METAL BIOACCUMULATION AND PRECIOUS METAL REFINERY WASTEWATER TREATMENT BY *PHOMA GLOMERATA*

THESIS

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ABSTRACT

The biosorption of copper, nickel, gold and platinum from single metal aqueous solutions by the nickel hyperaccumulator *Berkheya coddii* plant biomass was investigated. Potentiometric titrations of the biomass and determination of optimal sorption pH for each metal showed that nickel ions were released from the biomass into solution. The presence of free nickel ions interfered with the uptake of the other three metals and further biosorption investigations were discontinued. Three fungal isolates found colonising metal solutions were cultured and screened for their ability to remove 50 $mg.l^{-1}$ of copper, nickel, gold and platinum from solution and to survive and grow in precious metal refinery wastewaters. One isolate was selected for further studies based on its superior metal uptake capabilities (35 and 39 $mg.l^{-1}$ of gold and platinum, respectively) and was identified as *Phoma glomerata*. Copper, nickel, gold and platinum uptake studies revealed that nickel and gold were the most toxic metal ions, however, toxicity was dependent on pH. At pH 6 more biomass growth was achieved than at lower pH values and metal uptake increased by 51 and 17 % for copper and nickel, respectively. In addition, the production of extracellular polymeric substances played a role in base metal interaction. Precious metals were observed to be preferentially removed from solution, complete removal of gold and platinum was observed at all initial pH values, 89 % of copper was bioaccumulated at an initial metal concentration of 55 $mg.l^{-1}$ (pH 6) and only 23 % of nickel was removed from solution under the same conditions. Metal bioaccumulation was confirmed through transmission electron microscopy and micro particle induced X-ray emission. The effect of *P. glomerata* immobilised in a packed bed reactor on precious metal refinery wastewaters was investigated. It was found that the fungal isolate was not able to remove the high salt and chemical oxygen demand concentrations found in the wastewaters, however due to its ability to survive and grow in undiluted wastewater and remove metal ions from solution it may be utilised as a metal detoxification step in the treatment process train.

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LIST OF ABBREVIATIONS

In alphabetical order:

AAS	Atomic absorption spectrophotometer
AFS	Atomic flame spectrophotometer
ATP	Adenosine triphosphate
BLAST	Basic local alignment and search tool
BS	Back scattering
BSA	Bovine serum albumin
C_{acc}	Accumulated metal
COD	Chemical oxygen demand
C_{rem}	Remaining metal
Cys	Cysteine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleic triphosphates
DWAF	Department of Water Affairs and Forestry
EDTA	Ethylenediamine tetraacetic acid
EPS	Extracellular polymeric substances
FTIR	Fourier transform infrared
g	Gravity
Glu	Glutamic acid
Gly	Glycine
HCl	Hydrochloric acid
HRT	Hydraulic retention time
ICP-AES	Inductively coupled plasma atomic emission spectrophotometer
ITS	Internal spacer sequence
MeV	Mega electron volts
Micro-PIXE	Micro particle induced X-ray emission
MLSS	Mixed liquor suspended solids
NCBI	National Center for Biotechnology Information
OUR	Oxygen uptake rate
PCR	Polymerase chain reaction
q_m	Specific uptake
RA	Relative activity
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecylsulphate
SEM	Scanning electron microscopy
SOU	Specific oxygen uptake
Sp.	Species
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
Tris HCl	Tris hydrochloric acid
UV	Ultraviolet
V	Volts
WDCS	Waste Discharge Charge System
X_m	Dried cell mass

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CHAPTER 1

INTRODUCTION

1.1 Problem statement

Anglo Platinum precious metal refining generates approximately 18000 m^3 of wastewater on a daily basis. These wastewaters originate from two processes, and form an acidic and a caustic stream. Each stream has distinct characteristics as a result of the different processes in which the wastewaters are produced. The acidic stream contains fewer salts than the caustic stream and very low organic compound concentrations. The caustic stream contains the bulk of the recalcitrant compounds utilised during the refining process and high organic compound concentrations due to precious metal solvent extraction. The wastewaters are stored in large reservoirs, known as dams, to be atmospherically evaporated, however, the high salt and organic compound concentrations have impeded the evaporation and as a result the volume of untreated wastewater is increasing.

In addition to the environmental concerns regarding the accumulation of recalcitrant wastewater, both streams contain base and precious metals which have financial value and may be recovered. The environmental impact, in conjunction with the potential economic gain from recycling the wastewater has prompted Anglo Platinum to investigate suitable wastewater treatment strategies.

1.2 Synopsis

The foremost biological systems for metal recovery from solution include biosorption and bioaccumulation. Biosorption involves the sequestering of metal ions from solution by a non-viable biomass sorbent through chemical, physical and electrostatic interactions with the metal ion and sorbent surface. Bioaccumulation is defined as metal removal due to active uptake across the cell membrane by a living organism and metal ion interactions with the cell surface (biosorption).

Each process will be investigated with the plant biomass of the hyperaccumulator *Berkheya coddii* and the fungal plant pathogen *Phoma glomerata* to determine which is more suitable for the recovery of copper, nickel, gold and platinum from solution and to establish under which conditions maximum recovery is achieved. In addition, the toxic effects of each metal ion on *P. glomerata* will be determined based on inhibition of respiration, biomass growth and production of extracellular polymeric carbohydrates in the presence of the metal ions. Metal uptake will be confirmed through the use of qualitative techniques such as transmission electron microscopy (TEM) and micro particle induced X-ray emission (micro-PIXE).

The effect of *P. glomerata* on the two wastewaters in a reactor system will be investigated to determine if the fungal isolate can suitably survive and grow and alter the wastewaters for reuse elsewhere in the precious metal refinery.

CHAPTER 2

LITERATURE REVIEW

2.1 Water in South Africa

2.1.1 Water management

South Africa is a semi arid country with an annual average rainfall of 450 mm, only half of the global average (860 mm per year) and is considered water stressed (Otieno and Ochieng, 2004). Only 8.6 % of the rainfall in South Africa is available as surface water and the mean annual run off estimated at 50 million cubic metres per annum. In addition, rainfall is inconsistent over time and is distributed unevenly throughout the country with 80 % of the run off being localised to the eastern seaboard and very little available to the western areas. Similarly, when compared to world averages, South Africa's groundwater resources are relatively limited (Walmsley *et al.*, 1999). High variation of precipitation, evaporation and run off during a single year period exacerbates management challenges of both surface and ground water systems (Schulze, 2005).

South African priorities and approaches to water resource management have evolved significantly in the last fifteen of years. Prior to 1994, water resource management focused on development of water resources, such as construction of dams, irrigation schemes and inter-basin transfers (for agricultural, urban and mining sectors). Post 1994, political focus shifted and social equity became a priority. These changes became the foundation for the reformation of policies regarding water resources and water services and culminated in the development and implementation of the Water Services Act (1997) (WSA) and the National Water Act (1998) (NWA) (Ashton *et al.*, 2005).

Some of the main objectives of the WSA highlight that a basic water supply and basic sanitation is a right of every individual, that standardised tariffs for water usage must be put in place and that effective resource management and conservation of water needs to be undertaken. The latter includes the industrial use of water and the Act states that industrial effluent may not be disposed of in any manner other than that approved by the water services provider (WSA: Republic of South Africa, 1997). While the WSA is concerned with the socio-economic use and distribution of water, the NWA recognises that ecologically, South African water resources occur in different forms of the hydrological cycle and that each component needs to be suitably managed and protected in order to achieve sustainable use of water for all users, including the industrial sector (NWA: Republic of South Africa, 1998). In conjunction these two Acts provide the guidelines required for good governance and sustainable use of water and its conservation. It is rapidly becoming essential to follow these guidelines as accountability in industry with regard to water usage and effluent

disposal has increased substantially in the past few decades (Zbontar and Glavic, 2000; Ashton *et al.*, 2005).

2.1.2 Wastewaters in industry

Due to spatial variations in water resources and the scarcity of water throughout the country, in many areas demand for water exceeds the supply, a situation likely to worsen with time. Lack of water availability is aggravated by the pollution of surface and groundwater by industrial wastewaters, sewage, acid mine drainage and agricultural runoff. In addition, water requirements of mining and industrial activities have been projected to increase by 111.5 % between 1996 and 2030 (Walmsley *et al.*, 1999). Combined, these factors leave no question as to the importance of the development of effective water resource management and reuse.

Waste management principles have been established by the Department of Water Affairs and Forestry (DWAF) which have far-reaching implications for the industrial sector. The waste producer has a legal obligation to ensure that all wastes generated are handled and disposed of in a legal and environmentally conscious manner. The fundamental principles involved in this process as outlined by Brice *et al.* (2006) include the following:

Duty of Care Principle: The organisation which produces the waste owes society acknowledgement of and responsibility for the fate of the waste in terms of handling, transportation and disposal, according to legislative and environmental guidelines.

Polluter Pays Principle: The waste producer is accountable for any costs involved in remediation and rehabilitating the effects of the waste on the environment.

Precautionary Principle: The producer assumes all waste is hazardous and toxic until proved otherwise.

Sustainable Development Principle: All relevant aspects are considered including avoidance of waste production, minimisation and reuse/recycling where possible; and lastly disposal in a legally and environmentally responsible manner.

Based on these waste management principles (particularly the Sustainable Development Principle) a waste hierarchy (Figure 2.1) may be developed for the production and management of wastes. The most favoured scenario is the complete avoidance of waste production, after which waste minimisation may be undertaken; this involves reduction in the volume of waste produced. The third option is reuse, recycling and energy recovery, either in-house or externally, followed by waste treatment to reduce the volume and hazardous nature of the waste, and finally waste disposal in an environmentally safe manner (Brice *et al.*, 2006).

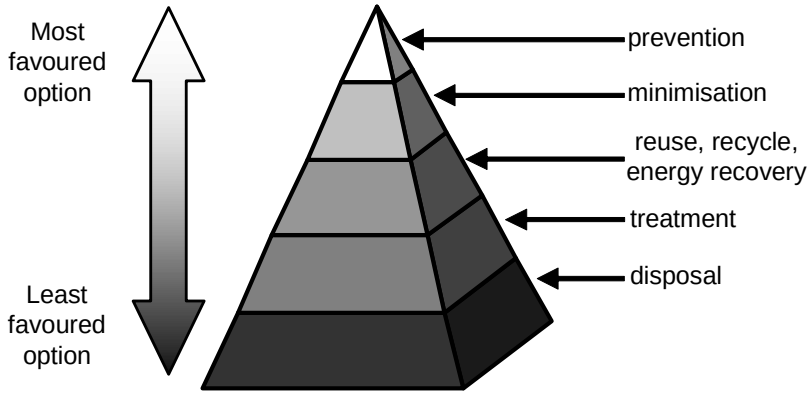

Figure 2.1 Schematic representation of the industrial wastewater hierarchy for water use and management of resources. Adapted from Brice *et al.* (2006).

Industry is strongly encouraged to adopt the Sustainable Development Principle in an effort to curb further unnecessary pollution and exploitation of water resources, particularly potable water, and to maintain social awareness and environmentally conscious use of natural water resources.

