels of 0, 10 and 190 and Cd^{2+} uptake rates *ca* 4 9, 5 0 and 17 0 nmol mg^{-1} h^{1} respectively However, after 4 days growth in the presence of 2 5mM of (i) PTU or (ii) PABA, the *C cladosporoides* had relative melanin levels of 121 and 97 and Cd^{2+} uptake rates of 9 1 and 8 6 nmol mg^{-1} h^{-1} respectively Also, cultures grown in the presence of PTU or PABA were green compared to the green-black colour of inhibitor-free cultures containing higher levels of melanin

This enhanced capacity of melanized *C cladosporoides* to remove more metal ion than non-pigmented *P digitatum* was also observed for Ni, Cu, Zn and Pb with uptake

rates of *ca* 52 0, 58 1, 51 7 and 23 2 nmol mg^{-1} h^{-1} for *C cladosporoides* compared to *ca* 10 5, 10 6, 9 4 and 8 5 nmol mg^{-1} h^{-1} for *P digitatum* Equilibrium loads of Ni, Cu, Zn, Cd and Pb at differing initial metal concentrations were also significantly greater for *C cladosporoides*

Rhizomorphs of *Armillaria* sp consist of an inner core, the medulla, and the cortex, which may contain up to three layers The cells of the cortex are melanized, with melanin also present in the intercellular spaces Rhizomorphs of four *Armillaria* species from three geographic locations had elevated levels of metal ions associated with the outer surfaces compared to the surrounding soil Mean concentrations of Zn, Al, Cu, and Cd from one particular site were, respectively, 96, 24, 30 and 40 times higher in rhizomorphs than in the associated soil Energy dispersive X-ray microanalysis of the rhizomorphs from this site demonstrated that the high concentrations of metal ions were located on the melanized outer surfaces and not in the medulla (Rizzo *et al* , 1992)

Fungal melanin has also a significant role to play in the biosorption of tributyltin chloride (TBTC), an organotin compound used in a variety of industrial processes (Cooney & Weurtz, 1989, Thayer, 1984) The melanic strain of *A pullulans* has been shown to adapt much more quickly to growth in the presence of 0 3µM TBTC, when compared to the albino strain Growth of the pigmented strain in the presence of an external concentration of 1mM TBTC resulted in the biosorption of *ca* 0 24mmol TBTC $(g\ dry\ wt)^{-1}$ (Gadd *et al* , 1990) Adsorption isotherms of TBTC adsorbed per unit weight of biomass at various concentrations revealed that pigmented *A pullulans* removed more TBTC from solution than the albino strain (see Table 1 4) Extracellular melanin purified from the pigmented strain exhibited uptake levels of *ca* 22µmol TBTC $(mg\ dry\ wt)^{-1}$ at an equilibrium concentration of only 0 4mM (Gadd *et al* , 1990)

1.6.3. *Conclusion*

Fungal melanins have a high biosorptive capacity for a variety of metal ions The presence of melanin in pigmented cell walls is certainly responsible for the higher levels of biosorption in comparison with albino cells, with purified extracellular melanin showing greater biosorption than intact biomass Metal desorption is also of the order albino biomass > pigmented biomass > extracellular melanin, indicating the strength of the melanin-metal bond

Melanins have a significant role in reducing levels of toxic metal ions external to the fungal cell, leading to better growth in toxic environments Adsorption of metal ions by melanized outer surfaces may also protect fungi from antagonistic microbes (Rizzo *et al*, 1992) High concentrations of metal ions on outer surfaces of fungi may be directly toxic to antagonists or may interfere with the activity of extracellular hydrolytic enzymes Complexation of metal ions may also reduce the availability of essential microelements to antagonistic microorganisms (Bell & Wheeler, 1986, Rizzo *et al*, 1992) Most fungal biomass in soils is melanized (Bell & Wheeler, 1986) so melanin-metal ion interactions are also ecologically significant in terms of soil pollution and plant nutrition (Senesi *et al*, 1987) The use of purified melanin and pigmented biomass for the removal of toxic and/or strategic ions from polluted waters, and recovery of precious metal ions from solution, warrants further study

1.7 Research overview.

The initial phase of this research was concerned with interactions between a range of metals and the yeast *S cerevisiae* The value of H^+ extrusion in assessing and understanding metal interactions with yeast was examined for a range of metals, namely Cu^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+} and Sr^{2+} On account of its role in possibly stabilising membrane structure and also its similar functions to Mg^{2+} which has previously been observed to reduce harmful effects of Cu^{2+} and Co^{2+} toxicity towards *S cerevisiae* (Aoyama *et al* , 1986, Karamushka & Gadd, 1994), interactive effects with Ca^{2+} for possible alleviation of metal toxicity was also examined The effect of Cu^{2+} and Co^{2+} on *S cerevisiae* growth was studied in order to gain a better understanding of their toxicity, and the findings related to the H^+ extrusion results Intracellular localisation of Cu^{2+} was examined at concentrations of 25 and 100μM to further understand the metal-yeast interactions

Subsequent work involved examining the abilities of *S cerevisiae*, *Amorphotheca resinae* and *Azolla filiculoides* to biosorb various metals and also the concomitant K^+ and Mg^{2+} release and pH change in certain situations Reactors employing a column configuration may offer greater metal binding capacity, higher purity effluents and are more readily adapted to automation than batch reactors (Bedell & Darnall, 1990) Consequently, biomass with the greatest biosorption characteristics was selected for use in packed bed column studies both in native form and after immobilisation

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS.

2.1 Organisms, media and culture conditions.

2.1.1 *Saccharomyces cerevisiae*: Maintenance, growth and preparation for batch studies

S cerevisiae was maintained on malt extract agar (Oxoid) at 28°C and grown in liquid medium comprising (g l^{-1}) D-glucose, 20, KH_2PO_4, 2 72, K_2HPO_4, 3 98, $(NH_4)_2SO_4$, 2, $MgSO_4\ 7H_2O$, 0 5, $FeSO_4\ 7H_2O$, 0 0022, $ZnSO_4\ 7H_2O$, 0 004, $MnSO_4\ 7H_2O$, 0 004, $CuSO_4\ 5H_2O$, 0 004, yeast extract, 1 A 10ml quantity of this medium was loop inoculated and grown at 28°C on an orbital shaker at 100 rpm for 48 h Experimental flasks were inoculated with these starter cultures and grown for a further 20 h The biomass was harvested by filtration through Whatman No 1 filter paper, washed several times with deionized, distilled water (DDH_2O) and pressed dry between sheets of Whatman No 1 filter paper Samples of biomass were dried at 60°C overnight for dry weight measurement

2.1.2 *Amorphotheca resinae* Parberry: Maintenance, growth and preparation for batch studies.

Amorphotheca resinae Parberry (IMI 296262) was maintained on malt extract agar at 28°C and grown in liquid culture comprising (g l^{-1}) D-glucose, 20, $(NH_4)_2SO_4$, 5, KH_2PO_4, 0 5, $MgSO_4\ 7H_20$, 0 2, $CaCl_2\ 6H_20$, 0 05, NaCl, 0 1, $FeCl_3\ 6H_20$, 0 0025, $ZnSO_4\ 7H_2O$, 0 004, $MnSO_4\ 4H_2O$, 0 004, $CuSO_4\ 5H_2O$, 0 0004 Cultures were grown at 28°C on an orbital shaker at 100 rpm for 7 days The biomass was harvested as described for *S cerevisiae* and mildly homogenised using a Sorvall Omni-Mixer (Du Pont

Instruments, Connecticut, USA) Samples of biomass were dried at 60°C overnight for dry weight measurement

2.1.3 *Azolla filiculoides* Lamarck: Preparation for batch studies.

Azolla filiculoides Lamarck used was obtained from Dr Elisha Tel-Or, Department of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot 76100, Israel This biomass was used in a range of different forms either in its native state or following immobilisation Prior to use the *Azolla* was washed extensively with deionized, distilled water, oven-dried at 60°C overnight and milled to uniform particle size The resulting particles were then sieved to a size range of between 0 1 and 0 5 mm in diameter (denoted milled-sieved *Azolla* in the following sections) The biosorbent was then either used in this form or immobilised using one of the following methods

(i) *Immobilisation with tetraethoxysilane (TEOS)* A procedure modified from Braun *et al* , (1990) was employed using a TEOS Methanol H_20 weight ratio of 9 5 20 6 73 These components were mixed for 2 h and the methanol was allowed to evaporate during 2 days at 25°C, until the mixture reached a consistency of oil Milled-sieved *Azolla* biomass was then added to a concentration of either 5g or 2g per 100ml (classified as TEOS (i) and TEOS (ii) respectively) and the solution dried at 90°C for 5 h

(ii) *Immobilisation with tetramethoxysilane (TMOS)* A procedure again modified from Braun *et al* (1990) was employed using a TMOS Methanol H_20 NaOH weight ratio of 6 84 4 74 2 4 10^{-4} These components were mixed and cooled to -5°C A 10g quantity of milled-sieved *Azolla* per 100ml was then added and the biosorbent dried at 90°C for 24 h

(iii) *Immobilisation with epichlorhydrin* A procedure modified from Dostalek *et al*, (1994) and Patzak *et al*, (1997) was employed Milled-sieved *Azolla* (2g) was added to 10ml of deionized, distilled water After 10 min, 1ml of 40% NaOH was added, followed 20 min later by 0 75 ml of epichlorhydrin, with mixing after each addition The suspension was left in a closed vessel for 60 min and then heated to 50°C for 60 min Following this, the suspension was neutralised in 0 35M acetic acid and washed several times with deionized, distilled water The biosorbent particles were then mixed with water in a high speed homogeniser, filtered and rinsed successively with methanol, acetone and diethyl ether The biomass was finally dried at room temperature for 24 h

Each of the above-mentioned biomass types were then milled to uniform particle size and filtered to a size range of between 0 1 and 0 5mm These immobilised biosorbents were subsequently examined for their Cu^{2+} uptake

2.2 Metal Analysis

2.2.1 Metal Analysis by Anode Stripping Voltammetry (ASV).

Concentrations of Cu^{2+} and Cd^{2+} were determined by Anode Stripping Voltammetry (ASV) using a Polarographic Analyser PA3 connected to a Static Mercury Drop Electrode SMDE1 with electrolysis time of 40 s, scan rate of 20 mV s^{1} and modulation amplitude of 50mV Duplicate readings were taken and the mean value determined

2.2.2 Metal analysis by Atomic Absorption Spectrophotometry (AAS).

Concentrations of Cu^{2+}, Cd^{2+}, Sr^{2+}, K^{+} and Mg^{2+} were determined by Atomic Absorption Spectrophotometry (AAS) using a Perkin Elmer 3100 AAS with reference

to appropriate standards prepared from commercial solutions and from the appropriate metal salts

2.3 Measurement of pH.

A WTW pMX 3000 Microprocessor pH/ION Meter was used to determine pH

2.4 Measurement of Optical Density (OD) during *S. cerevisiae* cell growth.

A Unicam 8625 UV/VIS Spectrometer was used to measure optical density at 550nm (OD_{550}) of *S cerevisiae* cultures

2.5 Toxicity of metals towards S. cerevisiae.

2.5.1 Organism and culture conditions: A brewing strain of *Saccharomyces cerevisiae* was routinely maintained on malt extract agar and grown in defined medium as described in *Section **2.1.1*** A 10ml quantity of this medium was loop inoculated and grown as a starter culture at 28°C on an orbital shaker at 100 rpm for 48 h

2.5.2 H^+ release after glucose uptake by *S. cerevisiae*. Inhibition by Cu^{2+}, Cd^{2+}, Co^{2+}, Sr^{2+} and Pb^{2+} and and examination of the protective effect of Ca^{2+}.

Cells from a 48 h starter culture were used to inoculate growth cultures which were grown in defined medium, as described in *Section **2.1.1***, at 28°C for 20 h in 250ml flasks on an orbital shaker at 100 rpm. These cells were then harvested by centrifugation, washed twice with, and finally resuspended in, PIPES buffer, pH 6 5 A 1ml quantity of this *S cerevisiae* suspension was added to a 250ml flask containing 100ml of 0 5mM PIPES buffer, pH 6 5 The cells were allowed to equilibrate for 20 min with magnetic stirring at

25°C after which time glucose was added to a final concentration of 1% Stock solutions of Cu^{2+}, Cd^{2+}, Sr^{2+}, Pb^{2+} and Co^{2+} were prepared from $Cu(NO_3)_2\ 3H_2O$, $Cd(NO_3)_2\ 4H_2O$, $Sr(NO_3)_2$, $Pb(NO_3)_2$ and $CoCl_2\ 6H_2O$ respectively The appropriate volumes of metal were added from stock solutions 5 min prior to glucose addition to give final concentrations of 10, 25 and 100µM Cu^{2+}, 50, 100 and 500µM Cd^{2+}, 0 1, 0 5 and 100mM Sr^{2+}, 0 1 and 0 5mM Pb^{2+} and 0 1, 1 0 and 5 0mM Co^{2+} Ca^{2+} was added from stock solution of $CaCl_2\ 6H_2O$, where required, either 1 min or 5 min prior to metal addition The cell suspensions were stirred continuously at 25°C and the pH was recorded using the WTW pH meter (see *Section 2.3*) connected to a computer Data was stored via Multi-Achat software and transferred to sigma plot for processing and graphing

2.5.3 Effect of Co^{2+} and Ca^{2+} on growth of *S. cerevisiae*.

A series of 250ml experimental flasks was prepared containing 100ml of defined medium (described in *Section 2.1.1*) to which Co^{2+} was added to concentrations of 100µM, 500µM, 1mM and 5mM The protective effect of 0 5 and 5mM Ca^{2+} (from stock solution of $CaCl_2\ 6H_2O$) towards Co^{2+} toxicity was also examined by adding Ca^{2+} to each of two series of experimental flasks containing Co^{2+} All flasks were inoculated with starter culture to an initial OD_{550} of 0 1 and grown on an orbital shaker at 100 rpm and 28°C for 21 h during which time samples were removed from each flask and OD_{550} recorded with reference to appropriate blanks comprising uninoculated medium. A set of control flasks was also included

2.6 Toxicity and localisation of Cu^{2+} in *S. cerevisiae.*

2.6.1 Organism and culture conditions: A brewing strain of *Saccharomyces cerevisiae* was routinely maintained on malt extract agar and grown in defined medium as described in Section **2.1.1** Cells from a 48 h starter culture were used to inoculate growth media to the required cell density (OD_{550} = 0 1)

2.6.2 Effect of Cu^{2+} on growth of *S. cerevisiae.*

A series of experimental flasks was prepared containing 100ml of defined medium to which Cu^{2+} was added to concentrations of 1, 10, 50, 100 and 500µM All flasks were inoculated with starter culture to an initial OD_{550} of 0 1 and grown for 30 h during which time samples were removed from each flask and OD_{550} recorded with reference to appropriate blanks A set of control flasks was also included

2.6.3 Subcellular localisation of Cu^{2+}: Cells were harvested by centrifugation after 20 h growth and were subsequently washed twice with, and finally resuspended in 5ml of, 10mM MES buffer pH 5 5 A quantity of 1 ml of biomass suspension was added to 100ml of 10mM MES, pH 5 5 After preincubation for 20 min with rotary shaking (150 rpm), Cu^{2+} was added to the cell suspensions, to the desired final concentrations, from stock solution After 10 min and 2 h, three types of samples were removed (i) 2 x 15ml samples were removed into universal containers, centrifuged, washed twice with 10mM MES and the pellets retained for analysis of intracellular Cu^{2+} levels (supernatant was retained for K^+ analysis), (ii) 1ml was removed, centrifuged, washed and resuspended in 10mM MES for methylene blue staining (see *Section* ***2.6.4.***), (iii) 2 x 100µl samples were

diluted 1 / 100 twice in MES, 100µl plated in triplicate onto malt extract agar and incubated for 48 h at 25°C

The two cell pellets at each time point were resuspended in 4 5ml of 10mM Tris/MES buffer, pH 6 0, and combined Subsequently, 1 5ml of this cell suspension was placed into each of 6 eppendorfs 3 for extracts and 3 for total digests 'Total digest' pellets were digested for 1h in 0 5ml of 6M HNO_3 at 100°C followed by addition of 3ml of distilled, deionised water Solutions were retained for Cu^{2+} determination

The subcellular compartmentation of Cu^{2+} was determined using procedues described by White & Gadd (1987 *a*) which were modified from Huber-Walchli & Wiemken (1979)

The level of Cu^{2+} in the wash fraction was determined by centrifuging each extract sample at 13,000 rpm for 3 min, removing and retaining the supernatant and resuspending in 1 5ml of Tris/MES buffer, pH 6 0 at 0 to 4°C, followed again by centrifugation These two supernatants were combined and retained for Cu^{2+} analysis

The cytoplasmic membrane of the cell pellets was permeabilised by resuspending the pellets in 1ml of 10mM Tris-MES buffer, pH 6 0, with 0 7M sorbitol at 25°C DEAE-dextran (40µl, 10mg ml^{-1}) was added, mixed and incubated for 30s at 25°C After centrifugation (13,000 rpm, 30s) the supernatant was removed and retained The cells were subsequently washed 3 times with 0 7M sorbitol in 10mM Tris-MES buffer, pH 6 0, at 0 to 4°C, with incubation for 1 min at each wash Supernatants were removed and retained after centrifugation at each wash and were combined with that from the initial permeabilisation step

The vacuolar membrane was permeabilised by suspending the pellet in 1ml of 60% (v/v) methanol at 0°C to 4°C After incubation for 30s, the solution was centrifuged

(13,000 rpm, 30s) and the supernatant removed and retained This process was repeated twice and the supernatants from the three steps retained and combined The pellet was then resuspended in 1ml of Tris-MES buffer, pH 6 0 at 0 to 4°C, incubated for 1 min, centrifuged (13,000 rpm, 30s) and the supernatant removed and retained This was repeated twice more and the three supernatants combined with the three from the initial permeabilisation steps

The resultant cell debris was digested in HNO_3 as described earlier, to release remaining bound Cu^{2+} Wash, cytoplasmic, vacuolar and "bound" fractions were subsequently analysed for Cu^{2+} content