Industrial waste management principles will soon be enforced by DWAF through the development of the Waste Discharge Charge System (WDCS). The WDCS is based on the polluter pays principle outlined above and strives to "promote the sustainable development and efficient use of water resources, promote the internalisation of environmental costs by impactors, recover some of the cost of managing water quality and create financial incentives for dischargers to reuse water and use water resources in a more optimal way" (Republic of South Africa, Department of Water Affairs and Forestry, 2003). Certain pollutants will be selected and methods for measuring them will be assessed, currently total dissolved solids, total nitrogen and total orthophosphate/phosphorus have been proposed as indicators for pollution. The polluter will then be charged a fee based on the concentration of each compound in the wastewater and the volume of wastewater released.

Of the potential water pollutants produced in industrial processes, heavy metal contamination is probably one of the most serious (Volesky, 2001). Metal ions are renowned for their mobility in liquid environments within the ecosystem and their toxic effects at low concentrations. Their recalcitrant nature renders them persistent in the environment and results in accumulation of metals in water, sediments and living systems (Al-Qodah, 2006; Malkoc, 2006). Due to our position in the food chain, the pollution by heavy metals has severe implications on human health as we are exposed to pre-concentrated sources of metal ions in the foods which we eat (Volesky, 2001).

Industries such as metal plating and metal and mineral processing generate large volumes of metal-contaminated wastewater and sludge (Ahluwalia and Goyal, 2007) and are responsible for the majority of metal pollution (Zafar *et al.*, 2007). Between 1987 and 1993, almost 12.25 million kilograms of nickel were released into the environment due to these industrial processes (Magyarosy, *et al.*, 2002). Treating metal pollution once it has entered the ecosystem is a difficult and time consuming process, furthermore, the toxic effect of the metals will be experienced by the environment as the metals become mobilised. Metal ion removal is better achieved at the source of the emission, before contamination of the environment can occur (Volesky, 2001).

In addition to the toxic effects metal contamination may have on the environment, many metals have considerable commercial value and industries involved in production of these metal materials aim to recover as much metal from their waste by-products as possible (Malik, 2004).

2.1.3 Traditional metal recovery techniques

Conventional technologies utilised for metal recovery from wastewater include ion exchange, chemical precipitation, oxidation and reduction, evaporation and membrane filtration, amongst others (Malik, 2004; Ahluwalia and Goyal, 2007), the principles, advantages and disadvantages of which are outlined in the following sections.

Ion exchange

Ion exchange is the process whereby one charged particle is displaced from its position on a molecule by another ion (Ebbing and Gammon, 2002). The predominant ion exchange resins are synthetically produced fixed ionic groups on a hydrocarbon backbone. This allows them to be tailored in terms of chemical and mechanical resistance, exchange capacity and application (Gomes *et al.*, 2001). An ion from solution displaces a similarly charged ion on the resin surface. Resins may be cationic exchangers, where the exchanging ions are positively charged, or anionic, where the ions are negatively charged (Ahluwalia and Goyal, 2007). As more charged particles come into contact with the resin, it becomes saturated and the exchanger capacity diminishes. Including a regeneration step displaces the bound ions with those from a strong solution of exchangeable active ions. The ion exchange process is affected by the concentration of competing ionic species, the concentration of contaminants, interfering organic or inorganic compounds and the presence of dissolved and suspended solids. The combination of these factors imposes a life span on the number of saturation-regeneration cycles the resin can withstand and eventually the resin can no longer be used (Gomes *et al.*, 2001).

Complete removal of copper, nickel, chromium and cadmium from electroplating wastewaters has been reported with the use of synthetic zeolites (Álvarez-Ayuso *et al.*, 2003). The authors

demonstrated that synthetic zeolite had a greater uptake efficiency than natural zeolite (clinoptilolite). Total chromium removal from solution by the resins Amberlite IR-120 and IRN77 were also reported (Sapari *et al.*, 1996; Rengaraj *et al.*, 2001). In addition, Ali and Bishtawi (1997), Keane (1998) and Juang *et al.* (2003) all confirmed that the adsorption capacity of the resin was dependant on the complex agent used and the pH. Recovery of precious metals involves ion exchange with liquid solvents. Gold and platinum undergo solvent extraction with methyl-*i*-butylketone and amine respectively (Cole *et al.*, 2006).

Chemical precipitation

Precipitation is probably the most widely used technique for the removal of metal from solution. The pH of the solution is adjusted to above 10 (Kurniawan *et al.*, 2006) and hydroxides, sulphites, carbonates and carbamates are added to solution and react with the metal ions to form insoluble complexes which can then be recovered (Mauchauffée and Meux, 2007). Lee *et al.* (2007) reported removal efficiencies of zinc and cadmium to be 95 and 93 % respectively from contaminated ground water by precipitation with lime and calcium carbonate. Lim *et al*. (2005) utilised ethylene diaminetetraacetic acid (EDTA) to extract cadmium from metal contaminated soil for further recovery, similarly, the mobilisation of cadmium and zinc with addition of EDTA was reported by Lai and Chen (2006).

Oxidation and reduction

Electrochemical recovery of metals involves a charge transfer between the electrically conductive metal and the ionic conductor resulting in the deposition of the metal ion at the anode or cathode (Chen and Lim, 2005). Electrochemical treatments may include electrodialysis, membrane electrolysis and electrochemical precipitation (Kurniawan *et al.*, 2006). A number of authors have shown almost complete removal of metal ions such as nickel, chromium, copper and cadmium from wastewaters through oxidation and reduction of the metal ions (Orhan *et al.*, 2002; Tzanetakis *et al.*, 2003; Rana *et al.*, 2004)

Evaporation

The use of atmospheric evaporation is mainly employed in industrial settings to form a metal concentrated sludge. Large amounts of space are required for this process, making it a viable option only where land is available; in addition, wastewater reservoirs or dams are required to be sealed in order to prevent ground water contamination. The evaporation may be accelerated with the use of industrial vacuums which vaporise water at low temperatures; however this requires the use of costly machinery (Cowan, 1998).

Membrane filtration

There are three main membrane filtration techniques for the removal of metal ions from solution: reverse osmosis, ultrafiltration and nanofiltration. Reverse osmosis involves the pressure driven passage of water through the membrane, while the metals are retained (Kurniawan *et al.*, 2006). Reverse osmosis membranes have a configuration defined as a thin film composite with a polyamine active layer and pore sizes as small as 10^{-4} µm have been successfully used in water purification (Kurniawan, 2006; Sagne *et al.*, 2008). Almost complete removal of copper and nickel with ultra-low-pressure reverse osmosis was reported by Ozaki *et al.* (2002). Similarly, 97 % of nickel was removed with the use of a polyamide membrane for reverse osmosis (Quin *et al.*, 2002).

Ultrafiltration makes use of a membrane to separate metals and other molecules from solution based on the pore size (5–20 nm) of the membrane and the molecular weight of the compounds. Membrane materials include cellulose acetate, silica and alumina and polyamide (Kurniawan, 2006). Juang and Shiau (2000) found that enhancing the membrane with chitosan significantly increased the removal of copper and zinc, most likely due to the amino groups on the chitosan molecule. These results were supported by Yurlova *et al.* (2002) who achieved compete removal of nickel with the use of micellar-enhanced ultrafiltration.

The properties of nanofiltration membranes include steric and electrical mechanisms. The small pores (10^{-8} to 10^{-9} m) and surface charge of the membrane allow for charged solutes smaller than the membrane pores to be separated with the larger neutral solutes (Kurniawan *et al.*, 2006). Nanofiltration used to be called Leaky Reverse Osmosis and achieves separation through a combination of mechanisms: sieving, solubility diffusion and charge rejection (Stephenson *et al*., 2000). Qdais and Moussa (2004) achieved 90 and 97 % removal of copper and cadmium from solution with the use of nanofiltration, however, the authors achieved improved removal of the same metals with the use of reverse osmosis. Ahn *et al.* (1999) demonstrated that the removal of metals from solution through nanofiltration was dependent on the initial metal concentration and the pressure applied across the membrane. Although nanofiltration can treat solutions with metal concentrations up to 2000 $mg.l^{-1}$, and a pH range of 3 – 8 and pressure of 3 – 4 bar, ultrafiltration and reverse osmosis are still more intensely investigated for application in metal removal (Kurniawan *et al.*, 2006).

In most cases these conventional metal recovery process are ineffective in removing metal ions in the concentration range between 1 and 100 $mg.l^{-1}$ and each is associated with significant drawbacks (Table 2.1) (Zafar *et al.*, 2007).

The techniques described in Table 2.1 do have advantages. Ion exchange, membrane technologies and evaporation may generate pure effluent which can then be reused, chemical oxidation or reduction results in mineralisation of the metal ions and precipitation is a simple and cheap process (Volesky, 2001). However, in most cases the disadvantages outweigh the advantages and in light of this, biological materials have become an economic and environmentally friendly option for the recovery of metal ions from solution (Ahluwalia and Goyal, 2007).

Table 2.1 Conventional metal recovery technologies and their associated disadvantages (Volesky, 2001; Swalaha *et al.*, 2002; Hanif *et al.*, 2007).

Recovery process	Drawback
Ion exchange	expensive, sensitive to particles
Precipitation	difficult metal separation, sludge production
Evaporation	expensive, energy intensive, sludge production
Oxidation/reduction	additional chemicals required, climate sensitive
Membranes	expensive, high pressure, fouling

2.2 Biological metal recovery

2.2.1 Biosorption

Biosorption can be broadly defined as the sequestering of metal ions from solution by biological material. The biosorbent may be living or dead biomass or in some cases cellular products such as polysaccharides (Wase and Forster, 1997).

Binding of a specific metal is dependent on its properties such as ionisation potential, redox potential, electronegativity, ionic radius and whether it is classified as a hard or soft acid or base, according to Pearson's Hard and Soft Acid Base Theory (Ebbing and Gammon, 2002) . Hard acids (SO_3, Na^+, K^+) interact more readily with hard bases (OH^-, Cl^-, PO_4^{3-}) as both keep hold of their electrons tightly and primarily form electrostatic interactions. Soft acids (Au^+, Pt^{2+}, Cd^{2+}) and bases (CO, $S_2O_3^{2-}$, H^-) do not hold electrons as tightly and can easily form covalent bonds. The degree of hardness or softness is based on the concept that a stronger acid/base will displace a weaker acid/base from its compounds. Therefore, identifying a particular metal as a hard or soft acid/base is a relative term and a number of cations and anions are considered borderline (Ni^{2+}, Cu^{2+}, $:NO_2^-$, $C_5H_5^-$) (Douglas *et al.*, 1994; Ebbing and Gammon, 2002). In addition, the charge and ionic radius also impact on the binding of the acids and bases (Douglas *et al.*, 1994). The more electronegative and the smaller the ionic radius of a metal, the faster it is sorbed onto the biomass surface, hence in most cases metals such as zinc and lead bind more readily than copper (Singh *et al.*, 2000).