2.6.4. Viability determination using Methylene Blue staining.

The citrate methylene blue staining technique was used to determine cell viability (Pierce, 1971) A quantity of 0 01g methylene blue (BDH, Poole, England) was dissolved in 10ml of DDH_2O, followed by the addition of 1g of tri-sodium citrate (BDH, Poole, England) A further 60 ml of DDH_2O was added, the resultant solution stirred until dissolved, filtered through Whatman no 1 filter paper and made up to 100 ml with DDH_2O Cells were diluted where necessary to give a countable number of cells and a 1 ml quantity of this solution was then mixed with an equal volume of the appropriate cell suspension and allowed to incubate for 5 min Viable cells appeared colourless whereas dead cells stained blue having taken up the stain The cells were counted using a haemocytometer and the number of colourless cells to total number of cells was then expressed as a percentage viability

2.7 Toxicity of Mn^{2+} towards *Saccharomyces cerevisiae*.

A starter culture of *S cerevisiae* was loop inoculated and grown at 25°C for 48 h This culture was used to inoculate two growth flasks to OD_{550nm} 0 1 Biomass was harvested aseptically after 18 h by centrifugation in sterile tubes This was subsequently washed twice with, and finally resuspended in, sterile DDH_2O A quantity of 1 5ml of biomass was added aseptically to three experimental flasks containing 100ml DDH_2O Before adding metal, at time t_{-5} min, 100µl was removed from each flask, diluted twice by 1/100 to 10^3 cells per ml and 0 1ml plated out in triplicate onto malt extract agar and incubated for 48 h at 25°C Two 5ml samples were also removed for K^+ analysis by AAS At t_o, Mn^{2+} was added from 1M stock to 2 flasks to final concentrations of 1 and 10mM After 5, 70 and 160 min 5ml samples were removed, for K^+ analysis by AAS, and 100µl samples for dilution and plating

A haemocytometer was used to calculate the number of cells in each flask so K^+ release per 10^9 cells could be calculated

2.8 Metal adsorption.

2.8.1 Effect of biomass concentration on native *A. filiculoides* biosorption.

A series of batch solutions was prepared in duplicate from Cu^{2+}, Cd^{2+} or Pb^{2+} stock solutions and deionised water with an initial metal concentration of 5 mg l^{1} After pH adjustment to 5 50 ± 0 05 using dilute HNO_3 or NaOH, quantities of 0 1, 0 5 or 1 0g (dry weight, corresponding to 1, 5 and 10g l^{-1} respectively) of *Azolla* was added to each flask The *Azolla* systems were left shaking overnight (*ca* 16 h) by which time complete saturation of the biomass with test ion was expected At equilibrium, duplicate 5ml samples were removed from each flask and mixed with 5 ml of either

0 5M HNO_3 for Cu^{2+} analysis or 0 5M HCl for Cd^{2+} and Pb^{2+} analysis Metal concentrations were determined using ASV A set of biomass-free flasks was also prepared and samples were removed and analysed in a similar fashion to determine initial metal concentrations Metal uptake was expressed for biomass dry weight in all cases

2.8.2 Adsorption isotherms using *S. cerevisiae*, *A. resinae* and *A. filiculoides* Lamarck.

A series of batch solutions was prepared in duplicate from Cd^{2+}, Cu^{2+} or Sr^{2+} stock solutions and deionised water with initial metal concentrations ranging from 0-200 mg l^{-1} After pH adjustment to 5 50 $\pm$ 0 05 using dilute HCl, HNO_3 or NaOH, 0 3g (wet weight) of *S cerevisiae*, 0 5g (wet weight) of *A resinae* or 0 1g (dry weight) of *Azolla* was added to each flask Wet weight quantities of 0 3g for *S cerevisiae* and 0 5g for *A resinae* correspond to dry weights of *ca* 0 1g so biomass concentrations were the same for all three biosorbents The *S cerevisiae* and *A resinae* systems were allowed to contact for 60 min on an orbital shaker at 150 rpm at room temperature after which time a sample was removed from each flask and centrifuged at 5,000 rpm for 15 min and the pH values noted The final metal concentrations of the supernatants were analysed using a Perkin-Elmer 3100 AAS, which was also used to determine supernatant K^+ and Mg^{2+} concentration The *Azolla* systems were left shaking overnight (*ca* 16 h) after which time 5ml samples were removed from each flask and mixed with 5 ml of either 0 5M HNO_3 for Cu^{2+} analysis or 0 5M HCl for Cd^{2+} analysis Metal concentrations were determined using ASV Metal uptake was expressed for biomass dry weight in all cases

A series of biomass-free flasks was also set up for each isotherm, and samples were removed and analysed in a similar fashion to determine initial metal concentrations

2.8.3 Cu^{2+} adsorption using immobilised and non-immobilised milled-sieved *A. filiculoides* Lamarck.

A series of flasks was prepared with 100ml of Cu^{2+} at concentrations of 50 and 100 mg l^{-1} Cu^{2+} Metal-free flasks were also prepared The initial pH was adjusted to 5 50 $\pm$ 0 05 using dilute solutions of HNO_3 and NaOH Immobilised or non-immobilised milled-sieved *Azolla* (0 1g) was added to each flask and left shaking overnight (*ca* 16 h) Duplicate samples were removed from each flask and metal concentrations were determined by AAS Biomass-free flasks were also included in all cases Metal uptake was expressed for biomass dry weight in all cases

2.8.4 Metal time-course uptake using *A. filiculoides* Lamarck.

To examine kinetics of uptake, 0 2g of dried *Azolla* was added to a 1 litre flask containing 199ml of deionised, distilled water, and agitated for 60 min to ensure complete wetting A 1 ml volume of 1000 mg l^{1} test ion (Cu^{2+} or Cd^{2+}) was then added to the flask to give a metal concentration of 5 mg l^{-1} At various time intervals, 1ml samples were removed and mixed with 10ml of either 0 5M HNO_3 or 0 5M HCl for Cu^{2+} or Cd^{2+} analysis respectively using ASV Metal uptake was expressed for biomass dry weight in all cases

2.9 Column Study with *A. filiculoides*

2.9.1 Column Set-Up for native, milled-sieved and epichlorhydrin-immobilised *Azolla*.

Initially all columns and fittings were thoroughly acid-washed in 10% HNO_3 and rinsed with deionised distilled water A 2 5g quantity of native *Azolla* was packed lightly into columns 50cm in length with an internal diameter of 1cm Due to their Cu^{2+} binding capacity, milled-sieved *Azolla* and epichlorhydrin-immobilised milled-sieved *Azolla* were also selected for use in column studies and quantities of either 1 or 2 5g of each were packed lightly into columns The dimensions of these columns were 22cm in length for the 1g columns and 40cm in length for the 2 5g columns, each with an internal diameter of 1cm A quantity of 2 1 of DDH_2O was pumped through each column prior to use in order to wash the biomass and also to reduce the risk of air pockets forming during pumping of metal ion This ensured the maximum biomass surface area was available for interaction with the influent metal solution Influent test ion solutions of concentration 5mg l^{-1} Cu^{2+} or Cd^{2+} were prepared using deionised, distilled water with the pH adjusted to 5 50 $\pm$ 0 05 using dilute HNO_3 or NaOH Feed solutions were pumped through the columns in a downflow direction in the case of native *Azolla* and in an upflow direction for milled-sieved and epichlorhydrin-immobilised *Azolla* Flow rates were either 1 or 3 5ml min^{-1} and samples were collected at regular intervals for metal analysis

2.9.2 Desorption of Cu^{2+} from milled-sieved *Azolla* column using Chelaton 3.

Chelaton 3 ($C_{10}H_{14}O_8N_2Na_2\ 2H_2O$), pH 5 50 ± 0 05, at a concentration of 5mM was used to desorb the accumulated Cu^{2+} from a column containing 1 g of milled-sieved *Azolla* through which 18 95 l of 5mg l^{-1} Cu^{2+} solution had previously been passed The eluting agent was pumped through the columns by a peristaltic pump at a flow rate of 1 ml min^{-1} and samples were collected at regular intervals for metal analysis

2.9.3 Determination of native *Azolla* biosorbent characteristics (Wet Density, Void Volume Fraction and Sorbent Bulk Density).

Swelling Volume of Biosorbent A quantity of 2 5g of native *Azolla* was placed into a 100ml graduated cylinder and weighed (weight = a) The cylinder was then filled with water and allowed to soak for 3 h with slight mixing Excess water was poured off and the cylinder + wet biomass weighed (weight = b) Weight of accumulated water, m = b - a

100ml volumetric flask filled with water, weight = M_1

Wet biomass was placed into flask and filled to 100ml mark with water, weight = M_2

Wet Density of Biosorbent

$\rho = \rho_v\ m/(m + (M_1-M_2))$

where m, M_1 and M_2 are as defined earlier and

ρ = wet density of Azolla

ρ_v = density of water at 20°C

Void Volume Fraction, $\varepsilon = 1 - ((m/Vs) / \rho)$

where m and ρ are as defined earlier and

ε = Void Volume Fraction

Vs = Bed Volume = $\pi r^2 h$

Sorbent Bulk Density

G / V = (dry weight / Vs) 1000

2.9.4 Mathematical model for prediction of column breakthrough curves for Cu^{2+} and Cd^{2+} using native *Azolla*.

A mathematical model for prediction of column breakthrough was developed by Dr J Votruba at the Academy of Sciences, Prague, Czech Republic This model was utilised in the present study and the results compared with present experimental data

Mathematical description of column type biosorber dynamics can be shown by mass balance

Balance of substance in liquid phase $\varepsilon (\delta c/\delta t) = (-F/V)\ \delta c/\delta \xi + \beta(C^*-c)\ G/V$ (1)

where $(\delta c/\delta t)$ represents the change in concentration of metal with respect to time,

$(-F/V)\ \delta c/\delta \xi$ represents the change in concentration of metal at various stages in the column as it flows through,

$\beta(C^*-c)\ G/V$ represents the adsorption of metal onto the biomass

Consequently, the balance of substance in solid phase may be shown by

$\delta q / \delta t = -\beta(C^*-c)$ (2)

where $\delta q / \delta t$ is dependent only on the adsorption of metal onto the surface of the biomass

Initial and boundary conditions $t = 0, c = q = 0$ for $\xi \in <0,1>$

$t > 0, c(0) = c_o$

F = Volume flowrate, C* = Equilibrium concentration of metal in solution calculated from Freundlich sorption isotherm, β = mass transfer coefficient, G/V = sorbent bulk density, c = concentration of metal in solution, V = bed volume, ξ = dimensionless length coordinate, q = concentration of metal based on sorbent mass, ε = void volume fraction, c_o = input concentration of metal in solution

The experimental sorbent characteristics and adsorption isotherms were used by Dr Pavel Dostalek at the Institute of Chemical Technology, Prague, Czech Republic to develop a computer model for predictive calculation of metal sorption The programming and simulation language PSI/c was used for these calculations The original set of partial differential equations (Eqs 1 and 2) was discretized in length coordinate by means of finite differences The resulting set of ordinary differential equations was integrated using the 4^{th} order Runge-Kutta method implemented in PSI/c and is shown in Appendix A The data obtained using this computer model were graphed and compared with results obtained experimentally

CHAPTER 3

RESULTS

CHAPTER 3: RESULTS

3.1 Toxicity of metals towards *S. cerevisiae*.

3.1.1 H^+ release after glucose uptake by *S. cerevisiae*. Effect of toxic metals and protection by Ca^{2+}.

Addition of glucose to a solution of *S cerevisiae* cells led to a progressive pH drop from 6 50 to *ca* 4 25, 90 min after the addition of glucose (Figure 3 1 1) Addition of Cu^{2+} inhibits this glucose-dependent H^+ efflux which is observed as a reduction in the pH drop (Figure 3 1 2) Higher concentrations of Cu^{2+} led to corresponding larger inhibitions in change of pH (Figure 3 1 2) After addition of 25, 50 and 100µM Cu^{2+}, the pH dropped to *ca* 4 9, 5 35 and 6 4, respectively, 90 min after glucose addition The effect of Ca^{2+} on Cu^{2+} toxicity was subsequently examined Addition of 0 5mM Ca^{2+} 1 min before 100µM Cu^{2+} led to a H^+ release once again as the pH drops to *ca* 5 85, 10 min after glucose addition (Figure 3 1 2) This H^+ efflux is then followed by a slight gradual influx during the following 40 min, resulting in a small pH increase to *ca* 6 0 The significance of increasing the time between Ca^{2+} addition and addition of Cu^{2+} was examined to see if it increased the protective effect, leading to greater metabolic activity as manifest by H^+ efflux A quantity of 0 5mM Ca^{2+} added 5 min before 100µM Cu^{2+} addition results in a slight increase in H^+ release to *ca* pH 5 75, before gradually rising once again over the following 40 min to *ca* 5 85 (Figure 3 1 2)

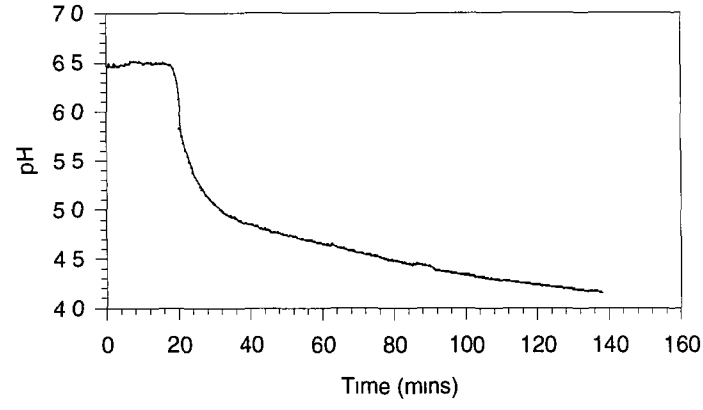


Figure 3.1.1 H^+ release after glucose uptake by *S cerevisiae* Glucose added at t_{20}

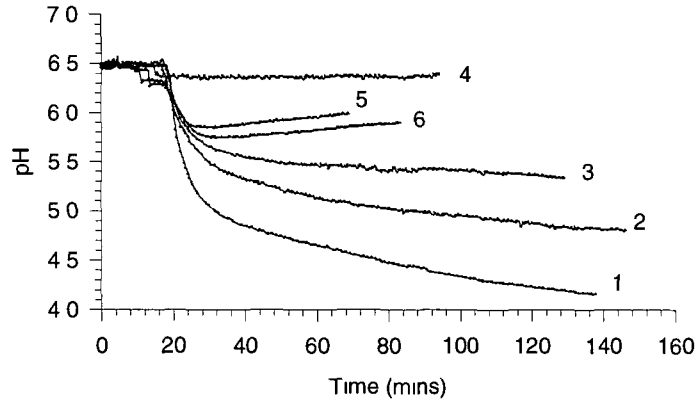


Figure 3.1.2 Toxicity of Cu^{2+} towards H^+ release by *S cerevisiae* H^+ release after addition of (1) 1% glucose, (2) 10μM Cu^{2+}, (3) 25μM Cu^{2+}, (4) 100μM Cu^{2+}, (5) 0 5mM Ca^{2+} followed 1 min later by 100μM Cu^{2+}, (6) 0 5mM Ca^{2+} followed 5 min later by 100μM Cu^{2+} All Cu^{2+} additions 5 min before 1% glucose Glucose added at t_{20}

The toxicity of Cd^{2+} was also demonstrated, with concentrations of 50, 100 and 500µM inhibiting the H^+ release (Figure 3 1 3) The decrease in pH was inhibited with pH dropping to *ca* 5 26, 5 69 and 5 98 after contact with 50, 100 and 500µM Cd^{2+} respectively The effect of 0 5mM Ca^{2+} on this toxicity was subsequently examined with the results shown in Figures 3 1 4 to 3 1 6 Addition of Ca^{2+}, to a concentration of 0 5mM, 5 min before 50µM Cd^{2+} was observed to reduce the toxic effect of the Cd^{2+}, with the pH decreasing to *ca* 4 94 after 90 min compared to ca 5 26 in the absence of Ca^{2+} (see Figure 3 1 4) A similar response was observed upon addition of 0 5mM Ca^{2+} 5 min before 100µM Cd^{2+} with the pH decreasing to 4 67 after 90 min compared to 5 69 in the absence of Ca^{2+} (see Figure 3 1 5) The pH was observed to decrease to *ca* 5 42 after 90 min when 0 5mM Ca^{2+} was added 5 min before 0 5mM Cd^{2+} compared to pH 5 98 in the absence of Ca^{2+} (see Figure 3 1 6)

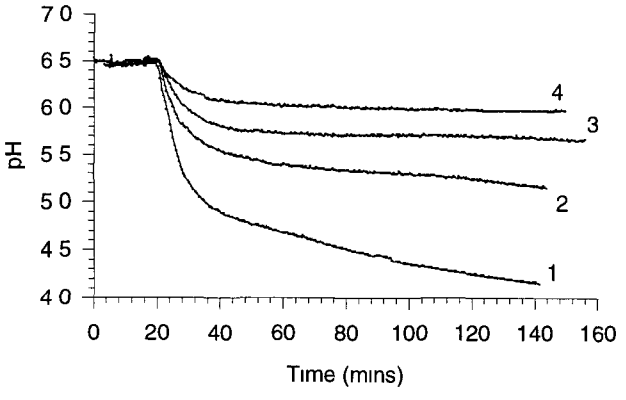


Figure 3.1.3 Toxicity of Cd^{2+} towards H^+ release after glucose uptake by *S cerevisiae* H^+ release after addition of (1) 1% glucose, (2) 50µM Cd^{2+} followed 5 min later by 1% glucose, (3) 100µM Cd^{2+} followed 5 min later by 1% glucose, (4) 0 5mM Cd^{2+} followed 5 min later by 1% glucose Glucose added at t_{20} min