A variety of ligands are involved in metal binding on the cell surface, these include amine, carboxyl, sulphydryl, phosphate and hydroxyl groups (Modak and Natarajan, 1995). In order to gain knowledge as to whether a particular biomass has the potential to be utilised as a biosorbent for metal recovery, it is important to know which functional groups are available for metal ion binding, the number of possible sites and the effect environmental changes, such as pH and temperature, may have on these groups (Naja *et al.*, 2005).

Factors influencing biosorption

Biosorption of metal ions in solution is dependant on many external factors, each of which will have a unique consequence on the rate of metal uptake.

The effects of temperature depend on whether the overall biosorption process is exothermic or endothermic in nature, as this will influence the equilibrium capacity of the biomass. In addition, an increase in temperature will result in a decrease in the viscosity of the liquid and increase the rate of diffusion of metal ions to the biomass surface (Al-Qodah, 2006). The nature of biological material should also be considered, as at high temperatures cellular structures may be permanently damaged and a decrease in biosorption may be observed above a certain threshold (Modak and Natarajan, 1995) in opposition to the general trend of higher temperatures promoting more rapid sorption in the case of endothermic reactions.

The response of the system to different temperatures may also give an indication as to which biosorption process is taking place. Malkoc (2006) demonstrated that nickel binding by the cones of the Ce Bai tree, *Platyciadus* (formerly *Thuja*) *orientalis*, was an endothermic reaction, as removal of metal ions from solution increased with increasing temperature (a possible ion exchange mechanism), while Öztürk *et al.* (2004) showed that binding of nickel by *Streptomyces coelicolor* A3(2) was an exothermic reaction, as maximum metal removal occurred at 25 ℃ after which uptake declined rapidly, indicating a physical adsorption mechanism. Al-Qodah (2006) proved that as temperature increased, the desorption rate of cadmium from activated sludge increased more significantly than the adsorption rate, resulting in a net reduction in adsorption capacity of the biomass, suggesting the possibility of physical adsorption predominating. Temperature did not affect the binding of cadmium to the microalga, *Chlamydomonas reinhardtii*, immobilised in alginate beads between 5 and 40 ℃, instead the mechanism of biosorption was dependent on the pH of the system (Bayramoğlu *et al.*, 2006).

The pH affects both the metal-binding sites on the biomass and the chemistry of the metal in solution. A general biosorption trend across all biomass and cationic metals can be observed at each pH. At pH values below 2 metal uptake is impeded, most likely due to the competition for

binding sites between the metal cations and excess protons in solution. Metal uptake will improve with increasing alkalinity, since more binding sites with negative charges become exposed with the increase. This is due to ligands on the biomass surface becoming deprotonated at pH values higher than their isoelectric point (Modak and Natarajan, 1995; Akar and Tunali, 2006). Metal sorption will reach a maximum, after which binding will begin to decrease. The reduction is due to metal behaviour in solution: as an increase in alkalinity increases the concentration of OH^- ions in solution, metal solubility is reduced and precipitation occurs (Modak and Natarajan, 1995; Akar and Tunali, 2006).

Anionic metals behave similarly. At low pH functional groups on the biomass are protonated, allowing for metal binding. However, at a pH lower than 2, the increase in chloride ions present due to pH adjustment compete for positive binding sites and lower metal ion uptake. In addition, metal solution chemistry is paramount to metal sorption, as the net charge of the ion is dependent on pH. The more electrons the molecule possesses, the larger it becomes and the number of functional groups able to accommodate the molecule are reduced (Niu and Volesky, 2003).

It has been shown that increased metal removal occurs if the metal to biomass ratio remains high. At low metal concentrations, the number of available binding sites on the biomass is high, and hence the removal capacity is greater than when the metal is more concentrated. When the metal concentration is increased, there is greater competition for binding sites and they rapidly become saturated, leaving metal ions in solution. Increased initial metal concentrations have been demonstrated to reduce the maximum biosorption capacities for single metal solutions of lead (Sekhar *et al.*, 2003; Akar and Tunali, 2006), nickel (Selatnia *et al.*, 2004; Malkoc, 2006) and copper (Pamukoglu and Kargi, 2007; Dahiya *et al.*, 2008).

Increasing the biomass concentration increases the number of available binding sites. However, it has been found that increasing biomass concentrations to a great extent impedes biosorption. The outer layer of cells may act as a screen, preventing the metal ions from reaching potential binding sites. A fine balance between biomass density and initial metal dosing needs to be found in order to achieve maximum metal removal (Selatnia *et al.*, 2004).

Nitrate salts have been commonly utilised in biosorption studies due to their high solubility and low affinity to readily form complexes with metals. This provides an inert solution in which all metals are in free form; owing to this, the effect of counterions is limited in the results obtained (Diniz and Volesky, 2005). In addition, most biosorbent studies are carried out with single or double metal ion species in aqueous solutions. Very few studies mimic the industrial wastewaters they are intended to treat and when they do, metal removal efficiency is greatly reduced (Malik, 2004).

Industrial wastewaters are usually comprised of a complex mixture of metals and other ions, which will influence the sorption of a specific metal. Most functional groups on the biomass bind cations non-specifically and metal uptake in mixed solutions is usually greatly reduced when compared to a single-species solution due to the competition for binding sites (Modak and Natarajan, 1995).

The presence of anions has a considerable effect on the biosorption efficiency of the metal in solution. The resulting metal complexes may be only weakly adsorbing or completely nonadsorbing. Pulsawat *et al.* (2003) found that the presence of anions reduced the removal of manganese from aqueous solution with decreasing availability of the metal when bound to the following; SO_4^{2-}, NO_3^- and Cl^-. Diniz and Volesky (2005) later proved that the binding of lanthanum by the marine alga, *Sargassum polycystum*, was affected similarly by nitrate and chloride salts, but that sulphate salts inhibited the uptake of metal ions greatly. These results were reiterated when testing the effect of the acid used for pH adjustment.

A number of pre-treatment methods enhance metal ion uptake, these include alkali, acid and heat treatments. Alkali treatment of the biomass solubilises lipids and proteins which may be masking additional binding sites. Treatment with acid is much more variable, depending on the metal and biomass being investigated, in some cases there is a decrease in metal binding, and other, no effect, or a slight increase. Heat treatment is usually carried out in conjunction with alkali or acid treatment (Akar and Tunali, 2006; Tunali and Akar, 2006).

Immobilisation of the biomass to form a particulate or granular structure can substantially improve the physical integrity of the biomass (Volesky, 2003). Immobilisation techniques include flocculation, encapsulation in polymer gels, entrapment in polymer matrices and covalent bonding to carriers (Kiran *et al.,* 2006). Zhang and Banks (2006) successfully immobilised maize, seaweed, sunflower waste and *Sphagnum* moss in polyurethane through encapsulation for the removal of nickel, lead, copper and zinc in continuous flow packed columns. The authors showed that during initial stages of column operation, all metals bound to the immobilised biomass, after which metals with lower affinity were progressively displaced, creating a chromatographic concentrating effect. Iqbal and Saeed (2006) entrapped fungal hyphae in the structural fibrous network of papaya wood. They showed that immobilised fungi had an increased uptake of zinc of > 40 %. In addition to enhancing biomass integrity, immobilisation provides an economical mechanism for the separation of biomass from the process waters following treatment (Wase and Forster, 1997).

Mechanisms of biosorption

In the case of living biomass, biosorption of metals may be metabolism-dependent, or metabolism-independent, while dead biomass can only bind metal independently of metabolism. Metabolism-independent binding usually occurs in a number of ways. The first, ion exchange, occurs on the polysaccharide components of cell walls by swapping bivalent metal ions with counterions and is the dominant form of metal-biomass interaction (Schneider *et al.*, 2001; Pradhan *et al.*, 2007). Counterions involved in ion exchange can include calcium, magnesium, sodium, potassium and zinc, with a large portion of the reaction occurring with H^+ ions. Due to the release of protons into solution a drop in pH can be observed after biosorption, this pH change indicates that ion exchange is the predominant biosorption mechanism taking place (Chojnacka *et al.*, 2005). Through infra-red spectroscopy Pradhan *et al.*, (2007), confirmed that iron biosorption by *Microcystis* was an ion exchange interaction with carboxyl groups on the cell surface, while amino groups were responsible for the biosorption of nickel in a mono- and multi-metal layer system. The functional groups responsible for ion exchange biosorption may also be identified by blocking each group and monitoring the effect on biosorption. Chojnacka *et al.* (2005), reported that chromium biosorption was reduced significantly when phosphate and carboxyl groups on a *Spirulina* sp. were esterified, while the change in biosorption of methylated hydroxyl and amine groups was insignificant, demonstrating precisely which groups were responsible for metal binding.

The second binding mechanism is through complexation and chelation of metals in solution by active groups on the cell surface (Veglio and Beolchini, 1997). The biosorption mechanism of chromium by the alga, *Chlorella miniata*, was shown to be predominately complexation with phosphate, amine and carboxyl groups involved in the reaction, the latter being responsible for biosorption and the formation of an acid insoluble complex (Han *et al.*, 2006). The authors also demonstrated that nickel bound specifically to the carboxyl groups on the same biomass, but through an ion exchange mechanism rather than complexation.

Precipitation of the metal may occur and this process may be metabolism-dependent or independent in the case of living biomass (Veglio and Beolchini, 1997). Precipitation by living biomass is most likely to be a consequence of a defensive mechanism against metal toxicity (Malik, 2004). The organism may produce compounds that promote precipitation, while metabolism-independent precipitation may be attributed to a chemical interaction between the cell surface and the metal ion. Surface precipitation of metal ions from solution occurs at a slower rate than ion exchange or chelation and in most cases takes place once a monolayer of metal ions has bound to the biomass surface.

Although the metal ion concentration in solution is much less than its solubility limit, if the net charge on the biomass is negative, this limit is exceeded near the sorption surface and precipitation takes place (Scheider *et al.*, 2001). In addition to this mode of precipitation, compounds released from the biomass during sorption may bind directly to metal ions and cause precipitation. Cho and Kim (2003), verified that in addition to lead binding to the yeast *Rhodotorula glutinis* through ion exchange, phosphate released from the biomass caused precipitation of the metal and accounted for the majority of biosorptive action.

Lastly, physical adsorption of the metal species to the biomass surface occurs through weak ionic forces and electrostatic interactions of the metal in solution with the cell surface, but contributes only slightly to the overall biosorption capacity of the biomass (Veglio and Beolchini, 1997). Chojnacka *et al.* (2005) proved conclusively that physical adsorption of chromium by *Spirulina* sp. accounted for < 4 % of the total biosorption capacity of the cells. If physical adsorption was predominant, the ratio of biomass surface area to bound metal ion should be larger than that found experimentally, clearly indicating that ion exchange, precipitation and chelation mechanisms are dominant in biosorption (Chojnacka *et al.*, 2005).

The mechanisms of ion exchange, chelation, precipitation and physical adsoption do not exist autonomously, biosorption is a specific combination of these binding processes and is completely dependent on the biosorbent, metal ion and conditions under which the reaction occurs (Veglio and Beolchini 1997).

Advantages and disadvantages of biosorption

Perhaps the main advantage of biosorption of metals is the use of non-living biomass which is unaffected by the toxicity limits experienced by living systems. Operating parameters such as pH, metal concentration and temperature are not limited by toxicity thresholds of living cells. There are no costly nutrient regimes in place and hence disposal requirements of excess metabolic material do not exist.

Due to the nature of the biosorption mechanisms the process is very rapid, within minutes to an hour the majority of metal ions in solution will be sorbed onto the biomass surface and in some cases, the ions may be desorbed and recovered allowing for regeneration of the biomass. In addition, waste biomass from existing agricultural or fermentation industries may be used as a biosorbent (Modak and Natarajan, 1995; Ahluwalia and Goyal, 2007).

The most prominent disadvantage of biosorption includes early saturation of the biomass, from which metal ions must to be desorbed before further removal can take place. The metal valence

state and chemistry cannot be altered as it can be in a living biological system. Metals cannot be altered to become less soluble, or extracted from complex organometallic molecules, which is more likely to be the norm for industrial wastewaters, rather than a single metal in aqueous solution. And lastly there is no opportunity for process development, genetic improvement of biomass or control over the biosorbent characteristics, particularly if waste biomass is to be used (Malik, 2004; Ahluwalia and Goyal, 2007).

Performance with real wastewater

Biosorption has yet to prove its mettle with regard to the treatment of industrial wastewaters. Outstanding results have been obtained with single and dual metals in aqueous solutions, but there is little evidence to support successful removal of metal ions from real wastewater (Malik 2004). Biosorption may in time become a feasible option for heavy metal treatment, but while it has yet to demonstrate its efficacy, other treatment technologies, such as bioaccumulation, cannot be ignored.

2.2.2 Bioaccumulation

Bioaccumulation is a process which involves two aspects; active metal uptake and passive metal uptake (biosorption) and may be carried out by any living organism with the ability to withstand the toxic effects of a particular metal ion (Malik, 2004). The interactions between toxic metals and fungi have been of particular scientific interest as metals form the basis of fungicides used to control plant pathogens (Gadd, 1993). Observations that fungi were able to resist toxic metals and adapt to their presence in the environment sparked interest with regard to fungal bioremediation of metal contaminated soils and wastewaters. As a result, a number of researchers have investigated the potential of various fungi to remove heavy metals from the environment through bioaccumulation. The study of metal uptake by microorganisms is not a new field of research, however. Recently there has been a considerable increase in interest in bioaccumulation, particularly with regard to biotechnological potential for metal recovery and remediation (López Errasquín and Vázquez, 2003).

Aspergillus niger has been shown to accumulate metals such as copper, nickel, lead and chromium. Dursun *et al.* (2003) reported specific uptake values for copper and lead with initial metal ion concentrations of 100 $mg.l^{-1}$ to be 15.6 and 34.4 $mg.g^{-1}$ respectively, these values corresponded to uptake efficiencies of 19.5 % for copper and 55.1 % for lead. The chromium ions proved to be slightly more toxic to the fungus and the highest specific uptake was 6.6 $mg.g^{-1}$, recorded at an initial concentration of 50 $mg.l^{-1}$. The accumulation of nickel by *A. niger* was also investigated by Magyarosy *et al.* (2002) who reported 99 % nickel removal from an initial concentration of 6.5 mM (± 380 $mg.l^{-1}$) within 120 hours; however when the removal of copper and

cadmium was assessed, it was found that no significant copper or cadmium removal was achieved. This can most likely be attributed to the toxicity of copper and cadmium at these concentrations and the fact that the *A. niger* used in the latter instance was isolated from a nickel rich environment and selected specifically for its nickel tolerance (Magyarosy *et al.*, 2002).
Other biomass investigated for their bioaccumulative properties include *Rhizopus arrhizus* (Preetha and Viruthagiri, 2007). The authors reported successful removal of chromium from solution, while López Errasquín and Vázquez (2003) investigated the removal of copper, zinc and cadmium from solution by *Trichoderma atroviride*. The authors reported that components of the media had a direct effect on the ability of the fungal isolate to remove metal ions from solution.

Factors influencing bioaccumulation
Since bioaccumulation occurs within living systems, there are a number of environmental factors which will affect the ability of the microorganism to remove metal ions from solution.

The pH of the solution affects metal uptake in two ways. Firstly, the pH of the environment needs to be optimal for biomass growth. Optimal fungal growth generally occurs in slightly acidic media, however this may vary between species and even strains (Rai, 2000). If more biomass is produced, higher concentrations of metal ions may be removed from solution. Secondly, as mentioned in Section 2.2.1, the pH affects the solution chemistry of the metal ions, and therefore their interactions with the fungal cells. Cations such as copper and nickel exist in solution as free ions (Lee, 1991), however if the pH is increased, cation hydroxides may be formed which could precipitate if the hydroxide concentration increased enough (Volesky, 2003). Once precipitated, the cations would have to be mobilised from the hydroxide groups in order to facilitate removal from solution (Gadd, 1993). However, while the metals are precipitated, they are no longer bioavailable and the biomass has the opportunity to establish itself in the medium before uptake occurs. Anionic metal species such as gold and platinum chloride are affected similarly. As the pH increases, the chloride ions are displaced by hydroxide ions and the metal complex becomes increasingly stable, finally producing a precipitate (Nakajima, 2003).

Dönmez and Aksu (1999) assessed a variety of yeast species for their ability to bioaccumulate copper. The authors reported maximum growth and specific uptake values of *Saccharomyces cerevisiae* and a *Candida* species to occur at pH 4.0, in contrast when assessing the growth and copper uptake by *Schizosaccharomyces pombe* the maximum growth and metal removal occurred at pH 5.0. The authors corroborated these data in a follow up paper in which the removal of copper and nickel from solution was carried out by a *Candida* species (Dönmez and Aksu, 2000). The optimum pH for fungal growth and metal uptake in the presence of both copper and nickel ions was found to be pH 4. Similarly, Dursun *et al.* (2003) found that the optimum pH for growth of *A. niger* in

the presence of copper ions to be pH 4.5, however maximum copper removal occurred at pH 5.0. In direct contrast to the data reported above, Magyarosy *et al.* (2002) reported that solution pH had no effect on the bioaccumulation of nickel ions by *A. niger*. The authors investigated metal removal in buffered media, where the pH remained constant for the duration of the analysis, and unbuffered media in which the pH was significantly reduced by extracellular components secreted by the *A. niger* biomass. They found that nickel uptake was not affected by the pH change (Magyarosy *et al.*, 2002).

In addition to the effect of the initial pH of the solution, the initial metal ion concentration plays an important role in determining the capacity of metal removal by the biomass. Since bioaccumulation occurs within a metabolising cell, if metal ion concentrations are too high, a toxic effect may be exerted on the organism (Malik, 2004). The toxicity of a particular metal ion is completely dependent on the ion itself, the particular fungal species and the surrounding environment (Gadd, 1993). López Errasquín and Vázquez (2003) determined the toxic threshold of *T. atroviride* as a function of biomass dry weight at each initial metal concentration assessed. The authors found that substantial inhibition of growth by copper and cadmium occurred at 350 and 125 $mg.l^{-1}$, respectively and at concentrations of 400 and 300 $mg.l^{-1}$ no growth was detected. In contrast, the inhibition of growth in the presence of zinc was more gradual and *T. atroviride* growth still occurred in solution with an initial concentration of 750 $mg.l^{-1}$. The toxic effect of copper on *A. niger* appeared to be more severe than that on *T. atroviride* (Dursun *et al.*, 2003). Notable inhibition of *A. niger* growth could be observed at 75 $mg.l^{-1}$ of copper, and 150 $mg.l^{-1}$ completely inhibited fungal growth. The toxic effects of lead appeared more severe in terms of total biomass production at the end of the analysis, but the fungus maintained growth at initial lead concentrations as high as 250 $mg.l^{-1}$. In contrast, 75 $mg.l^{-1}$ of chromium completely inhibited the growth of *A. niger*. Preetha and Viruthagiri (2007) also reported the toxicity of chromium. The organism used for metal bioaccumulation in this case was *R. arrhizus* and fungal growth inhibition began at a concentration of 50 $mg.l^{-1}$ and complete inhibition was observed at 75 $mg.l^{-1}$ of chromium.

Metal toxicity may be overcome through acclimatisation of the biomass to the particular metal. As mentioned previously, *A. niger* isolated from a nickel rich environment (Magyarosy *et al.*, 2002) was able to withstand much higher concentrations of nickel than any of the other organisms reported. The effect of acclimatisation was illustrated effectively by Dönmez and Aksu (2000) when they compared the copper and nickel uptake ability of non-adapted and adapted *Candida* species. The growth of the non-adapted *Candida* species at each copper and nickel concentration analysed was approximately half that of the adapted species at the same metal concentrations. In addition, higher specific uptake of copper and nickel was achieved at higher initial metal concentrations by the adapted *Candida* species than the non-adapted strain.

Fungal tolerance of metals is also determined by the type of substrate in which the organism is growing. Valix and Loon (2003) reported that acclimatised *A. niger* was able to grow on solid media with nickel concentrations as high as 2000 $mg.l^{-1}$, this metal concentration is higher than any of the others listed above in which growth inhibition was observed. In addition, the components of the medium play an important role on metal uptake. White and Gadd (1995) demonstrated that addition of glucose to the media increased the calcium uptake capacity of *Ophiostoma ulmi* substantially.

Metal bioaccumulation in living systems is a complicated process which depends on a wide variety of environmental factors, however, probably the most important factor to consider when undertaking an investigation of this nature is the specific interaction of the particular organism and the metal ion.

Advantages and disadvantages of bioaccumulation

The main advantage of using a living system to recover metal ions from the environment is the ability of the microbe to self-replenish. This results in continuous metal uptake, even after biosorption sites have been exhausted. In addition, improved metal uptake can be achieved through acclimatisation and development of metal resistant species (Malik, 2004). Metal ions which are removed from the environment by living cells are transported into the cell and immobilised by binding to proteins and other biomolecules; this is an irreversible process and ensures metal ions are not released back into the environment (Gadd, 1993). The use of a mixed microbial culture may preclude a conventional multi step approach to heavy metal contaminated wastewater treatment as growing cells have unlimited capacities to cleave organometallic complexes and degrade a range of other organic and inorganic compounds (Malik, 2004).

There are however a number of limitations to the use of living systems for metal recovery, the most important of which is sensitivity to extreme environments. Extreme pH and high salt and metal concentrations may severely inhibit the ability of the microorganism to sequester metal ions, even to survive in the harsh environment (Gadd, 1993). These issues may be circumvented through strain selection and the utilisation of multispecies consortia to stabilise the environment and withstand the extreme conditions found in wastewaters (Malik, 2004).