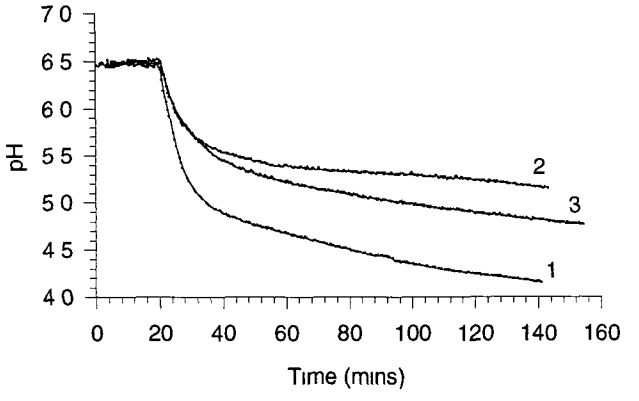


Figure 3.1.4 Toxicity of 50μM Cd^{2+} towards H^+ release by *S cerevisiae* and the protective effect of 0 5mM Ca^{2+} H^+ release after addition of (1) 1% glucose, (2) 50μM Cd^{2+} followed 5 min later by 1% glucose, (3) 0 5mM Ca^{2+} followed at 5 min intervals by firstly 50μM Cd^{2+} and then 1% glucose Glucose added at t_{20} min

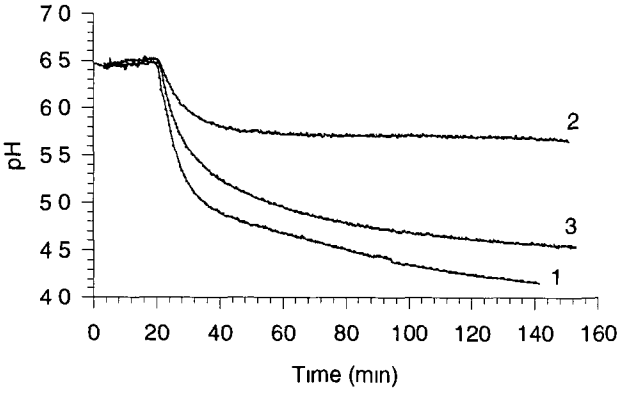


Figure 3.1.5 Toxicity of 100μM Cd^{2+} towards H^+ release by *S cerevisiae* and the protective effect of 0 5mM Ca^{2+} H^+ release after addition of (1) 1% glucose, (2) 100μM Cd^{2+} followed 5 min later by 1% glucose, (3) 0 5mM Ca^{2+} followed at 5 min intervals by firstly 100μM Cd^{2+} and then 1% glucose Glucose added at t_{20} min

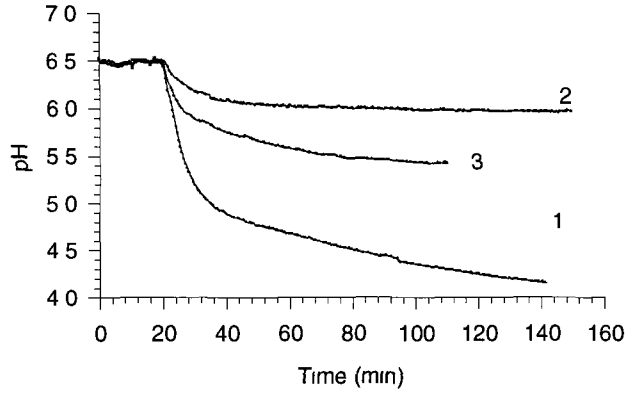


Figure 3.1.6 Toxicity of 0 5mM Cd^{2+} towards H^+ release by *S cerevisiae* and the protective effect of 0 5mM Ca^{2+} H^+ release after addition of (1) 1% glucose, (2) 0 5mM Cd^{2+} followed 5 min later by 1% glucose, (3) 0 5mM Ca^{2+} followed at 5 min intervals by firstly 0 5mM Cd^{2+} and then 1% glucose Glucose added at t_{20} min

Figure 3 1 7 shows results of the effect of Co^{2+} on *S cerevisiae* membrane function and the protective effect of Ca^{2+} Initially 100µM Co^{2+} was added and led to a corresponding pH drop to *ca* 4 31 after 90 min (Figure 3 1 7, plot 2) which was very similar to the control value of *ca* 4 25 in the absence of metal (Figure 3 1 7 plot 1) The corresponding viability after 90 min was 96 3% which again indicated the low toxicity of 100µM Co^{2+}

Increasing the Co^{2+} concentration to 1mM, however, leads to a reduction in H^+ release with the pH decreasing to *ca* 5 47, 75 min after glucose addition (Figure 3 1 7, plot 3) Viability decreased to 52 4% after 75 min which also indicates higher toxicity of Co^{2+} at 1mM

The possible protective effect of Ca^{2+} towards Co^{2+} toxicity was subsequently examined Ca^{2+} added to a final concentration of 0 5mM 5 min before addition of 1mM Co^{2+} leads to a cell viability of 96 0% and a pH decrease to *ca* 4 35, 90 min after glucose addition (Figure 3 1 7, plot 4)

Increasing the concentration of Co^{2+} to 5mM leads to a subsequent decrease in H^+ release leading to a pH of only 5 79, 90 min after glucose addition (Figure 3 1 7, plot 5) and a viability of 50 6% Addition of 0 5mM Ca^{2+} 5 min before the 5mM Co^{2+} appears once again to protect the cells against the toxic effects of Co^{2+}, with the pH dropping to *ca* 4 28 once again after 80 min (Figure 3 1 7, plot 6) and cell viability increasing to 98 2% The profile of the plot is almost identical to that of the H^+ efflux after addition of 100µM Co^{2+}

Toxicity of Pb^{2+} was also examined and at a concentration of 100µM Pb^{2+} reduced the H^+ release, with pH decreasing to *ca* 4 95 after 90 min (Figure 3 1 8) Pb^{2+} at a concentration of 100µM consequently appears to be toxic by slowing down transport by the plasma-membrane After 90 min the cell viability had decreased to 58 6% of the original viability at time zero, again indicating the toxic effect of the Pb^{2+} at a concentration of 100µM The Pb^{2+} concentration was then increased to 500µM resulting in a greater reduction in H^+ efflux, the pH decreasing to only *ca* 5 59 after 90 min

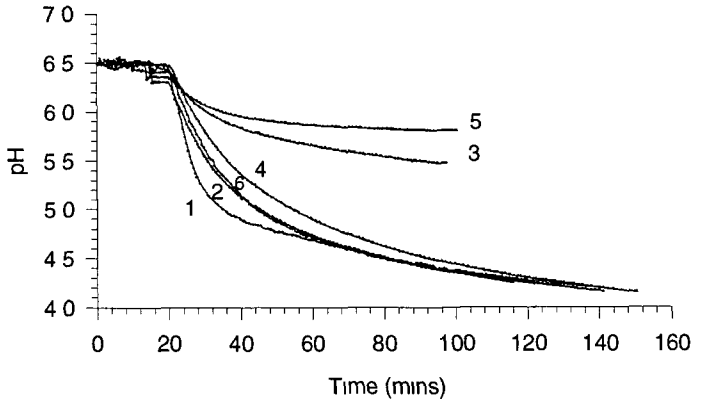


Figure 3.1.7 Toxicity of Co^{2+} towards H^+ release by *S cerevisiae* H^+ release after addition of (1) 1% glucose, (2) 100µM Co^{2+}, (3) 1mM Co^{2+}, (4) 0 5mM Ca^{2+} followed 5 min later by 1mM Co^{2+}, (5) 5mM Co^{2+}, (6) 0 5mM Ca^{2+} followed 5 min later by 5mM Co^{2+} Co^{2+} additions followed 5 min later by 1% glucose Glucose added at t_{20} min

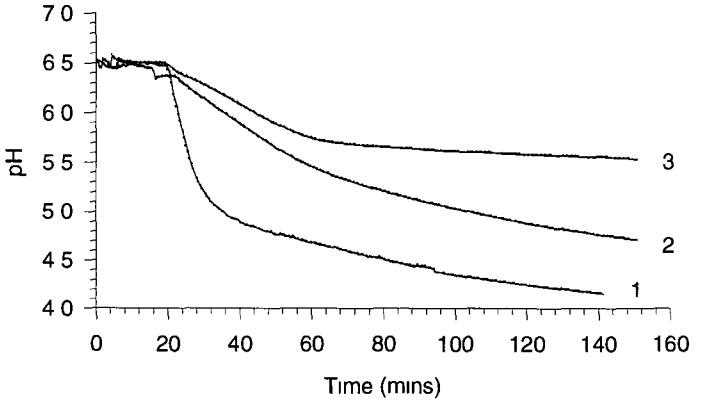


Figure 3.1.8 Toxicity of Pb^{2+} towards H^+ release by *S cerevisiae* H^+ release after addition of (1) 1% glucose, (2) 100µM Pb^{2+}, (3) 500µM Pb^{2+} Pb^{2+} additions followed 5 min later by 1% glucose Glucose added at t_{20} min

Sr^{2+} at concentrations of 100 and 500µM exhibits negligible toxicity towards the H^+ release from *S cerevisiae* with pH values of *ca* 4 44 and 4 42 after 90 min respectively, compared to the control value of *ca* 4 26 (see Figure 3 1 9) Similarly 100mM Sr^{2+} does not appear to affect H^+ release, leading to a pH of *ca* 4 27, 90 min after the addition of Sr^{2+} The corresponding cell viabilities after 90 min were *ca* 96 9%, 93 8% and 91 5% after contact with the 100µM, 500µM and 100mM Sr^{2+} respectively, again indicating the low toxicity of Sr^{2+} at these concentrations

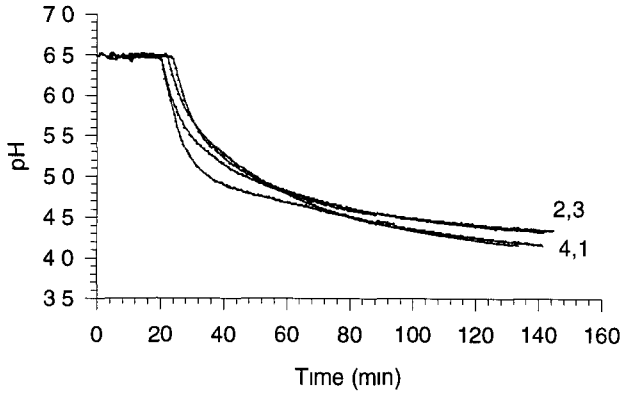


Figure 3.1.9 Toxicity of Sr^{2+} towards H^+ release by *S cerevisiae* H^+ release after addition of (1) 1% glucose, (2) 100µM Sr^{2+}, (3) 500µM Sr^{2+}, (4) 100mM Sr^{2+} Sr^{2+} additions followed 5 min later by 1% glucose Glucose added at t_{20} min

The effect of Co^{2+} on the growth *of S cerevisiae* was also examined (Figure 3 1 10 (a-c)) All four Co^{2+} concentrations studied inhibited cell growth (Figure 3 1 10 (a)) Toxicity increased with increasing Co^{2+} concentration, with growth after 21 h being reduced to 29 6, 17 5, 17 4 and 9 5% of the control value in the presence of 100µM, 500µM, 1mM and 5mM Co^{2+} respectively This is interesting as in Figure 3 1 7 no toxic effect was observed due to 100µM Co^{2+} Consequently, it appears that in the presence of 100µM Co^{2+} the cell membrane transport system in *S cerevisiae* is able to continue functioning, but actual cell growth is inhibited At concentrations of 1 and 5mM, however, both membrane function and cell growth are inhibited by Co^{2+}

Ca^{2+} at concentrations of 0 5mM and 5mM had a noticeable effect on the toxicity of Co^{2+} (Figure 3 1 10 (b) and (c)) The presence of 0 5mM Ca^{2+} increased cell growth after 21 h to *ca* 36 8, 22 5, 21 2 and 10 5% of the control after contact with 100, 500µM, 1 and 5mM Co^{2+} respectively which represent increases of *ca* 24 4, 28 6, 21 5 and 10 0% compared to corresponding growth in the presence of the same concentrations of Co^{2+} but in the absence of Ca^{2+} Addition of 5mM Ca^{2+} reduced Co^{2+} toxicity even further with growth after 21 h representing *ca* 36 9, 35 7, 36 7 and 20 0% of the control after contact with 100, 500µM, 1 and 5mM Co^{2+} respectively which represent increases of *ca* 24 6, 104 2, 110 7 and 110 4% compared to corresponding growth in the absence of Ca^{2+}

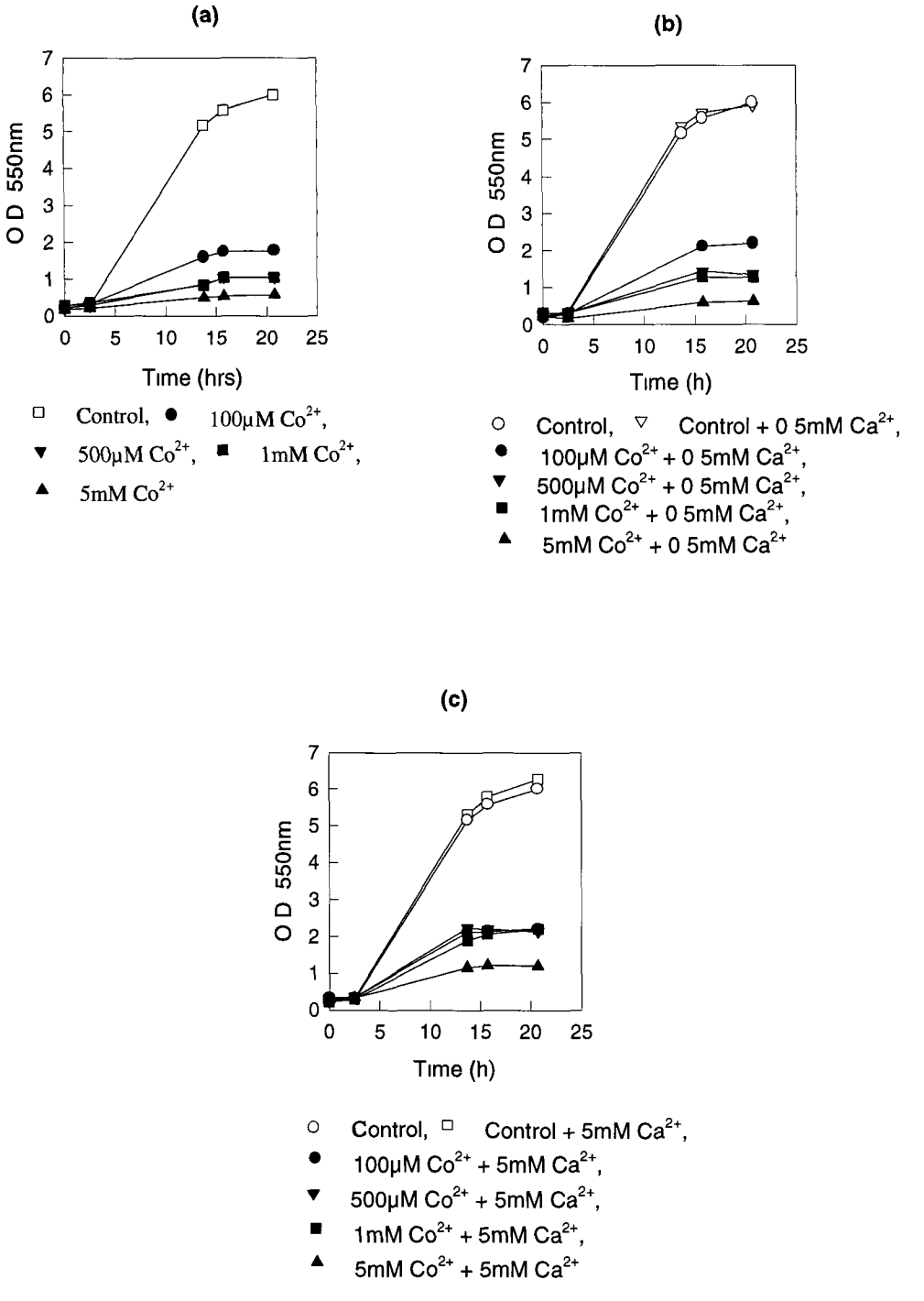


Figure 3.1.10 Effect of Co^{2+} on growth of *S cerevisiae* (a) and the possible protective effect of Ca^{2+} ((b) and (c))

3.1.2 Toxicity of Cu^{2+} and Mn^{2+} and localisation of Cu^{2+} in *S. cerevisiae*.

The effect of Cu^{2+} on the growth of *S cerevisiae* was examined and is shown in Figure 3 1 11 Growth was not inhibited in the presence of 1, 10 or 50μM Cu^{2+} However, Cu^{2+} at a concentration of 100μM causes a reduction in growth of 25 8% and 29 3% after 22 and 30 h respectively A concentration of 500μM Cu^{2+} has an even greater inhibitory effect, leading to a reduction in growth of 75 8% after 22 h and 79 6% after 30 h

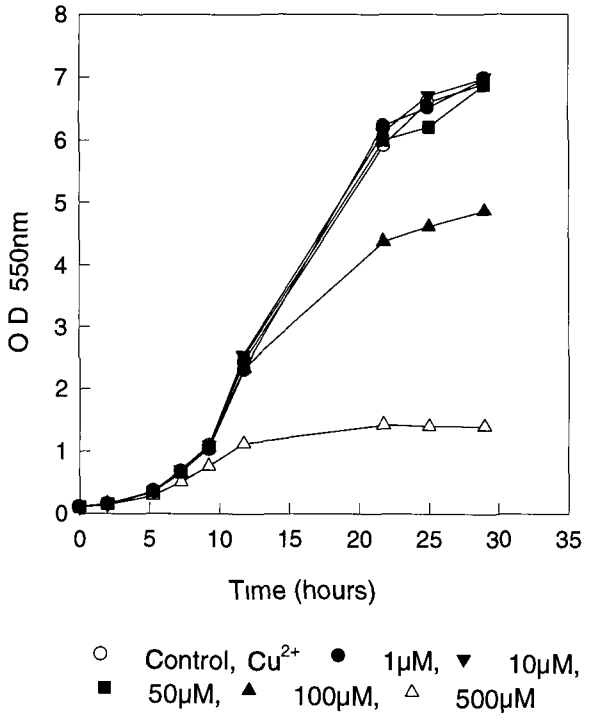


Figure 3.1.11 Effect of Cu^{2+} on growth of *S cerevisiae*

The intracellular localisation of sub-toxic and toxic cencentrations (25 and 100μM respectively) of Cu^{2+}, as determined from the *S cerevisiae* growth curve in Figure 3 1 11, was subsequently examined Results indicate that in the control cells there was no Cu^{2+} present in either the cytosol or vacuolar fractions after 10 min or 2 h (see