2.3 Fungal and metal interactions

2.3.1 Toxicity of metals

Certain heavy metals such as iron, calcium and magnesium are essential to fungal metabolism (Baldrian, 2003). Essential metals are generally classified as hard cations (Gadd, 1993). They are

small, have high polarizability and electronegativity and tend to interact in electrostatic reactions (Douglas *et al.*, 1994). In contrast, nonessential, toxic metals such as silver, cadmium and mercury are soft metals (Gadd, 1993). In high concentrations both essential and nonessential metals have the ability to induce a toxic response from the organism (Baldrian, 2003).

Microorganisms may transport metal ions across their cell membranes (flux) to control the intracellular concentration of both essential and nonessential metal ions in order to circumvent any toxic effect as a result of high environmental metal ion concentrations (Nies, 1999). A number of divalent heavy metal cations are structurally similar (Douglas *et al.*, 1994) and microorganisms need to bind these ions tightly in order to differentiate between essential and nonessential metals. However, the tight binding is an energetically expensive process for the cell (Nies, 1999). Organisms can overcome this problem through utilisation of two metal uptake systems. The first is rapid, unspecific and driven by the chemiosmotic gradient across the cytoplasm. The second is highly substrate specific and requires the use of adenosine triphosphate (ATP) hydrolysis to provide energy for the binding and transportation of the metal ion (flux). Since the second process is energetically expensive, it is most often utilised during periods of starvation or environmental stress (Nies and Silver, 1995).

Mechanisms of toxicity

The initial toxicity of metal ions is as a result of the unspecific uptake mechanism mentioned above. The transmembrane transporters responsible for the metal uptake are constitutively expressed and cannot be ‘closed’ (Nies, 1999). In addition, interactions with the cell wall may result in increased permeability and a loss of mobile cellular solutes (Gadd, 1993). Once the metal ions are inside the cytoplasm of the cell their toxicity to fungi may be experienced in a number of ways (Nies, 1999), but the underlying principle of toxicity is the strong coordinating abilities of the metal ions. Toxic metals ions have the ability to displace essential ions from biomolecules, denature essential enzymes, disrupt membrane integrity and block functional groups of important biomolecules, such as transport systems for essential nutrients and ions (Gadd, 1993).

In addition, metals exert toxicity on fungi through the formation of free radicals (Kendrick *et al.*, 1992), which have the ability to take part in chain reactions causing the breakdown of essential macromolecules. As a result, all aerobic organisms have produced specific enzymes such as superoxide dismutases as a method of protection against the free radicals produced in normal metabolism (Gadd, 1993). Superoxide dismutases are metalloenzymes containing manganese, iron or copper and zinc (Hwang *et al.*, 1999; Fréalle *et al.*, 2006).

Tolerance and resistance to metals

Two types of survival mechanisms may be defined when considering the effect of metals on fungi; tolerance and resistance. Tolerance may be considered as intrinsic properties possessed by the organism or the ability to modify the toxicity of the metal (Gadd, 1993). These mechanisms include the production of extracellular polysaccharides (Koukal *et al.*, 2007), precipitation of metals through binding to biomolecules such as organic acids (Di Palma and Mecozzi, 2007; Yuan *et al.*, 2007) or possession of thick, pigmented cell walls which may be impermeable to metal ions (Gadd, 1993). Resistance to metals may be defined as the ability of the organism to minimise metal toxicity through a metabolic mechanism stimulated as a direct response to the metal ions. The production of γ-glutamyl peptides and metallothioneins fall into this category (Reddy and Prasad, 1990; Daniele *et al.*, 1996;. Guimarães-Soares *et al.*, 2006).

A particular organism may rely on different strategies for survival of metal toxicity and it is not always possible to determine if the response to the presence of metal ions in the environment is intrinsic or acquired. This compounds the complexity of metal-microbe interactions as no two organisms will respond identically to the same metal ion and the same organism may elicit a different response to a particular ion under different environmental conditions (Nies, 1999).

2.3.2 Interactions between fungi and toxic metals

Six mechanisms of metal-microbe interactions have been postulated to overcome the toxicity of metal ions. These include: (1) extracellular precipitation, (2) metal exclusion by a permeability barrier, (3) modification of the metal to a less toxic form, (4) intracellular protein binding, (5) active transport of the metal out of the cell through efflux systems and (6) reduction in metal sensitivity (Bruins *et al.*, 2000).

Extracellular precipitation

Fungi produce a number of extracellular products with the ability to complex and precipitate heavy metals, these include organic acids such as oxalic and citric acid (Bruins *et al.*, 2000). Oxalic acid forms insoluble oxalate crystals, while citric acid is an efficient metal chelator (Gadd, 1993). Wilkolazka and Gadd (2003) demonstrated the production of oxalic acid and subsequent precipitation and immobilisation of zinc by the white rot fungi *Bjerkandera fumosa, Phlebia radiata* and *Trametes versicolor*. These results were corroborated by Sierra-Alvarez (2007) when a variety of white and brown rot fungi were screened for oxalic acid production in the presence of copper ions. The use of citric acid is well documented in the leaching of metal ions from soils for further recovery (Baldi *et al.*, 2007) and fungi producing this organic acid may mobilise metals present in the environment (Martino *et al.*, 2003). Many citrate-metal complexes are highly mobile and not

readily degraded (Gadd, 2004) and this may pose a drawback in the application of citric acid for bioremediation of metal contaminated soils and wastes.

In addition to the production of organic acids to neutralise the toxicity of metal ions, some fungi may produce low molecular weight peptides, such as glutathione (Bruins *et al.*, 2000). Glutathione has the ability to bind a wide range of compounds with reactive electrophilic centres. Guimarães-Soares *et al.* (2006) reported that *Flagellospora curta* had the ability to form extracellular copper- and zinc-glutathione complexes that were unable to cross the cell membrane.

Metal exclusion by a permeability barrier

The cell wall is the first site of cellular interaction between the fungus and the metal ion. Interactions at this level will be biosorptive, the characteristics of which have been outlined in Section 2.2.1. Fungal cell wall composition varies extensively between species, but they are predominantly made up of polysaccharide units with small amounts of proteins and lipids. The functional groups on these molecules provide ample binding sites for metal ions, preventing the entry of the ion into the cell (Gadd, 1993). In addition to cell wall binding, fungi may produce biomolecules which are capable of interacting with metals such as melanins and extracellular polysaccharides.

Melanins are formed through oxidative polymerization of phenolic compounds and are high molecular mass, dark pigments (Fogarty and Tobin, 1996). Melanised cell forms such as chlamydospores have been shown to have high metal binding capacities (Gadd and Mowll, 1985). Fungal melanins are located in the cell walls and extracellularly, however extracellular melanin may arise through the release of wall bound melanin or external synthesis (Fogarty and Tobin, 1996). Fungal melanins contain phenolic groups, carbohydrates, peptides, fatty acids and aliphatic hydrocarbons which are ideal for metal ion biosorption (Gadd, 1993). In addition to the production of wall-bound and extracellular melanin, fungi produce extracellular polymeric substances (EPS) which have a high carbohydrate concentration (Hu *et al.*, 2003). The carbohydrates within the EPS have an essential function in the transport, storage and detoxification of metal ions (Yang *et al.*, 2005). Several studies have confirmed the detoxification of metals and importance of metal interactions with EPS carbohydrates (Guibaud *et al.*, 2005; Comte *et al.*, 2006; Moon *et al.*, 2006).

Metal modification

Fungal metal transformations are not as well documented as bacterial modifications; however these processes may include redox transformations, methylation and dealkylation of the metal ion (Gadd, 1993). Most metal redox reactions have been reported in bacterial systems. Metal modification involves the reduction of the metal to a lower redox state, which alters the mobility of

the ion (mobilisation or immobilisation depending on the metal) and toxicity (Gadd, 2000; 2004). Fungal reduction of metalloids has been demonstrated in the reduction of tellurite to elemental tellurium and selenate to selenium (Gadd, 1993). During methylation, methyl groups are enzymatically transferred to the metal ion resulting in methylated metal compounds which vary in their solubility, toxicity and volatility (Gadd, 2004). While bacterial methylation of selenium, mercury and arsenic have been conclusively demonstrated (Turpeinen *et al.*, 1999; Hines *et al.*, 2006; Zhang *et al.*, 2007), there is unfortunately a lack of detail in the literature with regard to metal methylation by fungi (Gadd, 1993). Similarly, investigation of the role of fungi in dealkylation of organometal complexes has been very poorly reported and at this point the mechanism of disruption of carbon-metal bonds cannot be conclusively attributed to the action of fungi.

Intracellular fate of bioaccumulated metals

Metals which eventually find their way into the cell are invariably bound by polypeptides and metal-binding proteins. These may either be metallothioneins or γ-glutamyl peptides (Gadd, 2000). Peptides are considered metallothioneins if they have a low molecular mass, a high metal and cysteine content and lack histidine aromatic amino acids. In contrast, γ-glutamyl peptides are short metal-binding molecules with the general formula $(\gamma Glu\text{-}Cys)_n\text{-}Gly$) (Gadd, 1993). Jaeckel *et al.* (2005) reported the increased production of metallothioneins and γ-glutamyl peptides by the fungus *Heliscus lugdunensis* in response to the presence of high concentrations of cadmium. The metallothionein interactions with cadmium were corroborated by Guimarães-Soares *et al.* (2006). However, the authors found that copper and zinc were preferentially bound by the low molecular weight γ-glutamyl peptides. In addition to the binding of metal ions to polypeptides and proteins within the cell, often divalent metal ions are preferentially transported to vacuoles within the cell. Vacuoles are involved in cytosolic metal ion concentration regulation and have a pivitol role in controlling cytosolic calcium. Divalent cations are stabilised within the vacuole through binding to polyphosphate (Gadd, 1993).

Metal efflux

One of the largest metal resistance mechanisms of microbes is their ability to actively transport metal ions (Bruins *et al.*, 2000). As mentioned previously, flux systems may be ATP dependent or ATP independent (Nies, 1999) and may be classified as either a channel or a carrier system. Channels are characterised as a class of protein which behaves as a gated pore in the plasma membrane and ions flow down an electrical or chemical gradient. In contrast, carriers are linked to the H^+-gradient across the membrane and the proteins making up the carrier system undergo conformational changes which results in exposure of the transport binding sites to alternate between the cytosol and the external environment (Madigan and Martinko, 2006). Transport of metal ions relies heavily on the metabolic state of the cell. Metabolic inhibitors such as low

temperatures or lack of substrate result in reduced metal uptake, in addition, transport is concentration dependent and increased external ion concentrations may show uptake saturation (Gadd, 1993).

Reduction in sensitivity

Microorganisms may have the ability to adapt to the presence of metals in the environment by modifying essential cellular components, thereby reducing their sensitivity. Modification is achieved through mutations which decrease the sensitivity of the organelle without altering its basic function. In addition the organisms may produce alternate metabolic pathways to reduce the metabolic necessity of the sensitive component (Bruins *et al.*, 2000).

Whatever mechanism may be involved, fungi have the ability to either resist metal toxicity or to develop tolerance to metal ions which often results in the metal ion being removed from the environment. This has important bioremediation and biotechnological significance as metal pollution steadily increases.

2.4 Concluding remarks

The recovery and recycling of water in South Africa is essential if adequate and equitable water supplies are to be a reality for the general population. In addition, metal contamination is becoming an increasingly significant environmental concern. Both challenges can be met through the careful operation and use of water within industry and a conscientious effort to recover water from a variety of industrial processes. Currently physicochemical technologies are being employed to recycle water and recover economically important metals, however there are significant drawbacks associated with the use of these techniques. In many cases the cost of the process outweighs the benefits of metal and water recovery and in some instances the waste problem is merely shifted from one area to another. As a result of the limitations associated with traditional metal recovery processes to remove metals from wastewaters, the use of biological technologies has steadily gained interest. Biosorption and bioaccumulation may provide promising alternatives for the recovery of metals and may become integral elements of the metal recovery and wastewater treatment technologies used on a daily basis.

CHAPTER 3

AIMS AND OBJECTIVES

Two fundamental aims were identified for the purpose of this investigation. The first was to remove economically valuable metal ions (gold and platinum) and environmentally significant ions (copper and nickel) from solution in order to remediate precious metal refinery wastewaters and to recover economical loses experienced through incomplete metal extraction during the refining process. The following objectives were set to achieve this aim:

- Ascertain the optimum conditions for biosorption and bioaccumulation of copper, nickel, gold and platinum ions by the plant hyperaccumulator *Berkheya coddii* and the fungal plant pathogen *Phoma glomerata*, respectively.
- Investigate the role of extracellular polymeric substances (EPS) in the removal of metal ions from aqueous solution.
- Confirm the metal uptake by the biomass through qualitative visual techniques.

The second aim involved determining the bioremediative capacity of *P. glomerata* on two wastewaters generated during the precious metal refining process, objectives set to achieve this aim included:

- Determine of the optimal nutrient regime for *P. glomerata* growing in both wastewaters.
- Assess the effect of the growth of *P. glomerata* on various salt concentrations in each wastewater and propose possible reuse or further treatment options for the effluent.

CHAPTER 4

BIOSORPTION OF COPPER, NICKEL, GOLD AND PLATINUM BY *BERKHEYA CODDII*

4.1 Introduction

Biosorption and bioaccumulation strategies have been investigated as a means to remove metals from wastewaters (Ahluwalia and Goyal, 2006) and phytoremediation of soils is proving to be popular as an inexpensive, yet effective extraction technique. Phytoremediation is the use of living plants which have the ability to bind metals to their tissue to remediate metal contaminated soils (Yang *et al.*, 2005).

4.1.1 Berkheya coddii

Plants which have the ability to accumulate exceptionally high concentrations of metals within their structures are known as hyperaccumulators. Hyperaccumulation was first described in 1976 by Jaffré and Brookes (Mesjasz-Przybyłowicz *et al.*, 2004). Approximately four hundred hyperaccumulators have since been discovered with the ability to take up a variety of metals from the environment, the vast majority of these are nickel accumulators (Anderson *et al.*, 1999). Hyperaccumulators have been suggested as a potential remediation agent to remove metals from polluted soils by repeatedly growing hyperaccumulator crops on the contaminated site, harvesting and burning to produce a metal rich ash (Robinson *et al.*, 1997).

One of the most widespread nickel hyperaccumulators is *Berkheya coddii,* an asteraceous perennial found naturally only in serpentine outcrops in southern Africa (Robinson *et al.*, 1997). *Berkheya coddii* is an ideal candidate for hyperaccumulation-based bioremediation as it is fast growing, generating high biomass (300 g total dry weight per plant) and is perennial, so it may be harvested each year without the necessity of replanting. It has the ability to remove relatively high concentrations of nickel from contaminated soil; accumulated concentrations of up to 38 $mg.g^{-1}$ of leaf dry weight have been reported (Robinson *et al.*, 1997; 1999; Augustyniak *et al.*, 2002).

Presently, *B. coddii* is being used to recover nickel from metal contaminated soil at Anglo Platinum's base metals refinery situated in Rustenburg, South Africa. The plant is harvested, dried and smelted to extract the metal. Due to its current use, it was selected as a potential biosorbent material. High concentrations of various metals could potentially be recovered from other refinery processes if a sorption phase was to be included in the process between harvesting and smelting, with the harvested *B. coddii* plants being used as the sorbent.

The precious metals refinery generates wastewater containing precious and base metals of a recoverable concentration and *B. coddii* may be used as a biosorbent to recover these metals from the wastewater.

4.1.2 Biosorption

As described in Chapter 2, biosorption is the process of metal removal from solution by biological material. Biosorbents may include plant matter, bacterial, algal or fungal cells or animal products such as crab shells (Niu and Volesky, 2003; Al-Qodah, 2006; Ahluwalia and Goyal, 2007). Many factors may influence the efficiency of sorption such as sorbant and sorbent concentrations, temperature and interfering ions (Akar and Tunali, 2006; Al-Qodah, 2006). The most significant factor to consider is the effect of pH on biosorption and determination of the optimum pH for biosorption of each particular metal to each sorbent. At low pH values metal uptake is impeded, but it improves with increasing alkalinity until a sorption maximum is reached, after which metal sorption will decrease due to a reduction in metal solubility and increasing metal precipitation (Modak and Natarajan, 1995; Niu and Volesky, 2003; Akar and Tunali, 2006).

The optimum pH of the system is affected by the functional groups on the biomass surface. These include carboxyl, amine, hydroxyl, phosphate and sulphhydryl groups. The presence and concentration of these groups on the biosorbent surface should be elucidated in order to completely assess optimum pH and the mechanisms of metal binding (Modak and Natarajan 1995; Naja *et al.*, 2005). The heterogeneity of functional groups is analysed by carrying out an acid-base potentiometric titration. This information will assist in determination of optimum pH for sorption, identification of acid ionisable functional groups involved in ionic binding, estimation of the cationic exchange capabilities of the biomass and it describes the heterogenic reactivity of the biomass surface (Chojnacka *et al.*, 2005). The titrations usually generate classical sigmoidal-shaped curves, the result of plotting the volume of titrant against pH. It is possible to determine the nature of the functional groups available for metal binding on the biomass surface based on the pK_a values obtained during the titration. The pK_a values for carboxylic groups range between 2.5 and 5.2, the lower corresponding to a carboxylic group linked to an aromatic group, while higher values relate to the linking of an aliphatic chain. Amine groups have pK_a values which fall between 8.1 and 9.6, while phenolic groups range between 9.9 and 11.0 (Naja et al., 2005).

Information gained regarding functional groups on the biomass surface and optimum pH for sorption of various metals will guide further biosorption kinetic modelling and optimisation strategies.

4.2 Hypothesis

Berkheya coddii can be used as a biosorbent for recovery of mixed metals from precious metal refinery wastewaters after its utilisation as a phytoremediation agent in the extraction of nickel from mine dumps before final metal recovery by smelting. In addition, the nature of the biomass will allow for scale up operations and development of a fixed-bed reactor to attain a high volume wastewater throughput.

4.3 Aims and objectives

The aim of this chapter was to identify possible functional groups present on the dry *B. coddii* biomass surface, investigate the effect of particle size on functional group availability and determine the optimum sorption pH for copper, nickel, gold and platinum by *B. coddii*. In order to achieve this, the following objectives were set:

- Characterise functional groups on the *B. coddii* biomass surface through acid-base potentiometric titrations.
- Determine the effect of *B. coddii* biomass particle size on availability of the functional groups to ion binding.
- Elucidate of optimum sorption pH of copper, nickel, gold and platinum from single metal synthetic solutions by monitoring metal ions in solution by atomic absorption spectroscopy.

4.4 Materials and methodology

Unless otherwise stated, all growth media and reagents were reagent grade Biolab and Saarchem brand respectively, supplied by Merck Chemicals (Pty) Ltd (South Africa) and used as received. All glassware was acid washed in 5 % nitric acid HNO_3 for a minimum of 5 hours, rinsed in deionised water overnight and dried before use.

4.4.1 Potentiometric titration

The potentiometric titration was carried out according to Plummer (1971) and Deng and Ting (2005). *Berkheya coddii* biomass was dried at 60 ℃ until no further loss in mass was observed. The biomass was then homogenised and sieved to create three fractions, ≤ 599 µm, > 600 < 1000 µm and > 1000 µm < 20 mm, hereafter denoted as 599 µm, 1000 µm and 20 mm. One gram per litre of biomass was protonated in 0.1 M hydrochloric acid (HCl) for two hours and agitated at 120 rpm on a bench top Orbital Shaker (Labcon, South Africa). The biomass was filtered and rinsed with deionised water and stabilised for 12 hours in 50 ml of 0.01 M sodium chloride (NaCl). The pH of the solution was decreased to 2 and the titration was carried out with 0.1 M NaOH solution on a TritoLine Easy autotitrator (Schott Instruments, Germany). The volume of NaOH and

pH of the solution were recorded once equilibrium was reached. The titration was repeated with NaCl alone in order to correct for its buffering effect.

4.4.2 Optimum pH for biosorption of copper, nickel, gold and platinum

Atomic absorption spectrophotometer standard solutions (1000 $mg.l^{-1}$ copper in 0.5 M HNO_3, 1000 $mg.l^{-1}$ nickel in 1.0 M HNO_3, 1000 $mg.l^{-1}$ gold in 1.0 M HCl and 1000 $mg.l^{-1}$ platinum in 0.5 M HCl, EC Labs, South Africa) were used to investigate the biosorption of the various metals by *B. coddii*. Each solution was diluted to 100 µM (6.35, 5.86, 19.96 and 19.50 $mg.l^{-1}$ of copper, nickel, gold and platinum, respectively) and 100 $mg.l^{-1}$ (1573.66, 1703.86, 507.70, 512.61 µM of copper, nickel, gold and platinum, respectively) using deionised water as these two concentrations encompassed the maximum concentration of each metal in the wastewater. The pH was adjusted either with 1.0 M HCl or NaOH (Merck Chemicals) according to the following; copper and nickel: pH 2.0, 3.0, 4.0, 5.0 and 5.5; gold: pH 2.0, 2.5, 3.0, 3.5 and 4.0; platinum: pH 0.5, 1.0, 1.5, 2.0 and 2.5. All studies were carried out in triplicate. One gram per litre of 1000 µm biomass was introduced into each metal solution and agitated at 200 rpm on a bench top Orbital Shaker (Labcon). After 30 min and 60 min the 100 µM and 100 $mg.l^{-1}$ solutions were sampled respectively. Sample pH was adjusted to below 2 with 1.0 M HNO_3 and base and precious metals were filtered through 0.45 µm pore size nylon and cellulose acetate filters (Whatman) respectively. The metal concentration remaining in solution was determined by flame atomic absorption spectrophotometry on a 909 AAS (GBC Avanta, Germany). In addition the pH of each sample was measured using a pH meter (Cyberscan 2500, Eutech Instruments, Singapore). Biosorption studies were carried out at ambient temperature (approximately 25 ℃) with n o temperature change.