Table 3 1 1) The vast majority of Cu^{2+} was located in the membrane bound fraction (*ca* 695 and 445 nmol/10^9 cells after 10 min and 2 h respectively) This Cu^{2+} is that which was available in the unamended growth medium which was determined to be at a concentration of *ca* 15 µM

For cells contacted with 25µM Cu^{2+} the majority of the ions was once again found in the membrane fraction at concentrations of *ca* 1257 and 979 nmol/10^9 cells after 10 min and 2 h respectively (Table 3 1 1) There was also an increase in the amount of Cu^{2+} in the wash fraction with *ca* 89 and 193 nmol/10^9 cells after 10 min and 2 h respectively No Cu^{2+} was detected in the vacuolar fraction at either time point A trace amount of Cu^{2+} was detected in the cytosol fraction after 10 min but was not present after 2 h For cells contacted with 100µM Cu^{2+} however, the levels of Cu^{2+} increased in all four fractions with the majority still being present in the membrane fraction The total Cu^{2+} levels were within 0 2 and 3 0% of those for intact cells after 10 min and 2 h respectively

Compartment	Cu^{2+} content (nmol/10^9 cells)	
	Control	
	10 mn	2 h
Wash	10 2 ± 6 2	24 6 ± 9 5
Cytosol	0 0	0 0
Vacuole	0 0	0 0
Membrane bound	695 3 ± 47 9	444 8 ± 19 5
Total	705 5	469 4
Intact Cells	610 55 ± 16 1	367 4 ± 51 1
	25µM	
	10 mn	2 h
Wash	89 0 ± 3 6	192 9 ± 0 0
Cytosol	8 7 ± 0 0	0 0
Vacuole	0 0	0 0
Membrane bound	1257 3 ± 70 7	978 8 ± 6 4
Total	1355 0	1171 7
Intact Cells	1160 7 ± 30 4	1093 9 ± 3 7
	100µM	
	10 mn	2 h
Wash	536 4 ± 0 0	393 4 ± 3 6
Cytosol	239 2 ± 6 1	278 6 ± 3 0
Vacuole	42 9 ± 0 0	78 7 ± 7 2
Membrane bound	2208 2 ± 61 2	2682 1 ± 9 7
Total	2983 8	3181 8
Intact Cells	2979 7 ± 38 4	3277 4 ± 19 4

Table 3.1.1 Intracellular Cu^{2+} levels in *S cerevisiae* in the absence of Cu^{2+} or in the presence of 25µM or 100µM Cu^{2+} Mean values ± standard error of the mean from three determinations are shown

After contacting the *S cerevisiae* cells with 10, 25 and 100µM Cu^{2+}, K^+ release was observed to increase with increasing Cu^{2+} concentration and with time (Table 3 1 2) Toxicity was also examined by plate counts (Table 3 1 3) but there does not appear to be a significant drop in viability after addition of 10µM Cu^{2+} Viability as a % of the control subsequently decreased with both increasing Cu^{2+} concentration and time of contact for 25 and 100µM Cu^{2+} After contact with 25µM Cu^{2+}, viability decreased to *ca* 65 and 39% after 10 min and 2 h respectively and in the presence of 100µM Cu^{2+} decreased to *ca* 8 and 2%

Methylene blue staining however, indicated much greater reductions in viability In the presence of 10µM Cu^{2+} viability was reduced to 78 6 and 76 1% of the controls after 20 min and 2 h respectively (Table 3 1 4) A Cu^{2+} concentration of 25µM caused the viability to be reduced to 38 3 and 27 5% after 10 min and 2 h respectively while 100µM Cu^{2+} caused total loss of viability

The results of the K^+ release after contact with Mn^{2+} are shown in Table 3 1 5 As observed in the Cu^{2+} studies, an increase in Mn^{2+} gave rise to increased K^+ release After incubation for 48 h at 25°C the colonies on the malt extract plates were counted to give colony forming units (c f u) per ml (Table 3 1 6) Mn^{2+} at a concentration of 10mM caused a significant reduction in viability to *ca* 71 0 and 79 6% after 70 and 160 min respectively

	K^+ release ($\mu mol/10^9$ cells)			
Time	Control	10μM	25μM	100μM
10 min	5 02 ± 0 11	8 81 ± 0 19	20 63	23 44
2 hours	5 70 ± 0 78	10 42 ± 0 30	21 67	25 46

Table 3.1.2 Effect of Cu^{2+} on release of K^+ by *S cerevisiae*

	Viability (% of control)			
Time	Control	10μM	25μM	100μM
10 min	100%	93 5%	64 9%	8 4%
2 hours	100%	101 6%	39 0%	1 5%

Table 3.1.3 Viability of *S cerevisiae*, from plate counts (c f u x 10^7 ml^{-1}), after contact with Cu^{2+} at concentrations of 10, 25 and 100μM

	Viability (% of control)			
Time	Control	10μM	25μM	100μM
10 min	100 %	78 59 %	38 25 %	0 0 %
2 hours	100 %	76 10 %	27 47 %	0 0 %

Table 3.1.4 Viability determination by methylene blue staining for *S cerevisiae* after contact with Cu^{2+} at concentrations of 10, 25 and 100μM

	K^+ release (µmol/10^9 cells)		
Time (min)	Control	1mM Mn^{2+}	10mM Mn^{2+}
-5	5 33	5 50	7 01
70	5 37	12 61	15 88
160	5 29	11 66	16 67

Table 3.1.5 Effect of Mn^{2+} on release of K^+ by *S cerevisiae*

	Control	1mM Mn^{2+}		10mM Mn^{2+}	
Time (min)	cfu x10^7 per ml	cfu x10^7 per ml	% viability	cfu x10^7 per ml	% viability
-5	1 0667	0 9900	92 8%	1 0433	97 8%
70	1 2340	1 1167	90 5%	0 8767	71 0%
160	1 2433	1 1733	94 4%	0 9900	79 6%

Table 3.1.6 Viability of *S cerevisiae* after contact with 1 and 10mM Mn^{2+}

3.2 Metal Adsorption.

3.2.1 Effect of biomass concentration on native *A. filiculoides* biosorption.

Increasing biomass concentration in solution caused a corresponding decrease in Cu^{2+}, Cd^{2+} and Pb^{2+} biosorption (Figure 3.2.1). As *Azolla* concentration was increased from 1 to 5 to 10 g l^{-1}, Cu^{2+} biosorption per g (dry wt) of biomass decreased from 80.8 µmol g^{-1} to 17.9 and 9.0 µmol g^{-1}, biosorption of Cd^{2+} decreased from 51.6 to 10.5 and 5.3 µmol g^{-1} and Pb^{2+} biosorption decreased from 10.1 to 2.8 and 2.1 µmol g^{-1} respectively. Total test ion removal (expressed as a %) increased from 89.9 to 99.8 and 99.9% for Cu^{2+}, from 96.4 to 98.2 and 98.5% for Cd^{2+} and from 36.4 to 49.9 and 73.9% for Pb^{2+} as biomass concentration in solution was increased from 1 to 5 to 10 g l^{-1} respectively.

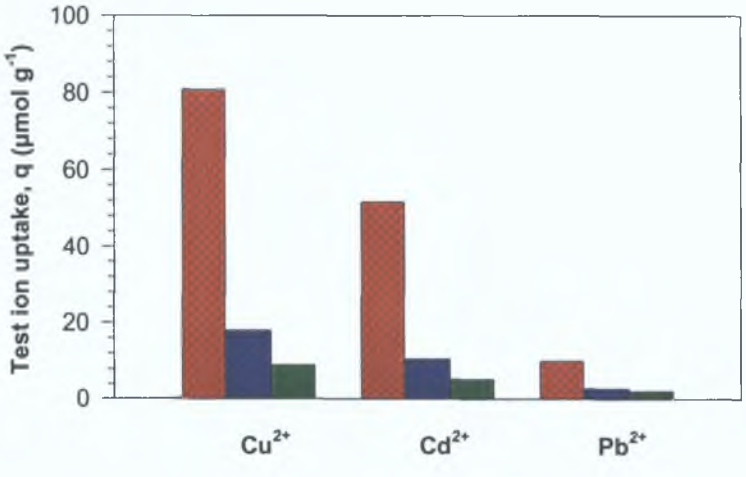


Figure 3.2.1 Biosorption of Cu^{2+}, Cd^{2+} and Pb^{2+} by native *Azolla* at biomass concentrations of 1 (red), 5 (blue) and 10 g l^{-1} (green). Initial test ion conc. = 5 mg l^{-1}.

3.2.2 Metal adsorption isotherms with *S. cerevisiae*, *A. resinae* and *A. filiculoides*.

Both *S cerevisiae* and *A resinae* exhibited uptake of Cu^{2+}, Cd^{2+} and Sr^{2+} from solution Cation adsorption levels were greater for *S cerevisiae* in each case Plotting uptake (q) against the final solution concentration (C_f) yields curvilinear isotherms, indicating saturation at higher cation levels (Figures 3 2 2 and 3 2 3) Sorption isotherms were also plotted for Cu^{2+} and Cd^{2+} uptake by *Azolla* and are shown in Figure 3 2 4

In the case of *S cerevisiae*, Cu^{2+} and Cd^{2+} were adsorbed to the highest levels, with maximum uptake levels of *ca* 150µmol g^{-1} Sr^{2+} was adsorbed to a maximum level of *ca* 90µmol g^{-1} Uptake levels of all three cations for *A resinae* were significantly lower than for *S cerevisiae*, with Cu^{2+}, Cd^{2+} and Sr^{2+} being adsorbed to maximum levels of *ca* 70, 40 and 45µmol g^{-1} respectively Of the three biomass types, *Azolla* exhibited the greatest adsorption with Cu^{2+} and Cd^{2+} being bound to maximum adsorption levels of *ca* 350 and 245µmol g^{-1} respectively

The uptake data were also tested for fit to the Langmuir adsorption model (Langmuir, 1918) $q = q_o bC_f/1+bC_f$

where q_o = maximum adsorption capacity at saturation

and b = Langmuir binding strength coefficient

Data conforming to Langmuir adsorption exhibit a straight-line relationship when plotted in the form 1/q versus $1/C_f$ A linear relationship was observed for all three test ions for *S cerevisiae* with r^2 values of 0 9863, 0 9947 and 0 9923 for Cd^{2+}, Cu^{2+} and Sr^{2+} respectively However, for *A resinae* a linear relationship was observed only for the Sr^{2+} data (r^2 value of 0 9782) and not for Cu^{2+} or Cd^{2+} (r^2 values of 0 8717 and 0 8629 respectively) The Scatchard transformation of the Langmuir model (Scatchard, 1949) is also useful for characterising metal-ligand interactions (Figures 3 2 5 (a) and (b)) Curved plots of q/C_f versus q resulted for Cu^{2+} and Cd^{2+} binding by *A resinae* However, a linear relationship was observed for both Cu^{2+} and Cd^{2+} binding by *S cerevisiae* and Sr^{2+} binding by *A resinae*, with a more scattered plot for *S cerevisiae* Sr^{2+} binding

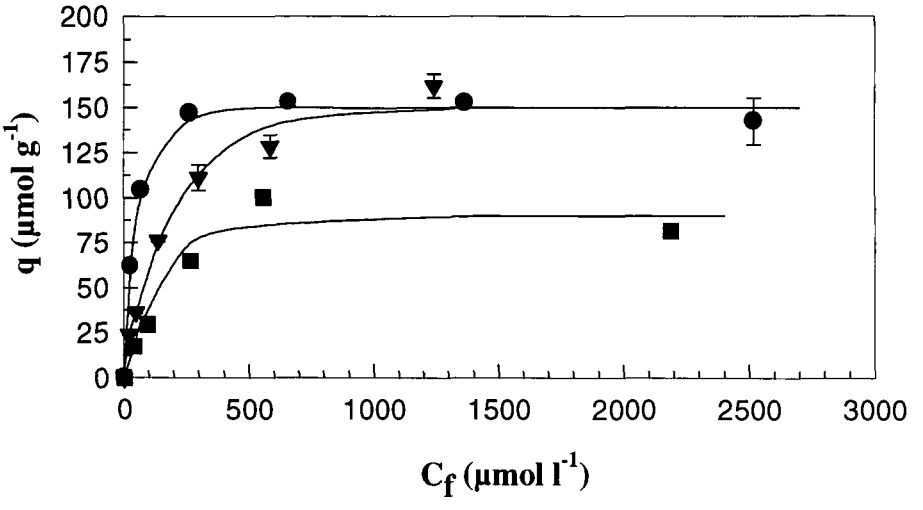


Figure 3.2.2 Isotherms for Cu^{2+}, Cd^{2+} and Sr^{2+} using *S cerevisiae* ● Copper, ▼ Cadmium, ■ Strontium.

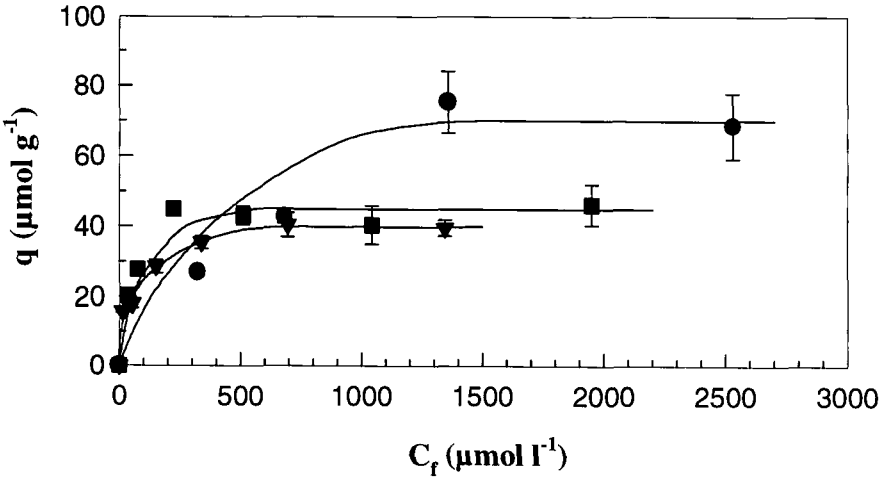


Figure 3.2.3 Isotherms for Cu^{2+}, Cd^{2+} and Sr^{2+} using *A resinae* ● Copper, ▼ Cadmium, ■ Strontium.

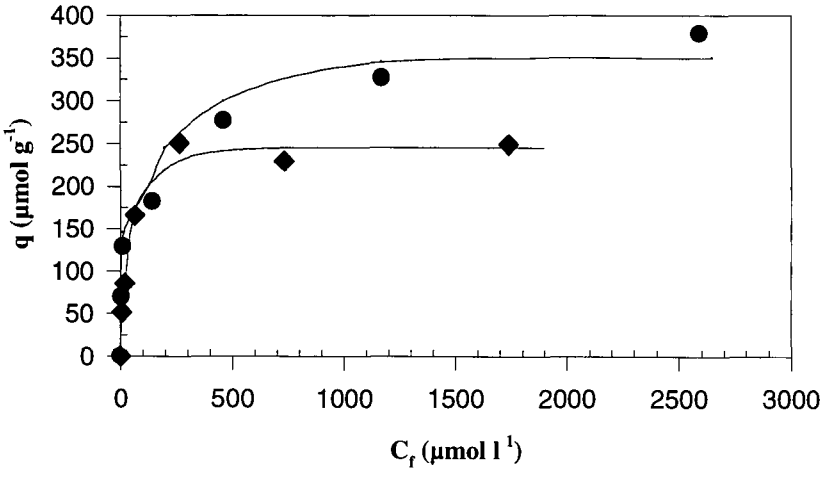


Figure 3.2.4 Equilibrium sorption isotherms for Cu^{2+} (●) and Cd^{2+} (◆) using native *Azolla*

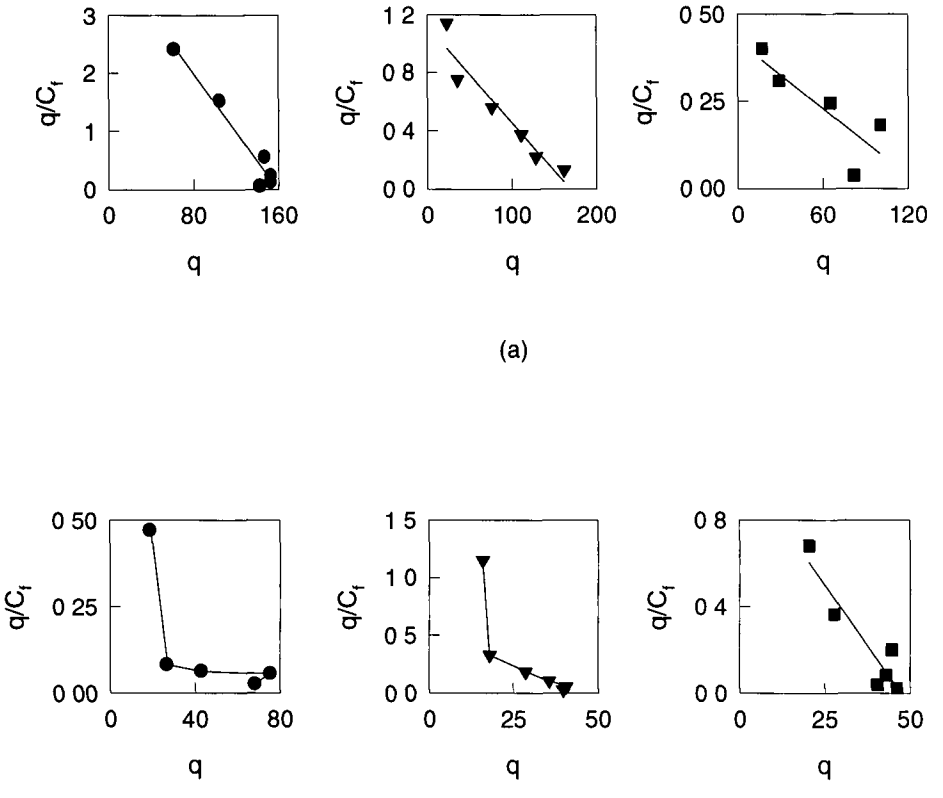


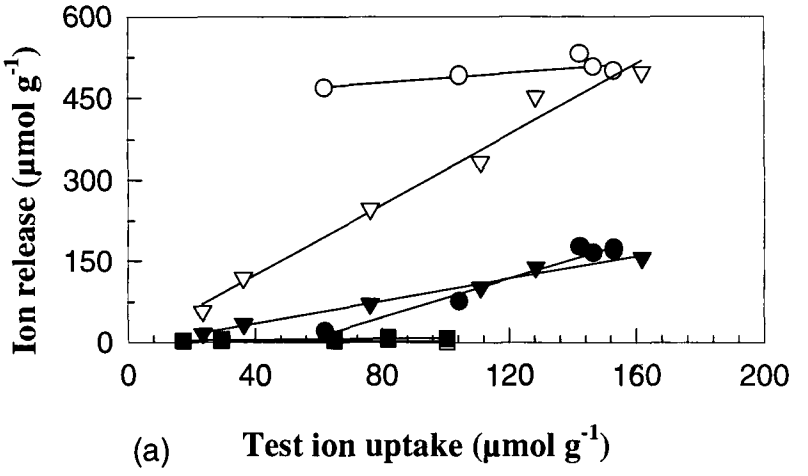
Figure 3.2.5 Scatchard plots for Cu^{2+}, Cd^{2+} and Sr^{2+} adsorption by (a) *S cerevisiae* and (b) *A resinae* ● Copper, ▼ Cadmium, ■ Strontium.