Metal uptake was calculated according to the following equation:

$$q = \frac{(C_i - C_f) \times V}{M} \qquad (4.1)$$

where q = metal ions sorbed onto biomass ($mg.g^{-1}$)

C_i = initial metal concentration ($mg.l^{-1}$)

C_f = final metal concentration ($mg.l^{-1}$)

V = volume of solution (l)

M = mass of biomass (g)

4.5 Results

4.5.1 Potentiometric titration

The purpose of the acid-base potentiometric titration was to elucidate the possible functional groups available for metal ion binding on the biomass surface, and utilise this information during

assessment of the optimal pH for metal sorption. The influence of the NaCl buffer on pH change during the titration needs to be taken into consideration when determining the presence of the various functional groups. This was done by titrating the buffer alone in order to correct for its effect. The resultant curves for the three biomass particle sizes and the buffer control were expected to be similar in shape, however the pH change of the buffer should occur with a lesser volume of NaOH utilised than in the biomass titrations. Under normal circumstances, once the buffer correction has been made, the resulting curve should appear as shown in Figure 4.1. In this example the first curve occurs at a pH of approximately 6 and represents amine functional groups, while the curve slightly above it at pH 9 is indicative of hydroxyl groups (Deng and Ting, 2005).

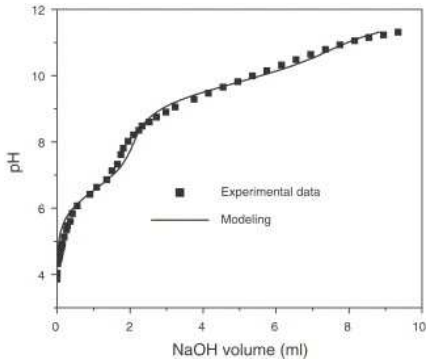

Figure 4.1 Example of a typical acid-base potentiometric titration once the effect of the buffer has been accounted for (Deng and Ting, 2005).

During the titration of the three *B. coddii* particle sizes and the NaCl buffer solution it was observed that the pH change occurred more rapidly in the presence of biomass than in its absence (Figure 4.2).

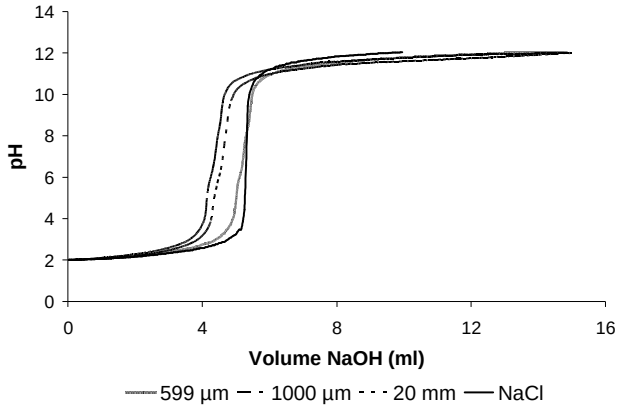

Figure 4.2 The acid-base potentiometric titration curve of the biomass particle sizes in relation to the NaCl buffer. *Berkheya coddii* concentration of 1 $g.l^{-1}$ was protonated in 0.1 M HCl for 2 hours and stabilised in 0.01 M NaCl for 12 hours before titrating with 0.1 M NaOH.

The increased pH change created a situation where it was unfeasible to compensate for the presence of the NaCl and hence determine the nature of the functional groups on the surface of the dried *B. coddii*. However, there did appear to be a slight resistance to pH change at pH 4.5, which may have indicated the presence of carboxylic groups on the biomass surface (Naja et al., 2005).

4.5.2 Optimum pH for biosorption of copper, nickel, gold and platinum

It was essential to determine the optimum pH of sorption of each metal (copper, nickel, gold and platinum) in order to maximise metal ion removal from solution by the biosorbent (*B. coddii*). The pH affects both the binding sites of the biomass and the metal chemistry in solution (Akar and Tunali, 2006). The optimum pH was determined by one point experiments immediately after equilibrium was reached. Monitoring the change in pH during the biosorption process may assist in determining the nature of the sorption reaction. For example, a pH decrease indicates a probable ion exchange mechanism due to the displacement of protons by the metal ions (Chojnacka *et al.*, 2005).

Biosorption of copper

Complete biosorption of copper ions with an initial concentration of 100 µM (6.35 $mg.l^{-1}$) occurred in 30 min at all pH values except pH 2.0 (Figure 4.3). There were too few metal ions in solution at this concentration for the pH to have a significant effect on functional group availability and hence metal uptake. In contrast, increasing the pH from 2.0 to 5.5 increased the specific uptake of copper ions from the 100 $mg.l^{-1}$ in solution from 405 to 556 $\mu M.g^{-1}$ (25.73 to 35.33 $mg.g^{-1}$) by *B. coddii*.

The presence of the biomass in aqueous solution increased the alkalinity of the solution. When in the presence of copper ions, a higher initial pH corresponded to a smaller pH change. This trend continued until the final pH had been reduced to below the initial value at pH 5.0 and 5.5 (Appendix A, Table A1).

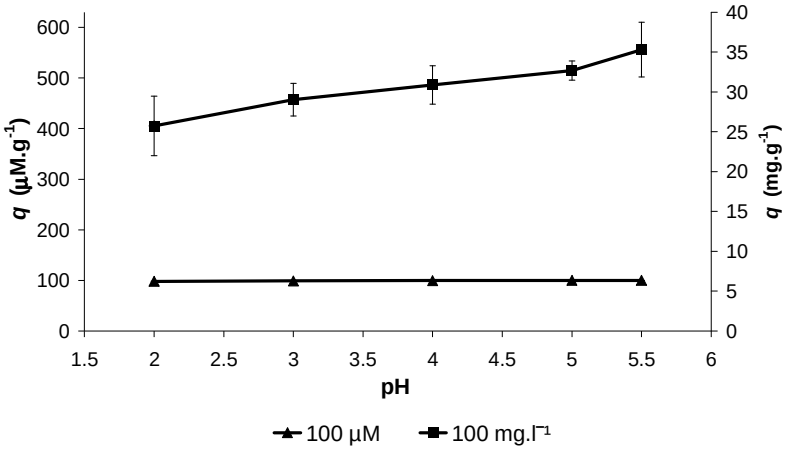

Figure 4.3 Specific uptake of copper ions from 6.35 mg.l^{-1} and 100 mg.l^{-1} solutions through sorption by 1 g.l^{-1} *B. coddii* after 30 min and 1 hour respectively. Error bars represent standard deviation and in some cases are too small to be visible.

Biosorption of nickel

As mentioned in section 4.1, living *B. coddii* is a nickel hyperaccumulator and is capable of actively depositing 38 mg.g^{-1} of nickel into its leaves. Since it only grows naturally in serpentine soils (Augustyniak *et al.*, 2002), it is highly probable that plants harvested in the wild will have nickel concentrated within their leaf matter. This was confirmed when biosorption studies of 100 mg.l^{-1} (1703.86 µM) initial nickel concentration resulted in higher final nickel concentrations in solution than those initially present in solution. However, at pH 3.0 and 4.0 nickel concentrations of 86 and 85 mg.l^{-1} (1465.24 and 1448.20 µM) remained in solution respectively (Figure 4.4). Standard deviations at these initial pH values were significantly high enough to exclude them as conclusive exceptions.

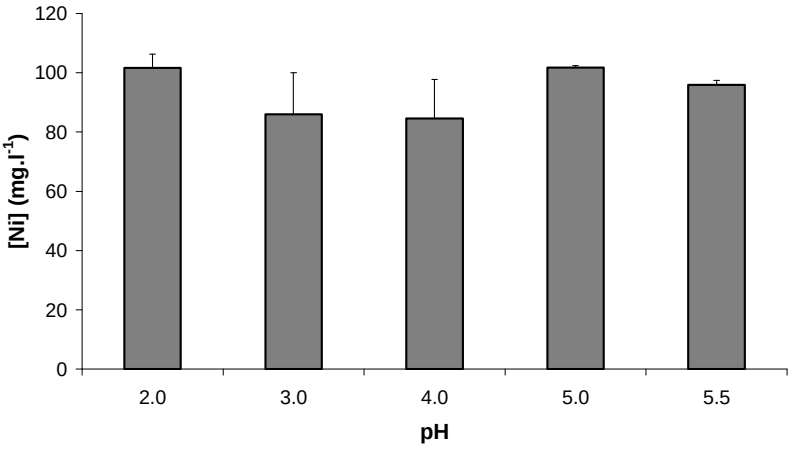

Figure 4.4 Nickel concentration remaining in solution after biosorption with 1 g.l^{-1} *B. coddii* after 1 hour with an initial concentration of 100 mg.l^{-1} (1703.86 µM). Error bars represent standard deviation.

Studies carried out with 100 µM (5.86 mg.l^{-1}) initial nickel concentration resulted in erratic and inconclusive final metal ion concentrations and in all cases more nickel was detected in solution after 30 min than was initially present. In all nickel sorption studies the pH after sorption increased, the extent of pH change appeared to be dependent on the initial nickel ion concentration (Appendix A, Figure A1.) After the biosorption studies of the other three metals, between 5 and 10 mg.l^{-1} of nickel was detected in solution.

Biosorption of gold

Gold exists in solution as an anion, and gold binding is significantly reduced under pH values at which ligands on the *B. coddii* biomass are deprotonated, as outlined in Section 4.1.2 (Akar and Tunali, 2006). Specific gold uptake values obtained after sorption of 100 mg.l^{-1} (507.70 µM) gold ions remained below 160 µM (31.51 mg.l^{-1}), while a maximum uptake of 508 µM was possible. Conversely, of the 100 µM (19.96 mg.l^{-1}) gold ions in solution of the lower concentration, 90 µM (17.72 mg.l^{-1}) was sorbed at pH 2.5 and 3.0. In Figure 4.5, both specific uptake curves follow the same trend with an optimum sorption pH of 3.0.

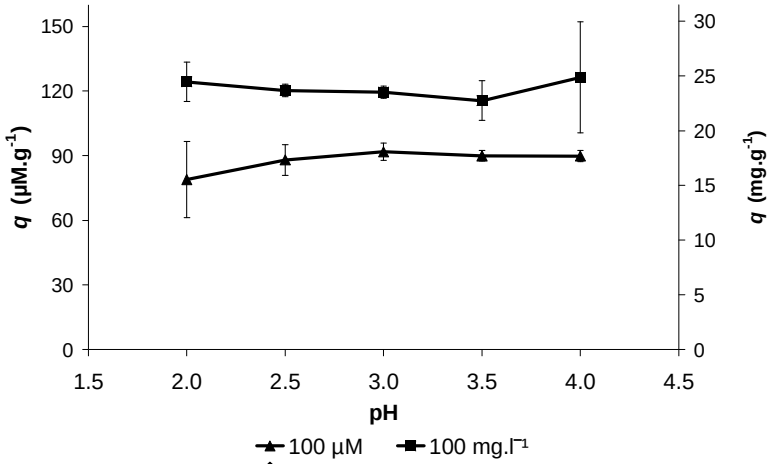

Figure 4.5 Specific uptake of gold ions from 100 µM (19.96 mg.l^{-1})and 100 mg.l^{-1} (507.70 µM) solutions through sorption by 1 g.l^{-1} *B. coddii* after 30 min and 1 hour respectively. Error bars represent standard deviation.