Concomitant to cation uptake was a release of K^+ and Mg^{2+} ions, with greatest ion release at higher cation adsorption levels (Figures 3.2.6 (a), (b) and Table 3.2.1). For *S. cerevisiae* Sr^{2+}, Cd^{2+} and Cu^{2+} displaced Mg^{2+} to maximum levels of 8.7, 156.3 and 176.1µmol g^{-1} respectively. The same test ions displaced K^+ maximally to 5.4, 497.1 and 531.6µmol g^{-1} respectively. Ion release was also observed for *A. resinae* with the test ions displacing Mg^{2+} maximally to 9.1, 7.0 and 11.3µmol g^{-1} respectively and K^+ to 11.0, 35.4 and 86.9µmol g^{-1} respectively.

During test ion uptake the solution pH decreased in the order $Sr^{2+} < Cd^{2+} << Cu^{2+}$ for *S. cerevisiae* and $Sr^{2+} < Cd^{2+} < Cu^{2+}$ for *A. resinae* (Figure 3.2.7). During Cu^{2+} uptake pH decreased from initial values of 5.50 to final values of 4.31 and 4.42 for *S. cerevisiae* and *A. resinae* respectively. While the change in solution pH for *S. cerevisiae* uptake of Cd^{2+} and Sr^{2+} was negligible, during uptake by *A. resinae* the pH dropped by 0.54 and 0.29 pH units respectively. Maximum H^+ displacement levels for Cu^{2+}, Cd^{2+} and Sr^{2+} were *ca.* 67.3, 1.3 and 0.5 µmol g^{-1} respectively for *S. cerevisiae* and 24.1, 6.7 and 5.6 µmol/g respectively for *A. resinae*.

For *S. cerevisiae*, K^+ was the main exchangeable cation during Cu^{2+} binding. At the lowest initial Cu^{2+} concentration relative percentages of K^+ and Mg^{2+} released (of the total exchanged cations) were *ca.* 95.9 and 4.1% respectively, with a slight decrease in H^+ ions. The relative percentages of released K^+ ions decreased to *ca.* 68.6% at the highest initial Cu^{2+} concentration, with Mg^{2+} and H^+ release accounting for *ca.* 22.7 and 8.7% respectively. Total ion release levels for *A. resinae* during Cu^{2+} uptake were *ca.* 52.9 and 122.3 µmol g^{-1} at the lowest and highest initial Cu^{2+} concentrations respectively, compared to *ca.* 487.6 and 774.9 µmol g^{-1} for *S. cerevisiae*. A decrease in the relative percentage of K^+ ions released, from *ca.* 88.9 to 71.1%, between the lowest and highest initial Cu^{2+} concentrations was also observed for *A. resinae*. The relative percentages of Mg^{2+} and H^+ also increased from *ca.* 5.4 to 9.2% and from *ca.* 5.7 to 19.7% for Mg^{2+} and H^+ respectively.

Total ion release increased from *ca.* 74.1 to 654.7 µmol g^{-1} for *S. cerevisiae* and from *ca.* 25.4 to 49.1 µmol g^{-1} for *A. resinae* for the lowest and highest Cd^{2+} concentrations. Once again, as observed for Cu^{2+}, for both organisms the relative percentages of released K^+ decreased and those of Mg^{2+} increased, albeit to lower degrees than for Cu^{2+}. The relative percentages of released H^+ ions also increased for both organisms at the highest initial Cd^{2+} concentration.



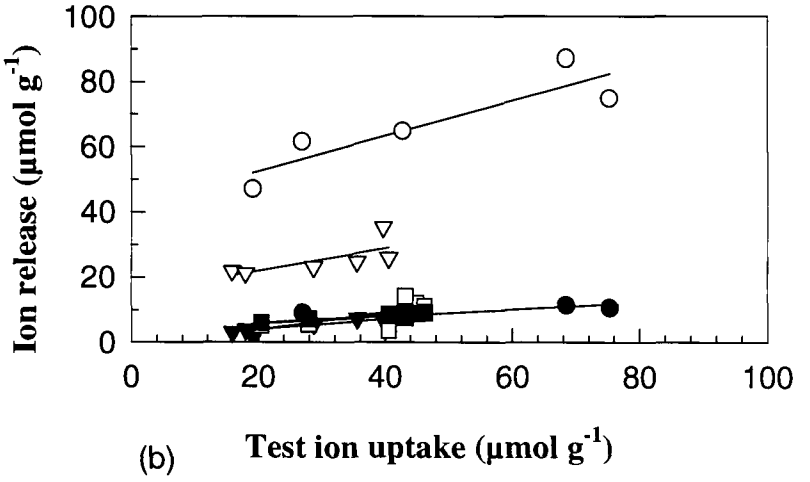


Figure 3.2.6 K^+ (open symbols) and Mg^{2+} (closed symbols) displacement resulting from Cu^{2+}(○ ●), Cd^{2+}(▽ ▼) and Sr^{2+}(□ ■) adsorption by (a) *S cerevisiae* and (b) *A resinae*

Initial Test Ion conc ($\mu mol\ l^{-1}$)	Ion	*S cerevisiae* Uptake/release ($\mu mol\ g^{-1}$)	*A resinae* Uptake/release ($\mu mol\ g^{-1}$)
78 290	Cu^{2+}	62 078	19 142
	K^{+}	467 439	47 013
	Mg^{2+}	20 151	2 848
	H^{+}	-1 736	3 030
2622 773	Cu^{2+}	142 519	68 475
	K^{+}	531 561	86 940
	Mg^{2+}	176 130	11 291
	H^{+}	67 256	24 076
44 258	Cd^{2+}	23 509	15 917
	K^{+}	58 086	21 874
	Mg^{2+}	15 995	3 043
	H^{+}	0 020	0 482
1482 668	Cd^{2+}	161 858	39 681
	K^{+}	497 080	35 414
	Mg^{2+}	156 335	6 971
	H^{+}	1 287	6 718
56 779	Sr^{2+}	17 329	20 416
	K^{+}	------	5 157
	Mg^{2+}	2 621	5 946
	H^{+}	0 480	0 352
1902 154	Sr^{2+}	81 771	46 224
	K^{+}	5 430	11 006
	Mg^{2+}	8 682	9 082
	H^{+}	0 470	5 572

Table 3.2.1 Test ion uptake and resulting K^{+}, Mg^{2+} and H^{+} displacement at minimum and maximum initial test ion concentrations for *S cerevisiae* and *A resinae*

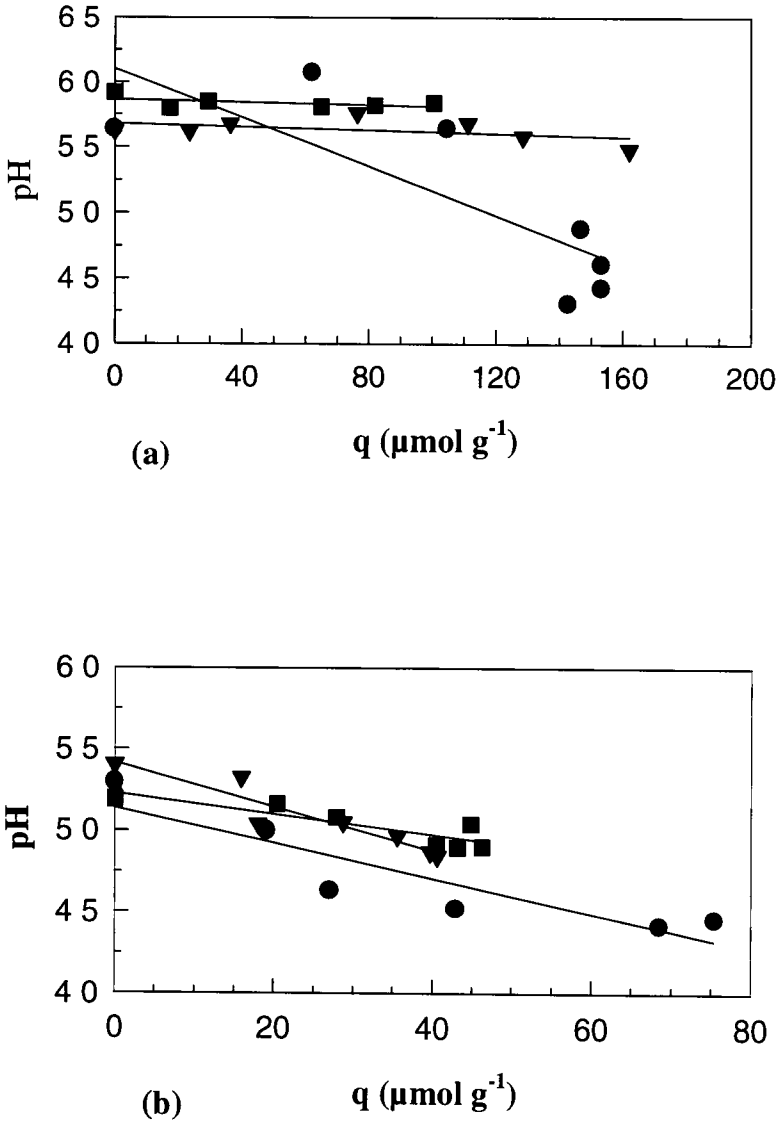
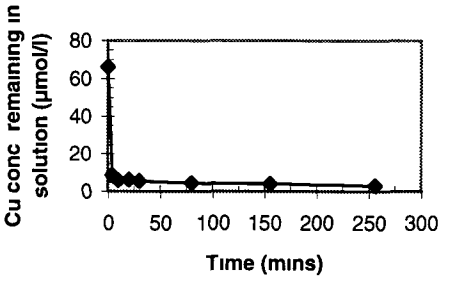


Figure 3.2.7 Equilibrium pH values after Cu^{2+}, Cd^{2+} and Sr^{2+} adsorption by (a) *S cerevisiae* and (b) *A resinae* ● Copper, ▼ Cadmium, ■ Strontium.

When Sr^{2+} uptake by both organisms is examined the levels of total ion release are lower than those observed for both Cu^{2+} and Cd^{2+} uptake At the lowest initial Sr^{2+} concentration, no K^{+} release was observed from *S cerevisiae* and levels of Mg^{2+} and H^{+} release were very low (*ca* 2 62 and 0 48 µmol g^{1} respectively) Even at the highest initial Sr^{2+} concentration the amount of total ion release was only *ca* 14 6 µmol g^{-1} Total ion release for *A resinae* was *ca* 11 5 and 25 7 µmol g^{-1} at the lowest and highest initial Sr^{2+} concentrations respectively

3.2.3 Metal time-course uptake using *A. filiculoides*.

Investigation of the kinetics of Cu^{2+} and Cd^{2+} uptake in batch systems revealed that 87% of Cu^{2+} was removed from solution after only 4 min, with uptake of Cd^{2+} being considerably slower taking 30 min for 67% removal (see Figure 3 2 8 (a) and (b)) A value for characteristic time τ was obtained from the first three points of each data set using linear regression to calculate the intercept with the x-axis (Volesky & Votruba, 1992) This value was applied to the computer model for prediction of breakthrough For Cu^{2+} and Cd^{2+} the τ values are 9 47 and 19 65 min respectively



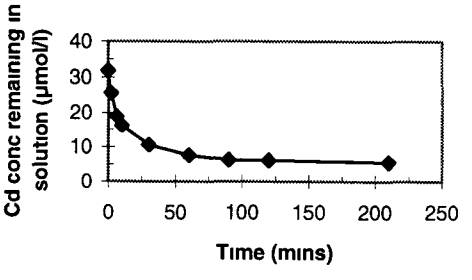


Figure 3.2.8 Kinetics of (a) Cu^{2+} and (b) Cd^{2+} uptake by *Azolla*

3.2.4 Cu^{2+} adsorption using immobilised and non-immobilised milled-sieved *A. filiculoides* Lamarck.

Immobilisation had differing effects on the biosorption of Cu^{2+} by milled-sieved *Azolla* In batch studies using solutions with initial Cu^{2+} concentrations of 50 and 100mg l^{1}, native milled-sieved *Azolla* exhibited the greatest uptake, with uptake levels of *ca* 297 and 363 µmol g^{-1} respectively, while epichlorhydrin-immobilised *Azolla* exhibited uptake of *ca* 287 and 320 µmol g^{-1} respectively (see Table 3 2 2) Consequently these two biomass types were selected and subsequently examined for their use in column removal of Cu^{2+} TEOS (i) biosorbent, by comparison, exhibited much lower uptakes of *ca* 121 and 155µmol g^{-1} from 50 and 100mg l^{1} solutions of Cu^{2+} while TEOS (ii) biosorbent exhibited extremely low biosorption levels of *ca* 64 and 68 µmol g^{-1} from similar solution concentrations TMOS biosorbent was capable of removing *ca* 106 and 121µmol g^{-1} from the 50 and 100mg l^{-1} solution respectively

	c50		c100	
Biomass Type	mg g^{-1}	µmol g^{-1}	mg g^{-1}	µmol g^{-1}
Milled-sieved Azolla	18 9	296 9	23 1	363 3
Epichlorhydrin Azolla	18 2	286 9	20 4	320 2
TEOS (i)	7 7	121 4	9 9	155 3
TEOS (ii)	4 1	64 3	4 3	67 7
TMOS	6 8	106 4	7 7	120 5

Table 3.2.2 Uptake of Cu^{2+} by native and immobilised milled-sieved *Azolla* c50 and c100 = uptake from an initial Cu^{2+} solution of 50 or 100mg l^{-1} respectively, TEOS (i) = 5g *Azolla* per 100ml TEOS solution, TEOS (ii) = 2g *Azolla* per 100ml TEOS solution Initial solution pH = 5 50 $\pm$ 0 05

3.2.5 Column study using *A. filiculoides*.

The *Azolla* isotherm data from *Section 3 2 2* (see Figure 3 2 4) were fitted to the Freundlich sorption model (Freundhch, 1926) using the Enzfitter program The Freundlich model takes the general form

$$q = KC_f^{(1/n)}$$

where q and C_f are as defined earlier and K and n are constants This can be linearised to give the form

$$\ln q = \ln K + 1/n \ln C_f$$

Enzfitter was used to calculate values for K and n For Cu^{2+} K = 7 6026 and n = 0 2298 while for Cd^{2+} K = 0 1064 and n = 0 1977 These values, along with those for the experimental sorbent characteristics (see *section 2 9 3*), were used with a previously written computer model for predictive calculation of metal sorption and the resultant breakthrough curves compared with results obtained experimentally The programming and simulation language PSI/c was used and the full program is listed in Appendix A

Experimental and predicted breakthrough curves for Cu^{2+} are shown in Figure 3 2 9 The computer predicted profile exhibits breakthrough starting after *ca* 5 5 litres of influent Cu^{2+} solution had passed through the column, with complete saturation of biomass predicted after *ca* 7 litres of Cu^{2+} Breakthrough in the experimental profile was initially very gradual until *ca* 5 litres and increased until *ca* 11 litres when the profile began to level off indicating approach of biomass saturation Complete saturation of the biomass did not take place until *ca* 19 litres

Figure 3 2 10 shows the corresponding experimental and predicted breakthrough curves for Cd^{2+} The model profile predicts breakthrough starting later than that for Cu^{2+}, at *ca* 6 5 litres, reaching saturation after *ca* 10 litres of influent Cd^{2+} Initial breakthrough of the experimental profile takes place much earlier than that for the predicted profile and, as in the case of Cu^{2+}, exhibits a much more gradual slope, not reaching saturation until *ca* 15 litres

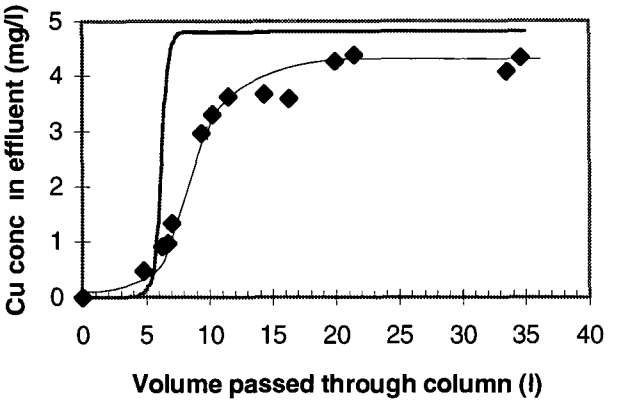


Figure 3.2.9 Computer predicted (——)and experimental (◆) breakthrough curves for Cu^{2+} from column containing 2 5g of native *Azolla* Influent conc = 5mg l^{-1} , influent pH = 5 50 ± 0 05

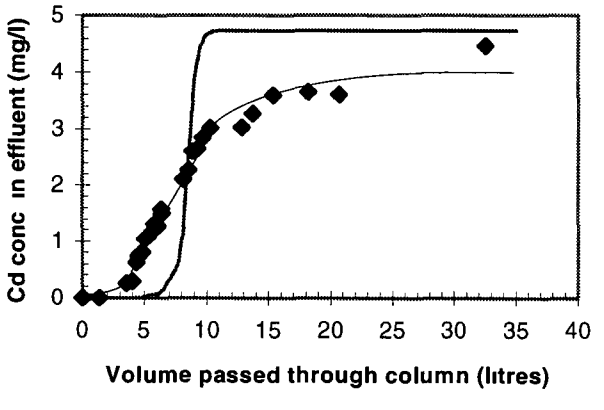


Figure 3.2.10 Computer predicted (——) and experimental (◆) breakthrough curves for Cd^{2+} from column containing 2 5g of native *Azolla* Influent conc = 5mg l^{-1} , influent pH = 5 50 ± 0 05

Breakthrough curves for columns containing 1g of milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* are shown in Figures 3 2 11 and 3 2 12 respectively Initially the milled-sieved *Azolla* column was efficient at removing Cu^{2+} from the influent solution with breakthrough not starting until *ca* 3 litres Breakthrough increased thereafter until *ca* 8 litres when the profile began to level off In the case of the epichlorhydrin-immobilised column, breakthrough started slightly later at *ca* 4 litres and was capable of removing Cu^{2+} from the influent solution until over 12 litres had passed through the column

The corresponding breakthrough curves for columns containing 2 5g of milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* are shown in Figures 3 2 13 and 3 2 14 respectively As expected, breakthrough in these systems starts much later than previously observed due to an increased amount of biomass in the columns, as is evident from curves plotted with respect to effluent volume Initially the milled-sieved *Azolla* column was efficiently removing Cu^{2+} from the influent solution with breakthrough not starting until *ca* 10 5 litres (Figure 3 2 13) The situation is similar for the epichlorhydrin-immobilised *Azolla* column in the early stages of the curve with initial breakthrough at *ca* 12 litres (Figure 3 2 14) The effects of biomass pre-treatment and quantity in each column can be observed more clearly in Figure 3 2 15 where each curve is plotted

Cu^{2+} was effectively desorbed from the column containing 1g of milled-sieved *Azolla* using 5mM Chelaton 3 (Figure 3 2 16) A quantity of 18 95 litres of 5mg l^{-1} Cu^{2+} was previously passed through the column (see Fig 3 2 11) and following desorption was concentrated into a volume of 345ml

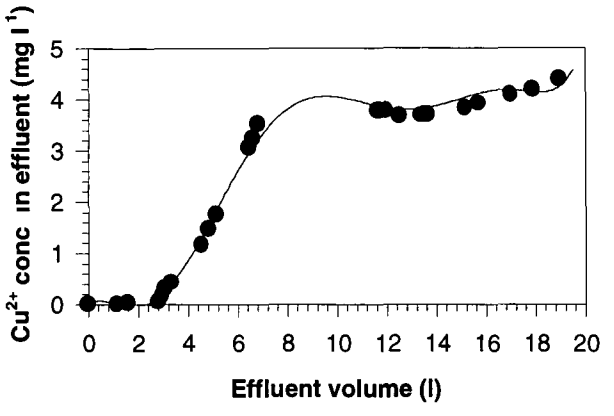


Figure 3.2.11 Experimental Cu^{2+} breakthrough curve for column containing 1g of milled-sieved *Azolla* Influent conc = 5mg l^{-1} Influent pH = 5 50 ± 0 05

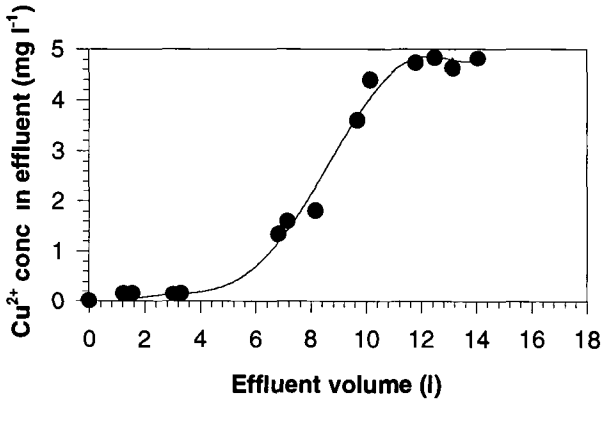


Figure 3.2.12 Experimental Cu^{2+} breakthrough curve for column containing 1 0g of epichlorhydrin immobilised *Azolla* Influent conc = 5mg l^{-1} Influent pH = 5 50 ± 0 05

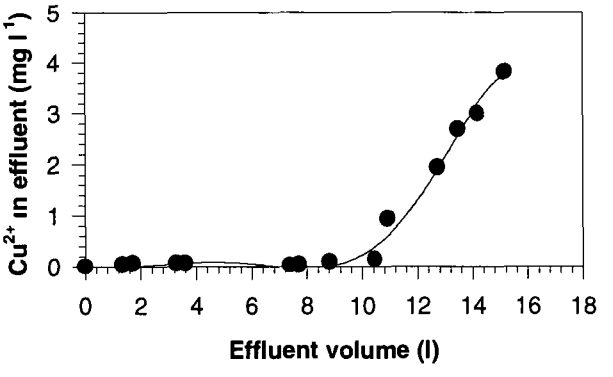


Figure 3.2.13 Experimental Cu^{2+} breakthrough curve for column containing 2 5g of milled-sieved *Azolla* Influent conc = 5mg l^{-1} Influent pH = 5 50 ± 0 05

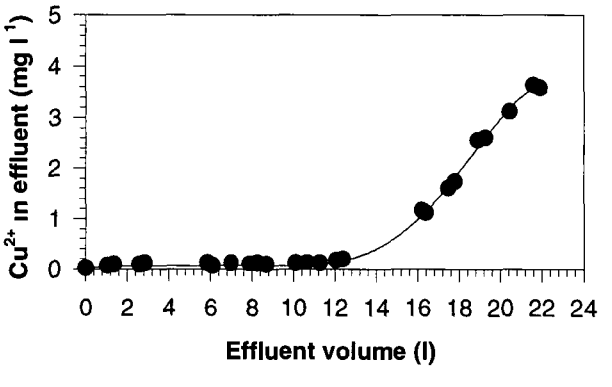


Figure 3.2.14 Experimental Cu^{2+} breakthrough curve for column containing 2 5g of epichlorhydrin immobilised *Azolla* Influent conc = 5mg l^{-1} Influent pH = 5 50 ± 0 05

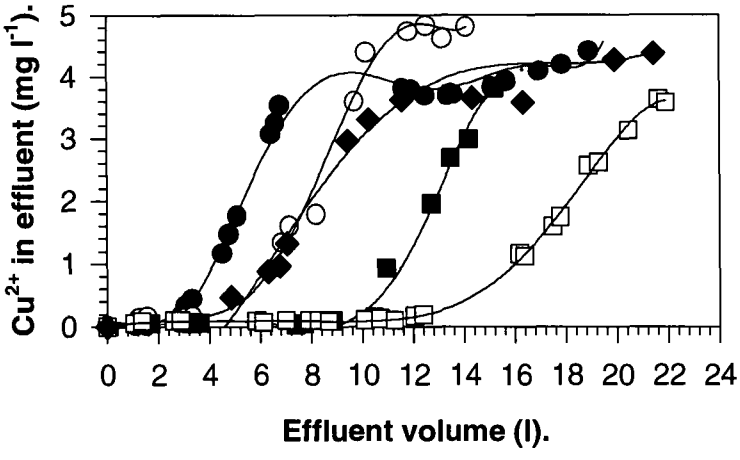


Figure 3.2.15 Experimental Cu^{2+} breakthrough curves for columns containing ◆ = 2 5g of native *Azolla*, ● = 1 0g of milled-sieved *Azolla*, O = 1 0g of epichlorhydrin immobilised *Azolla*, ■ = 2 5g of milled-sieved *Azolla*, □ = 2 5g of epichlorhydrin immobilised *Azolla* Influent conc = 5mg l^{-1} Influent pH = 5 50 ± 0 05

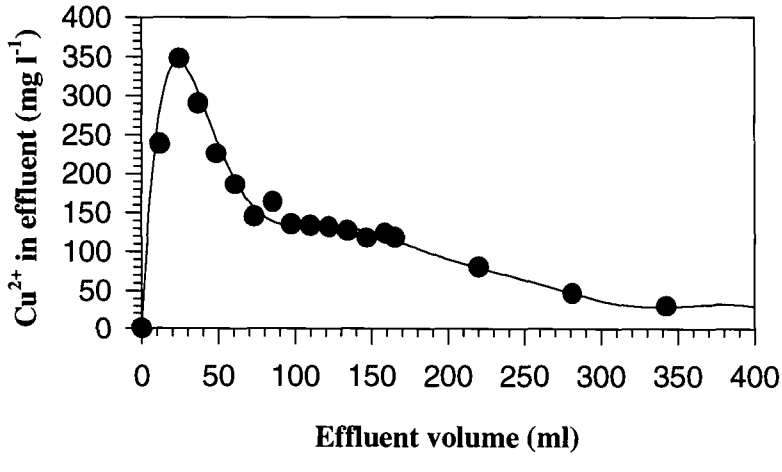


Figure 3.2.16 Desorption of Cu^{2+} from column using 5mM Chelaton 3 A quantity of 18 95 litres of 5mg l^{-1} Cu^{2+} was previously passed through the column which contained 1g of milled-sieved *Azolla* (see Fig 3 2 11)

CHAPTER 4

DISCUSSION

CHAPTER 4: DISCUSSION

4.1 Toxicity of heavy metals towards *S. cerevisiae*.

4.1.1 H^+ release after glucose uptake by *S. cerevisiae*. Effect of toxic metals and protection by Ca^{2+}.

The aim of the work in this section was to analyse the value of H^+ extrusion in assessing and understanding metal interactions with fungi The influence of a range of metals on growth and H^+ efflux in S cerevisiae was examined with the purpose of investigating the relationship with metal toxicity The interactive effects with Ca^{2+}, to possibly alleviate metal toxicity, were also examined

The plasma membrane is a selective barrier that can control both the influx and efflux of metal ions and is an important initial site of biological interaction between cells and external metal ions (Gadd, 1993, Karamushka & Gadd, 1994) The transport of a variety of organic and inorganic solutes across yeast and fungal plasma membranes is dependent on the plasma membrane H^+-ATPase activity which creates a transmembrane electrochemical proton gradient ($\Delta\mu_H^+$) which is negative and alkaline inside (Gadd, 1993, Jones & Gadd, 1990) This electrochemical gradient has an electrical and a chemical component, the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) respectively, which drive the transport of ionisable substances across membranes These secondary gradient-coupled transport systems are energized by

coupling with passive reflux of H^+ The solute and proton fluxes may be in the same (symport) or opposing (antiport) directions (Sanders, 1990)

Three main classes of H^+-pumping ATPases have been identified in fungi and yeasts (Serrano, 1984, 1985) in mitochondrial, vacuolar and plasma membranes, although a Ca^{2+}-ATPase located on the endoplasmic reticulum has now also been identified (Goffeau *et al* , 1990) The vacuolar and plasma membrane ATPases are associated with ion transport, regulation of intracellular pH and intracellular compartmentation (Jones & Gadd, 1990)

The addition of a carbon source to an *S cerevesiae* suspension leads to acidification of the external medium This is believed to be mainly due to H^+-efflux through a membrane bound H^+-ATPase (Ramos, 1985) rather than due to release of organic acids resulting from fermentation

The plasma membrane H^+-ATPase is a useful indicator of environmental stress, including that caused by potentially toxic metal concentrations, as it is clearly responsive to external physico-chemical factors (Gadd *et al* , 1986, White & Gadd, 1987 *b*) Consequently, it has many potential applications both in toxicity assessment and investigation into methods for the alleviation of such toxicity *i e* the interactions between different metals in the cell's environment

Upon addition of 1% glucose to the suspension of *S cerevisiae* cells the pH was observed to steadily decrease, reaching *ca* 4 25, 90 min after glucose addition (Figure 3 1 1) This glucose-dependent H^+ efflux results primarily from the proton pumping activity of the plasma-membrane bound ATPase (Serrano, 1980) This process depends upon the stability of both the plasma membranes and the ATPase

enzyme and also upon the presence of sufficient ATP as substrate Variations in the H^+ efflux may accordingly be connected with irregularities in membrane integrity, permeability and, ultimately, functionality

In the presence of 10, 25 and 100μM Cu^{2+}, H^+ efflux was reduced in proportion to the Cu^{2+} concentration, with almost total inhibition at 100μM Cu^{2+} (Figure 3 1 2) Cu^{2+} is toxic towards *S cerevisiae* by interfering with the glucose dependent H^+ efflux and it has previously been observed that Cu^{2+} may cause damage to the plasma membrane leading to dissipation of the transmembrane electrochemical proton gradient ($\Delta\mu_{H^+}$) (White & Gadd, 1987 *b*) Cu^{2+} is known to be a powerful inhibitor of plasma membrane H^+-ATPases and complete inhibition has been observed in *S cerevisiae* at 10-50μM (Vara & Serrano, 1982, Serrano *et al* , 1985)

The toxicity of heavy metals to microbes can be influenced by the presence of other cations in the environment, as a result of competition with the cationic forms of the metals for anionic sites on cell surfaces (Hughes & Poole, 1989) Magnesium and calcium reduce the toxicity of a range of metals to microbes for example magnesium reduced the toxicity of nickel towards various filamentous fungi (Babich & Stotzky, 1981, 1982, 1983), the yeast *Torula utilis* (Abelson & Aldous, 1950) and *Bacillus subtilis* (Webb, 1970 *a*, 1970 *b*) Increasing levels of magnesium also decreased the toxicity of manganese to *S cerevisiae* (Blackwell *et al* , 1997), copper and cobalt to *S cerevisiae* (Aoyama *et al* , 1986, Karamushka & Gadd, 1994), cadmium and zinc to *E coli* (Abelson & Aldous, 1950) and of zinc to *Anacystis nidulans* (Shehata & Whitton, 1982), and *Klebsiella pneumoniae* (Ainsworth *et al* , 1980)

On account of its similar functions to Mg^{2+}, and also its role in possibly stabilising membrane structure (Karamushka & Gadd, 1994), Ca^{2+} was examined in

the present study to determine its ability to alleviate metal toxicity Addition of 0 5mM Ca^{2+} before 100μM Cu^{2+} partially alleviated the inhibitory effect of the Cu^{2+}, with an interval of 5 min between Ca^{2+} and Cu^{2+} additions leading to a greater H^{+} release than that for a 1 min interval (Figure 3 1 2) Previously Karamushka and Gadd (1994) observed that 0 5mM Ca^{2+} completely alleviated the inhibitory effect of Cu^{2+} at concentrations of 5, 10 and 50μM and considerably reduced it at 100μM, where the effect was greater than that observed in the present study

Calcium has been shown to reduce the toxicity of copper to *S cerevisiae* (Karamushka & Gadd, 1994), zinc and mercury to *Chlorella vulgaris* (Rai *et al* , 1981) and zinc to *Hormidium rivulare* (Say & Whitton, 1977) and *Anacystis nidulans* (Shehata & Whitton, 1982) while zinc reduces the toxicity of nickel to *Achyla* species (Babich & Stotzky, 1982) In the presence of calcium the uptake of copper and lead by *Nostoc muscorum* was reduced as a result of competition for sites on the cell surface (Schecher & Driscoll, 1985)

Both Mg^{2+} and Ca^{2+} may interact with negatively charged functional groups and cross-link carboxylated and phosphorylated anionic polymers on cell surfaces and, like other metals, alter electrosurface properties of microbial cells (such as magnitude and distribution of surface charge, the structure of electric double layer *etc*) (Collins & Stotzky, 1992) At high Ca^{2+} concentrations, the cell surface becomes increasingly less electronegative leading to reduced interaction with Cu^{2+} and other metal ions (Gadd, 1993) Aoyama *et al* , (1986) reported that alleviation of Co^{2+} toxicity is due to the repression of Co^{2+} uptake by Mg^{2+} and, similarly, Gadd and Mowll (1985) observed Ca^{2+} to cause a reduction of up to 83% in the surface binding of Cu^{2+} to *Aureobasium pullulans*

A similar inhibition of H^+ efflux was also observed due to the presence of 50µM, 100µM and 0 5mM Cd^{2+}, with the reduction in H^+ efflux again proportional to the concentration of Cd^{2+} (Figure 3 1 3) However, unlike Cu^{2+}, 100µM Cd^{2+} did not completely inhibit the efflux of H^+, with the pH dropping to *ca* 5 69 after 90 min indicating the higher toxicity of Cu^{2+} towards the *S cerevisiae* plasma membrane Even in the presence of 0 5mM Cd^{2+} H^+ efflux was observed, with pH dropping to 5 98 after 90 min As in the Cu^{2+} studies, the presence of 0 5mM Ca^{2+} alleviated the toxic effects of the Cd^{2+} at all three Cd^{2+} concentrations examined (Figures 3 1 4 - 3 1 6)

The effect of Co^{2+} on H^+ efflux was subsequently examined at concentrations of 100µM, 1mM and 5mM (Figure 3 1 7) A concentration of 100µM Co^{2+} proved to be relatively non-toxic, leading to a corresponding pH drop to *ca* 4 31 after 90 min (Figure 3 1 7, plot 2) The H^+ release is almost identical to that observed in the absence of metal, where the pH after 90 min is *ca* 4 29 (Figure 3 1 7, plot 1) However, the initial slope of H^+ release in the presence of 100µM Co^{2+} is less steep which may indicate a certain degree of toxicity Viability after 90 min was 96 3%, also indicating only a very slight toxicity of Co^{2+} at 100µM

Increasing the Co^{2+} concentration to 1mM, however, does lead to a reduction in H^+ release with the pH decreasing to *ca* 5 47, 75 min after glucose addition (Figure 3 1 7, plot 3) Viability decreased to 52 4% which also indicates toxicity of Co^{2+} at 1mM A concentration of 5mM leads to a further decrease in H^+ release leading to a pH decrease to only 5 79, 90 min after glucose addition (Figure 3 1 7, plot 5) and a viability of 50 6%

The possible protective effect of Ca^{2+} towards Co^{2+} toxicity was subsequently examined Ca^{2+} added to a final concentration of 0 5mM 5 min before addition of 1mM

Co^{2+} leads to a pH decrease to *ca* 4 35, 90 min after glucose addition (Figure 3 1 7, plot 4) which is similar to the levels observed both in the absence of Co^{2+} and for 100μM Co^{2+} (Figure 3 1 7, plots 1 and 2 respectively) Viability increased to 96 0% compared to 52 4% in the absence of 0 5mM Ca^{2+}

Addition of 0 5mM Ca^{2+} 5 min before the 5mM Co^{2+} once again protects the cells against the toxic effects of Co^{2+}, with the pH dropping to *ca* 4 28 after 80 min (Figure 3 1 7, plot 6) and the viability increasing from 50 6% in the absence of Ca^{2+} to 98 2% The profile of the H^+ efflux in this case is almost identical to that after addition of 100μM Co^{2+} Consequently, Ca^{2+} at a concentration of 0 5mM appears to alleviate toxicity from Co^{2+}, at concentrations of 1 and 5mM, towards the *S cerevisiae* plasma membrane and preserves cell viability

Toxicity of Pb^{2+} was also examined and was observed to reduce the H^+ efflux, the effect being greater for the higher Pb^{2+} concentration At concentrations of 100μM and 0 5mM Pb^{2+} the pH's decreased to *ca* 4 95 and 5 59, respectively, 90 min after the addition of glucose (Figure 3 1 8)

Of all the metals examined, Sr^{2+} appeared to be the least toxic towards the *S cerevisiae* plasma membrane Concentrations of 100μM, 0 5mM and 100mM Sr^{2+} exhibit negligible effects towards the H^+ efflux and (Figure 3 1 9) Cell viabilities after contact with all three concentrations were also very high, though decreasing with increasing metal concentration, again indicating the low toxicity of Sr^{2+} towards *S cerevisiae* at these concentrations

Growth of *S cerevisiae* was inhibited by Co^{2+} at concentrations of 100μM, 500μM, 1mM and 5mM with toxicity increased with increasing Co^{2+} concentration (Figure 3 1 10 (a)) Since 100μM Co^{2+} reduced cell growth by 70 4% (Figure (3 1 10 (a))

but had a lesser effect on the glucose-dependent H^+ efflux (Figure 3 1 7, plot 2), it appears that Co^{2+} at this concentration is toxic towards cell growth but causes only slight damage to the plasma membrane Having previously observed that the presence of 0 5mM Ca^{2+} alleviated the toxic effects of Cu^{2+} and Cd^{2+} towards H^+ efflux (Figures 3 1 2 - 3 1 6), it was also examined for a protective effect towards Co^{2+}-inhibited cell growth Ca^{2+} at a concentration of 0 5mM was observed to significantly reduce Co^{2+} toxicity towards cell growth at all four Co^{2+} concentrations, with an even greater effect observed for 5mM Ca^{2+} (Figure 3 1 10 (b) and (c))

This chapter has examined the effect of various metal ions on the H^+ efflux from metabolising *S cerevisiae* as a rapid means of toxicity assessment, and demonstrated that toxic effects can be alleviated by external Ca^{2+} The reduction in metal toxicity is due mainly to competitive and stabilising interactions at the cell surface (Gadd, 1993) The extent of the toxicity for the range of metals examined in the present study appears quite different due to the chemical differences of the metal ions, with toxicity being of the order $Cu^{2+} > Cd^{2+} > Pb^{2+} > Co^{2+} > Sr^{2+}$

4.1.2 Toxicity of Cu^{2+} and Mn^{2+} and localisation of Cu^{2+} in *S. cerevisiae*.

Growth of *S cerevisiae* was not inhibited in the presence of 1, 10 or 50μM Cu^{2+} (Figure 3 1 11) However, Cu^{2+} at a concentration of 100μM does appear to inhibit growth and at a concentration of 500μM, Cu^{2+} has an even greater inhibitory effect

Intracellular localisation of a toxic (100μM) and sub-toxic (25μM) concentration of Cu^{2+}, as determined from growth of *S cerevisiae* in the presence of Cu^{2+} (Figure 3 1 11), was examined The vast majority of Cu^{2+} in the control cells was located in the membrane bound fraction (*ca* 695 and 445 nmol/10^9 cells after 10 min and 2 h respectively) (see Table 3 1 1) This Cu^{2+} is that which was available in the unamended growth medium at a concentration of *ca* 15 μM There was no Cu^{2+} present in either the cytosol or vacuole fractions at either time point The Cu^{2+} levels in intact cells after acid digestion (*ca* 610 and 367 nmol/10^9 cells) were *ca* 87 and 78% of those for the sum of Cu^{2+} in the four fractions after 10 min and 2 h respectively

In the presence of 25μM Cu^{2+} the majority of the ions is once again present in the membrane fraction (*ca* 93 and 84% of total ions present after 10 min and 2 h respectively) Cu^{2+} was detected in the wash fraction after 10 min with more than twice the amount present after 2h (*ca* 89 and 193 nmol/10^9 cells respectively) In the presence of 100μM Cu^{2+} however, the levels of Cu^{2+} increased in all four fractions with the majority still being present in the membrane fraction The amount of membrane-bound Cu^{2+} represented *ca* 74 0 and 84 3 % of the total Cu^{2+} present in the *S cerevisiae* cells (*ca* 2984 and 3182 nmol/10^9 cells after 10 min and 2h respectively) The total Cu^{2+} levels were within 0 2 and 3 0% of those for intact cells after 10 min and 2 h respectively

K^+ release was observed to increase with increasing Cu^{2+} concentration and with time after the *S cerevisiae* cells were contacted with 10, 25 and 100µM Cu^{2+} (Table 3 1 2) After contact with 10µM Cu^{2+} there does not appear to be a significant drop in viability (Table 3 1 3) However, methylene blue staining indicates a reduction in viability for cells in the presence of 10µM Cu^{2+} to 78 6 and 76 1% of the controls after 20 min and 2 h respectively (Table 3 1 4) This may be correlated with the increase in K^+ release from the same cells Viability (c f u x 10^6 per ml) decreased with both increasing Cu^{2+} concentration and time of contact with the 25 and 100µM Cu^{2+} (Table 3 1 3) After contact with 25µM Cu^{2+}, plate-count-viability (as percentage of control) decreased to *ca* 65 and 39% after 10 min and 2 h respectively Methylene blue staining also indicated a drop in viability to ca 38 and 27% after 10 min and 2 h respectively This may be compared to the K^+ release which also increased considerably in the presence of 25µM Cu^{2+} (Figure 3 1 2) In the presence of 100µM Cu^{2+} viability decreased to *ca* 8 and 2% after 10 min and 2 h respectively which correlates well with earlier observations where 100µM Cu^{2+} significantly reduced growth of *S cerevisiae* (Figure 3 1 1) Viability as determined by methylene blue staining indicates zero viabilities at both time points

Since the concentrations of Cu^{2+} were low *i e* 25 and 100µM, detection by A A S was difficult Cu^{2+} was not observed in the vacuolar fraction after contact with 25µM Cu^{2+} Increasing the concentration of Cu^{2+} to 100µM increased the amount located in the cytosol and vacuole but, as seen in the previous experiments (*Section 3 1 1*), increasing the Cu^{2+} concentration causes toxicity by damaging the plasma-membrane leading to little Cu^{2+} being located in the vacuole Cu^{2+} has proved to be very difficult to examine intracellularly due to its toxicity, with the majority of Cu^{2+} being membrane

bound Protection from this toxicity using Ca^{2+} and Mg^{2+} may be possible, leading to higher Cu^{2+} levels being detected in the cytosol and vacuole fractions

As observed in the Cu^{2+} studies, an increase in Mn^{2+} concentration also gave rise to increased K^+ release (Table 3 1 5) The amount of K^+ released after contact with 1 and 10mM Mn^{2+} (*ca* 11 7 and 16 7 µmol/10^9 cells after 160 min) (Table 3 1 6) was considerably less than that released after contact with 25 and 100µM Cu^{2+} (*ca* 21 7 and 25 5 µmol/10^9 cells respectively) indicating the higher toxicity of Cu^{2+} towards *S cerevisiae* At a concentration of 10mM, Mn^{2+} caused a significant reduction in viability to *ca* 71 0 and 79 6% after 70 and 160 min respectively However, as in the K^+ study, these values show that Mn^{2+} is not as toxic towards *S cerevisiae* cells as Cu^{2+} where viability was reduced to *ca* 39 and 1 5 % after 2 h contact with 25 and 100µM respectively

4.2 Metal Adsorption.

4.2.1 Effect of biomass concentration on native *A. filiculoides* biosorption.

Biomass concentration affected the amount of test ion recovered from solution, with uptake (expressed as μmol g^{-1}) of Cu^{2+}, Cd^{2+} and Pb^{2+} decreasing as *Azolla* concentration in suspension was increased from 1 to 5 to 10 g l^{1} The corresponding total ion removal (expressed as % removal) increased with increasing biomass concentration Increasing biomass concentration has been reported to reduce metal uptake per gram of biomass in a number of systems (Brady & Duncan, 1994 *a*, Fourest & Roux, 1992, Singleton & Simmons, 1996) Increasing the biomass concentration in solution from 1 to 8 mg cm^{-3} was previously reported to decrease silver biosorption by 60 2% (Singleton & Simmons, 1996) Fourest and Roux (1992) report that zinc uptake decreases when *R arrhizus* biomass concentration is increased They also comment that reduction in biomass concentration in the suspension at a given metal concentration enhances the metal/biosorbent ratio, and increases metal uptake per g of biosorbent, as long as the latter is not saturated Electrostatic interactions and interferences between binding sites will also be reduced at lower biomass concentrations, leading to increased biosorption (Fourest & Roux, 1992, Meikle *et al* , 1990)

4.2.2 Metal adsorption isotherms with *S. cerevisiae*, *A. resinae* and *A. filiculoides*.

The metal adsorption characteristics of *S cerevisiae* and *A resinae* differ considerably in terms of both uptake of the test ions and also the degree of ion exchange exhibited For *S cerevisiae*, uptake was generally of the order $Sr^{2+} < Cd^{2+} = Cu^{2+}$ and it was noticeable that at the lower concentrations examined Cu^{2+} uptake exceeded that of Cd^{2+} considerably, although their saturation uptake values were similar

These trends are consistent with previous work which found the order of binding to be $Sr^{2+} < Cd^{2+} < Cu^{2+}$ for *S cerevisiae* metal binding in 50µM solutions, with uptake levels of 14 9, 23 4 and 26 7µmol g^{-1} for Sr^{2+}, Cd^{2+} and Cu^{2+} respectively (Avery & Tobin, 1993) Earlier work by Norris and Kelly (1977) reported yeast Cd^{2+} uptake levels of *ca* 22µmol g^{-1} from 200µM $CdSO_4$ which is significantly lower than the present uptake (*ca* 35µmol g^{-1} from 89 3µM Cd^{2+}) Brady and Duncan (1994 *a*) report Cu^{2+} uptake of *ca* 700µmol g^{-1} at pH 6 5, while Gadd and Mowll (1983) observed Cd^{2+} uptake of *ca* 310µmol g^{-1}

Amorphotheca resinae (formerly called *Cladosporium resinae*) is a filamentous fungus that has been isolated from soil (Parberry, 1968), air (Parberry, 1969), fresh, estuarine and marine waters (Ahearn & Meyers, 1972) and hydrocarbon-rich environments (Parberry, 1969) and grows on glucose or any of several hydrocarbons as its sole carbon source (Carson & Cooney, 1989) The organism is a contaminant of light hydrocarbon fuels (Edmonds & Cooney, 1967) and is a potential source of operational problems in the fuel system of jet aircraft due to corrosion (Parberry, 1969, 1971) Uptake values for *A resinae* followed the same general order $Sr^{2+} < Cd^{2+} < Cu^{2+}$ although the

saturation uptake values for Sr^{2+} and Cd^{2+} were very similar As above this is in keeping with literature reports of biosorption of these metals by a number of fungal biomasses such as *Rhizopus arhizus* biomass, which also exhibits a $Sr^{2+} < Cd^{2+} < Cu^{2+}$ binding order (Brady & Tobin, 1994, 1995) However, the level of uptake of all three metals is in marked contrast with the present findings for *S cerevisiae* and also with earlier published studies For example, the maximum Cu^{2+} uptake exhibited by *A resinae* was *ca* 70µmol g^{-1} as compared to sequestration levels of *ca* 200 µmol g^{1} at a final solution concentration of *ca* 90uM reported by Gadd and de Rome (1988) Similarly, the present uptake values are considerably lower than those observed for *R arrhizus* where Sr^{2+}, Cd^{2+} and Cu^{2+} were adsorbed maximally to 174, 246, and 357µmol g^{-1} respectively (Brady & Tobin, 1994) and Cd^{2+} to *ca* 225µmol g^{-1} for inactive *R arrhizus* biomass (Fourest & Roux, 1992)

Metabolically, it seems that the filamentous fungus *Amorphotheca resinae* may be unique as it has the ability to utilise a number of unusual substrates which are restrictive because of their toxicity and/or their chemical complexity (Parberry, 1971) This, possibly environmentally significant ability (Parberry, 1969, Parberry 1971), may be a significant factor when comparing the biosorption of toxic metals Cu^{2+}, Cd^{2+} and Sr^{2+} with that of *S cerevisiae* Furthermore, the presence of the pigment melanin in the cell walls of *A resinae* has previously been shown to increase its biosorptive capacity (Gadd & de Rome, 1988)

As the pH and contact conditions of the present work were of the range and type usually considered optimum for metal biosorption, it is likely that the differences in uptake levels are attributable to different culture conditions which results in significant variations in cell wall structures (Remacle, 1990) thereby influencing passive adsorption of test ions

(Meikle, 1990, White & Gadd, 1995) The presence of carboxylate, thiolate, phosphate and amine groups results in the cell wall being net negatively charged under physiological conditions, (Collins and Stotzky, 1989, Chen and Ting, 1995) which is significant in terms of interactions with metal cations This charge varies depending on species of micro-organism, with those possessing a greater net negative charge also possessing a potentially greater ability to bind charged species, including metal cations This may be one reason why *S cerevisiae* binds Cu^{2+}, Cd^{2+} and Sr^{2+} to a greater degree than *A resinae* in the present study

Furthermore, the concentration of *A resinae* per flask was higher than previous studies involving this biosorbent (Gadd & de Rome, 1988, de Rome & Gadd, 1987) Increasing biomass concentration has been reported to reduce metal uptake per gram of biomass in a number of systems (Brady & Duncan, 1994 *a*, Fourest & Roux, 1992, Singleton & Simmons, 1996) as was discussed in *section 4 2 1*

Azolla filiculoides is a floating water fern common in many parts of the world and has recently gained attention as a potential metal biosorbent for use in the treatment of metal-bearing effluents (Sela *et al* , 1990, Tel-Or *et al* , 1996, Zhao & Duncan, 1997, 1998) In this work the characteristics of biosorption of Cu^{2+} and Cd^{2+} by *Azolla* were examined in terms of kinetics of uptake, equilibrium isotherms and column performance

Both Cu^{2+} and Cd^{2+} were adsorbed to high levels by *A filiculoides*, with maximum uptake levels of *ca* 350 and 245 μmol g^{-1}, respectively (Figure 3 2 4) These uptake values are significantly greater than those exhibited by either *S cerevisiae* (*ca* 150μmol g^{-1} for both test ions) or *A resinae* (*ca* 70 and 40 μmol g^{-1} for Cu^{2+} and Cd^{2+} respectively) The initial slopes of the *Azolla* isotherm curves were

steep indicating high Cu^{2+} and Cd^{2+} removal at the lower metal concentrations This trend of Cu^{2+} uptake being greater than that of Cd^{2+} has been previously observed for fungal/yeast systems both in this study for *S cerevisiae* and *A resinae*, and also by Avery and Tobin (1993) for *S cerevisiae* and by Brady and Tobin (1994, 1995) for *R arrhizus* Brady and Tobin (1994) observed maximal uptake levels of 357 and 246μmol g^{-1} for Cu^{2+} and Cd^{2+} by *R arrhizus* which are very similar to the levels observed in the present study Tel-Or *et al*, (1996) propose the major heavy metal binding groups for Cu, Cd, Ni, Zn and Pb in *Azolla* to be pectin, polyphosphates and metal binding peptides

Using reciprocal Langmuir plots to further examine metal binding by *S cerevisiae* and *A resinae*, the better fit of the *S cerevisiae* data, as evidenced by the higher r^2 values, is indicative that the binding conforms more closely to the monolayer, single-site type adsorption underlying the Langmuir model (Langmuir, 1918) However, the wider applicability of the model to various other kinds of sorption phenomena has been also recognised (Weber, 1972) The results suggest in general a more complex form of binding for *A resinae*

Scatchard analysis may be used to elucidate the mechanisms underlying microbial metal binding (Scatchard, 1949), although the limitations of the technique are well known (Alberts & Gresey, 1983, Perdue & Lytle, 1983, Shuman *et al*, 1983) In the present work, the clearly curved nature of the Scatchard plots for Cu^{2+} and Cd^{2+} uptake data for *A resinae* suggests binding to multiple, non-equivalent sites in the biomass In contrast, the linear/scattered plots for uptake of all three test ions by *S cerevisiae*, and Sr^{2+} uptake by *A resinae*, indicate a more simple mechanism likely comprising a single binding site or type (Brady & Tobin, 1994, Scatchard, 1949; Tobin *et al*, 1990) These observations are

consistent with the reciprocal Langmuir plots, with both models suggesting a single binding site or type for *S cerevisiae* adsorption and Sr^{2+} uptake by *A resinae* but a more complex type of binding for *A resinae* Cu^{2+} and Cd^{2+} adsorption

Nieboer and Richardson (1980) use the covalent index (Xm^2r) as an index of the degree of class B ("soft") behaviour to be expected, where Xm is the metal-ion electronegativity (Allred, 1961) and r its ionic radius (Shannon & Prewitt, 1969, 1970) Accordingly, the covalent index of Cu^{2+} is greater than that of Cd^{2+} and the Cu^{2+} ion is expected to possess a greater degree of class B character and enhanced potential to form covalent bonds with biological ligands Sr^{2+} is a class A ("hard") ion with a lower covalent index than either Cu^{2+} or Cd^{2+} and is predicted to form bonds that are principally ionic In the present study, the "softer" Cu^{2+} was bound to higher levels than the "hard" Sr^{2+} for both organisms, with Cd^{2+} bound to an intermediate level for *S cerevisiae* but to the least extent for *A resinae* Similarly, the "softer" Cu^{2+} was bound to greater levels than Cd^{2+} by *Azolla* This trend has been previously observed for *S cerevisiae* (Avery & Tobin, 1993) and *R arrhizus* (Brady & Tobin, 1994, 1995) where uptake in both cases was of the order $Cu^{2+} > Cd^{2+} > Sr^{2+}$

Cation release is an indication of an ionic interaction between metal and biomass (Avery & Tobin, 1992) However, ion release into the surrounding environment is frequently also the result of membrane damage and resulting ion leakage from the cell interior (Brady & Duncan, 1994 *c*, Gadd & Mowll, 1983, Passow & Rothstein, 1960) In this work both Cu^{2+} and Cd^{2+} uptake caused K^+ release which was generally greatly in excess of stoichiometric amounts for both organisms Similarly, Norris and Kelly (1977) observed *S cerevisiae* cellular K^+ levels decrease to 339 and 122µmol g^{-1} after incubation in 0 2mM $CdSO_4$ and $CuSO_4$ respectively Cellular K^+ levels of *ca* 550µmol g^{-1} have

previously been reported for *S cerevisiae* (Blackwell, 1998) Furthermore, as seen in Figures 3 2 6 (a) and (b), K^+ release is independent or only weakly dependent on the level of Cu^{2+} or Cd^{2+} adsorbed which suggests that ion release is due not to sorption directly but probably to damage resulting from sorption (Belde *et al* , 1988, Brady & Duncan, 1994 *c*, Gadd & Mowll, 1983, Joho *et al* , 1995, Passow & Rothstein, 1960) When Cu^{2+} and Cd^{2+} uptake is examined in both organisms, the relative percentage of released K^+ decreases as test ion concentration increases, with a concurrent increase in the relative percentages of Mg^{2+} and H^+ (Table 3 2 1)

In contrast, Sr^{2+} uptake causes little or no ion release in either organism. While this is consistent with the generally lower uptake of Sr^{2+} by both organisms and has been observed previously for *S cerevisiae* (Avery & Tobin, 1992), it contrasts with the behaviour reported recently for denatured *R arrhizus* (Brady & Tobin, 1994)

Brady & Tobin (1994) observed a H^+ release of the order $Cu^{2+}>Cd^{2+}>Sr^{2+}$ for *R arrhizus* which is comparable with the present study and H^+ displacement has been reported to be characteristic of covalent bonding (Avery & Tobin, 1992) Again, this trend is in keeping with the hard and soft principle whereby softer ions will tend to interact with biological ligands predominantly through covalent bonding (Brady & Tobin, 1995, Shuman *et al* , 1983), leading to a reduction in pH

Under the present experimental conditions, *S cerevisiae* proved to be a more efficient biosorbent of the test ions examined than *A resinae* However, literature studies indicate that by varying culture conditions and biomass concentrations, metal uptake g^{-1} of *A resinae* may be increased to the levels observed for other biomass types (Brady & Duncan 1994 *c*, Brady & Tobin, 1994, Fourest & Roux, 1992, Gadd & Mowll, 1983, Gadd & de Rome, 1988) Biosorption of Cu^{2+} and Cd^{2+} was significantly greater for

Azolla than either *S cerevisiae* or *A resinae* and for this reason *Azolla* was subsequently selected for further study to examine uptake kinetics, immobilisation and column performance

4.2.3 Time-course uptake using *Azolla filiculoides*.

Results of the time-course studies illustrate the rapid removal of Cu^{2+} from solution by *A filiculoides*, with 87% adsorption after 4 min (Figure 3 2 8 (a)) Cd^{2+} removal was much slower, with only 60% removal after 30 min (Figure 3 2 8 (b)) This order is similar to that observed for the equilibrium adsorption isotherms (Figure 3 2 4) where maximum Cd^{2+} adsorption was only 70% that of Cu^{2+}

4.2.4 Cu^{2+} adsorption using immobilised and non-immobilised milled-sieved *A. filiculoides* Lamarck.

Immobilisation had a profound effect on the biosorption of Cu^{2+} by milled-sieved *Azolla* In batch studies native milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* exhibited the greatest Cu^{2+} uptake, with uptake levels of *ca* 363 and 320 μmol g^{-1} respectively from solutions with an initial Cu^{2+} concentration of 100mg l^{-1} (see Table 3 2 2) These biomass types exhibited higher Cu^{2+} uptake levels than either *S cerevisiae* or *A resinae* (uptake levels of *ca* 153 and 75μmol g^{-1} respectively from 100mg l^{-1} Cu^{2+} solutions) and were comparable to native *Azolla* which exhibited uptake of *ca* 327μmol g^{-1} (Figures 3 2 2 - 3 2 4) Consequently the native milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla*, along with the

native *Azolla*, were selected and subsequently examined for their use in column removal of Cu^{2+} TEOS (i), TEOS (ii) and TMOS biosorbents, by comparison, exhibited much lower uptakes of *ca* 155, 68 and 121μmol g^{-1} from 100mg l^{-1} solutions of Cu^{2+}

4.2.5 Column studies using *Azolla filiculoides*.

The computer model used to predict continuous fixed-bed adsorption was not entirely successful in predicting the experimental breakthrough curves The initial computer-predicted breakthrough for the Cu^{2+} column corresponds closely to the actual experimental situation for the early section of the experimental profile with breakthrough starting after *ca* 5 5 litres had passed through the column, reaching complete saturation after *ca* 7 litres (Figure 3 2 9) Breakthrough in the experimental profile was initially very gradual until *ca* 5 litres, after which the breakthrough was more rapid until *ca* 11 litres when the profile began to level off indicating initiation of biomass saturation Complete saturation of the biomass did not take place until *ca* 19 litres The slope of the experimental breakthrough was not as great as that predicted by the model indicating that the biomass was able to continue removing Cu^{2+} ions from solution, not saturating until at least *ca* 19 litres of Cu^{2+} solution had passed through, as mentioned earlier

For Cd^{2+} the model predicts breakthrough starting later than that for Cu^{2+}, at *ca* 6 5 litres, reaching saturation after *ca* 10 litres of influent Cd^{2+} (Figure 3 2 10) The initial breakthrough of the experimental profile takes place much earlier than that for the predicted profile, after *ca* 3-4 litres, and also, similar to the Cu^{2+} experimental

column, exhibits a much more gradual slope, not reaching saturation until *ca* 15 litres

The main breakthrough for Cu^{2+} takes place later than that for Cd^{2+} indicating that the *Azolla* is able to remove more Cu^{2+} than Cd^{2+} from the influent solution These results agree with the batch studies where native *Azolla* was capable of higher maximum adsorption levels of Cu^{2+} (*ca* 350 μmol g^{-1}) than Cd^{2+} (*ca* 245 μmol g^{-1}) (Figure 3 2 4)

In contrast, the biosorption column containing 1 0g of milled-sieved *Azolla* (Fig 3 2 11) was the least successful column in removing Cu^{2+} ions from solution in a continuous system However, upon increasing the quantity of biomass in the column to 2 5g the performance was, as expected, greatly improved and was capable of continually removing Cu^{2+} much more efficiently than the native *Azolla* column (Fig 3 2 9) which contained the same quantity of biomass The 1 0g milled-sieved *Azolla* column was capable of complete biosorption of Cu^{2+} from *ca* 3 litres of influent solution whereas upon increasing the quantity of biomass to 2 5g, complete sequestration of test ion from *ca* 10 5 litres was possible (Figure 3 2 13) This compares to a breakthrough of *ca* 3-4 litres for the 2 5g native *Azolla* column Breakthrough increased thereafter until the profiles began to level off after *ca* 8 litres for the 1 0g milled-sieved *Azolla* and *ca* 11 litres for the 2 5g native *Azolla* column Even after *ca* 16 litres of influent solution had passed through the 2 5g milled-sieved *Azolla* column it was still at less than 80% saturation Clearly the pre-treatment utilised in this situation had a profound effect on the column performance and greatly enhanced its potential use for large scale effluent clean-up

The column containing 1 0g of epichlorhydrin-immobilised *Azolla* (Fig 3 2 12) was capable of removing Cu^{2+} to a similar level to that exhibited by the native *Azolla* column, which contained a much greater amount of biomass (2 5g) Initially both columns were efficient at continuously removing influent metal from solution, with breakthrough not starting until *ca* 4 litres for the epichlorhydrin-immobilised column compared to *ca* 3-4 litres for the column containing 2 5g of native *Azolla* Breakthrough increased thereafter in both columns until the profile began to level off after *ca* 11 litres for the native *Azolla* column whereas the 1 0g epichlorhydrin-immobilised *Azolla* column was capable of removing Cu^{2+} from *ca* 12 litres of influent solution

Upon increasing the quantity of biomass in the column to 2 5g the performance was once again greatly improved, as expected and observed earlier for the milled-sieved *Azolla* columns Continuous fixed bed adsorption of Cu^{2+} ions by a column containing 2 5g of epichlorhydrin-immobilised *Azolla* (Figure 3 2 14) was greater than in any of the previously examined systems This column was capable of complete sequestration of Cu^{2+} ions from *ca* 12 litres of influent solution Even after 22 litres of influent solution had passed through, the epichlorhydrin-immobilised *Azolla* column was still at less than 75% saturation

The effects of biomass pre-treatment and quantity in each column can be observed more clearly in Figure 3 2 15 where each curve is plotted Biomass pre-treatment and immobilisation was observed to have a significant effect on column sorption performance Both of the columns containing epichlorhydrin-immobilised *Azolla* were more efficient at removing influent Cu^{2+} from solution than those containing the same corresponding quantity of milled-sieved *Azolla*, which in turn

were more efficient than the native *Azolla* column Increasing the quantity of biomass in each case led to a corresponding and expected increase in column performance as continuous fixed bed adsorption of Cu^{2+} ions was greatest for columns containing 2 5g of epichlorhydrin-immobilised *Azolla* and milled-sieved *Azolla* An earlier study by Zhao and Duncan (1998) examined column removal of Zn^{2+} by *Azolla* from an influent Zn^{2+} concentration of 100mg l^{-1} and pH of 6 2 Breakthrough started at *ca* 0 4, 1 0 and 1 8 litres for columns containing biomass quantities of 2 5, 5 0 and 7 5g, and reached saturation after *ca* 2 0, 2 1 and 3 6 litres

In order to reduce operational costs of any continuous fixed-bed adsorption system, the biomass utilised should demonstrate potential for metal recovery once the metal has been removed from solution Cu^{2+} was effectively desorbed from the column containing 1 0 g of milled-sieved *Azolla* using 5mM Chelaton 3 (Figure 3 2 16) A quantity of 18 95 litres of 5mg l^{-1} Cu^{2+} was previously passed through the column (see Fig 3 2 11) and following desorption was concentrated into 345ml representing a *ca* 55-fold reduction in volume The desorption protocol utilised a minimum quantity of Chelaton 3 to yield a concentrated, low volume metal effluent This procedure should improve the process economics of a fixed-bed biosorption system (Volesky, 1990 *a*) Consideration of recycling the recovered metals is important due to increasing costs of solid waste disposal, with selective removal of metals from the metal-loaded biomass possibly being required (Eccles, 1995)

Silver ions have previously been desorbed from *Aspergillus niger* biomass using dilute HNO_3 (Akthar *et al* , 1995) Similarly acid elution using 0 1M HCl has been used to efficiently remove Cu^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} from *S cerevisiae* biosorption columns (Wilhelmi & Duncan, 1995) The high recovery of these metals

by mild acid elution suggests accumulation by passive binding to the yeast cell walls As in the present study, the recovered metals were concentrated in small volumes, which for Cu, Zn, and Co represented a 50 fold reduction from the initial volume Chromium was not eluted by 0 1 M HCl however, but when the concentration of HCl was increased to 1 M, 34% chromium recovery was achieved These differences may allow selective desorption of chromium and the other metals examined using HCl of differing concentrations, and the same principle could also apply to the use of different concentrations of Chelaton 3 in the present study Biosorbent reusability and selective recovery of metals are considered to be critical in the development of a viable, cost effective metal bioremediation technology (Wilhelmi & Duncan, 1995)

CHAPTER 5

CONCLUSION

CHAPTER 5: CONCLUSION

This present study investigated the toxicity and biosorption of a range of heavy metals for *Saccharomyces cerevisiae*, *Amorphotheca resinae* and *Azolla filiculoides*

The H^+ efflux from *S cerevisiae* was successfully used to assess the toxicity of a range of metals The extent of the toxicity for the range of metals examined appears quite different, with toxicity being of the order $Cu^{2+} > Cd^{2+} > Pb^{2+} > Co^{2+} > Sr^{2+}$ The toxic effects were alleviated by external Ca^{2+} Cu^{2+} and Co^{2+} had detrimental effects on *S cerevisiae* growth at concentrations of 100µM Future work could look at lower, less toxic Cu^{2+} concentrations, increasing the Ca^{2+} concentration while also increasing the time between addition of Ca^{2+} and Cu^{2+} The protective effect of other metal cations may also be considered

S cerevisiae, *A resinae* and *A filiculoides* all biosorbed various metals in batch studies, with *A filiculoides* exhibiting the highest uptake levels Both Cu^{2+} and Cd^{2+} were adsorbed by *S cerevisiae* to maximum levels of *ca* 150µmol g^{-1} and by *Azolla* to *ca* 350 and 245 µmol g^{-1} respectively Milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* exhibited Cu^{2+} uptake levels of *ca* 363 and 320 µmol g^{1} respectively from solutions with an initial Cu^{2+} concentration of 100mg l^{1}

Consequently the native *Azolla*, milled-sieved *Azolla* and epichlorhydrin-immobilised *Azolla* were selected for use in packed bed biosorption columns and effectively removed metals from aqueous solution Epichlorhydrin-immobilised *Azolla* exhibited the greatest column performance with complete metal sequestration from *ca* 4 and 12 litres of influent 5mg l^{-1} Cu^{2+} by columns containing 1 0g and 2 5g of biomass respectively The 2 5g column was still at less than 75% saturation even after

22 litres of influent solution had passed through Chelaton 3 was successfully used to desorb and recover the biosorbed Cu^{2+} from a milled-sieved *Azolla* column, with a corresponding *ca* 55-fold reduction in volume Columns containing epichlorhydrin-immobilised *Azolla* possess potential for use in large scale effluent treatment Mixed metal influent solutions and columns set up in series would further determine the suitability of this process for effluent clean-up

The use of microbial- or plant-based biosorbents for industrial applications has been hindered by problems associated with the physical characteristics of the material (McHale & McHale, 1994) Low mechanical strength of the biomass can cause difficulties associated with separation of the biomass from effluents which, in turn, contribute to limitations in process design A further problem is associated with fragmentation of the biomass causing flow restrictions in continuous-flow contact vessels Immobilisation of the biosorbent, for example with epichlorhydrin which was utilised in the current work, may overcome many of these problems by improving the physical characteristics of the biomass (McHale & McHale, 1994, Tobin *et al* , 1993, Volesky, 1990 *a*) and may also enhance the sorption performance (Leusch *et al* , 1995, Ting & Teo, 1994) Biomass immobilisation will also facilitate its use in column reactors and the reusability should improve the process economics (Volesky, 1990 *b*) Reactors employing a column configuration may offer greater metal-binding capacity and higher efficiency (*i e* , higher purity effluents) and are more readily adapted to automation than batch reactors (Bedell & Darnall, 1990)

Rather than being viewed as an alternative to conventional chemical methods for treating metal-polluted aqueous systems, plant- or microbe-based adsorption may be used in tandem with these techniques, possibly as an additional final purification step It may

also prove economical to direct research towards the utilisation of waste fungal biomass from industrial sources

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Appendix A

PSI/c model used for prediction of column breakthrough curves for Cu^{2+} and Cd^{2+}

f=3 3466

ep=0 1679

gv=96 4577

k=7 6026 for Cu, 10 6494 for Cd

a=0 2298 for Cu, 0 1977 for Cd

n=1/a

tau=9 4622 for Cu, 19 6492 for Cd

d=1

c0=4 8291 for Cu, 4 752 for Cd

l=33

v=3 1415*d^2/4*l

fv=f/v

h=0 2*ep

cr1=var((qr1/k)^n),

cr2=var((qr2/k)^n),

cr3=var((qr3/k)^n),

cr4=var((qr4/k)^n),

cr5=var((qr5/k)^n),

qr1=relay(q1,0 0001,q1),

qr2=relay(q2,0 0001,q2),

qr3=relay(q3,0 0001,q3),

qr4=relay(q4,0 0001,q4),

qr5=relay(q5,0 0001,q5),

q1=int((c1-cr1)/tau par 0 0001),

q2=int((c2-cr2)/tau par 0 0001),

q3=int((c3-cr3)/tau par 0 0001),

q4=int((c4-cr4)/tau par 0 0001),

q5=int((c5-cr5)/tau par 0 0001),

c1=int(-fv*(c1-c0)/h-gv*(c1-cr1)/ep/tau par 0 0),

c2=int(-fv*(c2-c1)/h-gv*(c2-cr2)/ep/tau par 0 0),

c3=int(-fv*(c3-c2)/h-gv*(c3-cr3)/ep/tau par 0 0),

c4=int(-fv*(c4-c3)/h-gv*(c4-cr4)/ep/tau par 0 0),

c5=int(-fv*(c5-c4)/h-gv*(c5-cr5)/ep/tau par 0 0),

where f = flow rate, ep = void volume fraction = ε, gv = sorbent bulk density, k and n are constants generated from mathematical modelling of isotherm data using the Freundlich model $q=K_fC^{(1/n)}$, tau = $1/\beta$, d = diameter of column = 1cm, c0 = initial concentration of influent metal ion, l = length of column, v = volume of column