No clear correlation between specific uptakes of gold ions by *B. coddii* and pH change exists (Appendix A, Table A1). The more alkaline the initial solution, the greater the increase in pH over time. Too large an increase may have inhibited the uptake of gold ions from solution to a larger extent at the higher initial pH solutions and resulted in the specific uptake decrease in Figure 4.5.

Biosorption of platinum

As with gold, if the majority of the ligands on the biomass surface are deprotonated, platinum ion binding is significantly reduced. The optimum pH for 100 mg.l^{-1} (512.61 µM) platinum ion sorption

onto the *B. coddii* biomass was pH 2.0 (Figure 4.6). At values more acidic than this, uptake was reduced by approximately 30 %. Of the 512.61 µM metal ions in solution, a maximum of 293 µM (57 $mg.l^{-1}$) was sorbed. At pH 2.5 the specific uptake of platinum ions began to decrease. Uptake at the lower concentration of 100 µM (19.50 $mg.l^{-1}$) decreased slightly as the initial pH of the solution increased, however approximately half the metal ions in solution were sorbed in 30 min at all pH values.

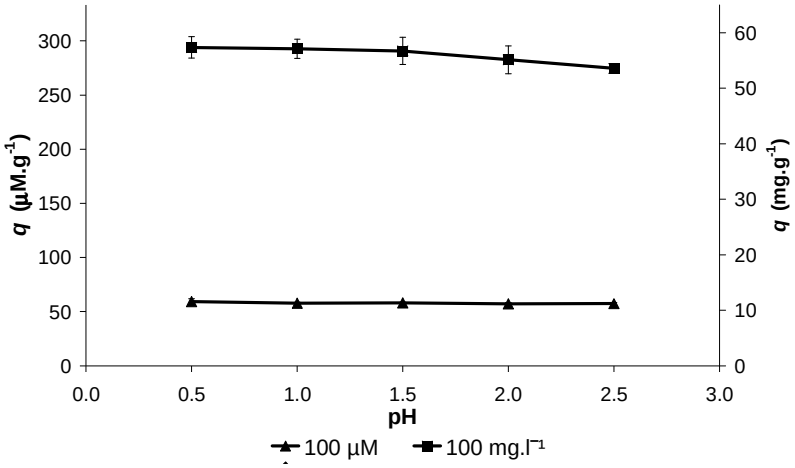

Figure 4.6 Specific uptake of platinum ions from 100 µM (19.50 $mg.l^{-1}$)and 100 $mg.l^{-1}$ (512.61 µM) solutions through sorption by 1 $g.l^{-1}$ *B. coddii* after 30 min and 1 hour respectively. Error bars represent standard deviation and in some cases are too small to be visible.

The general decrease in pH over the entire initial pH range may have been indicative of an ion exchange mechanism of biosorption. The lesser decrease at pH 2.5 corresponded with a decrease in platinum ion specific uptake, corroborating an ion exchange mechanism. The greatest pH change occurred at pH 0.5 with a specific uptake value of only 294 $µM.g^{-1}$ (57.35 $mg.g^{-1}$), which indicated that the pH changes did not occur solely due to sorption of the metal ions. If this had been the case, the greater decrease in pH would have resulted in an increased specific uptake value.

4.6 Discussion

4.6.1 Potentiometric titration

Due to the NaCl buffer requiring more NaOH to alter the pH than the *B. coddii* biomass during the potentiometric titration, it was not possible to correct for the effect of the buffer as it resulted in negative values of NaOH. Had the appropriate buffer correction been possible, functional groups with the following pK_a values may have been observed. Carboxylic groups have pK_a values between 2.5 and 5.2; pK_a values which occur at around 6.9 indicate the presence of phosphate

groups, while amine groups fall between 8.1 and 9.6. Lastly, phenolic or hydroxyl groups have the highest pK_a values ranging between 9.9 and 11 (Chojnacka *et al.*, 2005; Naja *et al.*, 2005).
A slight resistance to change in pH between pH 4 and 6 did occur (Figure 4.2). The functional groups with pK_a values in this range are carboxylic groups; this has been demonstrated using a variety of biomass types (Naja *et al.*, 2005) including algae (Chojnacka *et al.*, 2005) sunflower waste and maize (Zhang and Banks, 2006).

The greater amount of titrant required to obtain a given pH of the NaCl buffer when compared to the biomass titration may have been due to the release of nickel from the leaves of the *B. coddii*. If the nickel was bound within the leaves in an ion exchange fashion, cations such as the sodium ions and free protons in solution would bind to the newly available functional groups. If excess protons were removed from solution the relative concentration of hydroxyl ions would increase, resulting in the pH of the *B. coddii* biomass titrations to increase faster than it would have in the absence of biomass (Volesky, 2003). The unusual results obtained from the titration were the first indication that *B. coddii* might release nickel into solution, however it was decided to continue with sorption studies to obtain conclusive data to confirm that *B. coddii* was an unsuitable biomass for biosorption of metal ions from solution.

Despite not being able to determine the presence of more functional groups such as hydroxyl, amine and phosphate groups, it was possible to comparatively assess the three biomass particle sizes; 599 µm, 1000 µm and 20 mm. Decreasing the particle size of a given mass of biomass increased its surface area and hence more functional groups were available for metal ion binding. This effect has been reported previously by several researchers using a variety of biosorbents. Bhatti *et al.* (2007) found that decreased particle size of *Moringa oleifera* (horseradish tree) increased the biosorption of Zn ions from 24 to 37 $mg.g^{-1}$. Similar findings with regard to particle size and Zn uptake have been published using crab carapace (Lu *et al.*, 2007) and modified activated coconut shell carbon (Amuda *et al.*, 2007) as sorbents. The particle sizes of 1000 µm and 20 mm generated very similar titration curves, while the 599 µm particle size required a larger volume of NaOH to alter the pH. This is indicative of the presence of more sorption sites on the smaller particle biomass surface which bound the additional hydroxyl ions in the system and buffered any pH change (Naja *et al.*, 2005) and it may suggest a higher metal binding capacity in the 599 µm biomass than the two larger particle sizes.

The disadvantage of employing the smaller particle size becomes apparent when scale up of the system is considered. To date, the most effective scaled up sorption system designed is the fixed-bed column. The sorption bed must be porous to allow the metal laden liquid to percolate through it and maintain maximum sorption of the metal ions to the biosorbent. Particles should therefore be

an appropriate size to withstand the pressure drop across the sorption bed and yet present the maximum number of sorption sites to the sorbate. Conventional ion exchange columns used for this purpose typically have a particle size between 0.7 and 1.5 mm (Volesky, 2001; Vijayaraghavan *et al.*, 2006). Based on this information, further biosorption studies were carried out with a particle size of 1000 µm, which represented a compromise between functional group concentrations and column bed packing.

4.6.2 Optimum pH for biosorption of copper, nickel, gold and platinum

Biosorption of copper

Biosorption equilibrium occurs within 60 to 300 min of biomass and metal ion contact (Han *et al.*, 2006; Benaïssa and Elouchdi, 2007). Within 30 min of *B. coddii* and copper contact complete removal of copper ions from solution with an initial concentration of 100 µM (6.55 $mg.l^{-1}$) occurred. Conversely, a maximum of only 556 µM (43 $mg.g^{-1}$) were removed from the solution per gram of biomass, with an initial concentration of 100 $mg.l^{-1}$ at pH 5.5 (Figure 4.3). This specific uptake concurred with findings by Benaïssa and Elouchdi (2007), who determined that sorption of copper ions by sunflower leaves resulted in the maximum sorption occurring at pH 5.0 after 300 min with a specific uptake of 42 $mg.g^{-1}$.

However other authors have reported much higher copper specific uptake capacities using different biosorbents. Zhang and Banks (2006) determined biosorption by *Sphagnum* moss resulted in a specific uptake of copper of 229 $mg.g^{-1}$ at pH 4.0. Chojnacka *et al.* (2005) achieved a specific uptake of 390 $mg.g^{-1}$ with *Spirulina* sp. biomass. In contrast, maximum specific uptake of copper with an initial concentration of 100 $mg.l^{-1}$ by *Aspergillus flavus* was found to be only 10 $mg.g^{-1}$ at pH 5.0 (Akar and Tunali, 2006). There appears to be extensive variation of copper ion removal from solution based on the biosorbent used, most likely due to the heterogeneity of biomass functional groups. Some may have a higher affinity for copper ions than others (Zhang and Banks, 2006). Through Fourier transform infrared (FTIR) spectrophotometry, Nakbanpote *et al.* (2007) determined that the binding of copper to rice husk and its derivatives occurred predominantly on oxygen-containing functional groups. Dundar *et al.* (2007) and de Carvalho *et al.* (2003) showed that copper ions bound to functional groups containing C–O and C=C bonds when investigating the biosorptive capacity of leaf litter of *Populus tremula* and *Maitenus truncata* biomass respectively. The removal of metal ions from solution may be directly related to the concentration of these functional groups on the biomass surface.

While the specific copper uptake of 43 $mg.g^{-1}$ by *B. coddii* is superior to some biomass types, it cannot compete favourably with other biomass, such as *Sphagnum* moss and blue-green algae and therefore would not be selected as a biomass for copper ion recovery in a real world situation.

There is slight variation in the optimum pH for copper sorption reported in literature, pH values between 4.0 and 7.0 have been used successfully to sequester the metal ions from solution with the most common optimum being pH 5.0 (Chojnacka *et al.*, 2005; Zhang and Banks, 2006; Benaïssa and Elouchdi, 2007). Variations in optimal sorption pH are based on the specific biomass used and the functional groups present on its surface (Naja *et al.*, 2005).

Biosorption of nickel

The presence of nickel in the leaves of the *B. coddii* resulted in elevated concentrations of the metal ion in solution at the end of sorption. This occurrence demonstrated that utilising *B. coddii* as a biosorbent after a period of phytoremediation of metal contaminated soils will result in poor nickel removal from wastewater and may even release nickel ions into solution. The pH at which the least nickel was released into solution, or the highest concentration of metal ions sorbed (85 $mg.l^{-1}$), was pH 4.0 (Figure 4.4). This result corresponds with data presented by Malkoc (2006) and Zhang and Banks (2006). Malkoc (2006) reported the specific uptake value of nickel by cone biomass of *Thuja orientalis* to be 12.42 $mg.g^{-1}$, while Zhang and Banks (2006) obtained a specific uptake value of 121 $mg.g^{-1}$ with *Sphagnum* moss biomass, also at an optimum pH of 4.0. However, as with biosorption of copper, the optimum pH appears to be biomass dependent, as while this study corroborates findings by Zhang and Banks (2006), it contradicts those of Zafar *et al.* (2007), who reported that the optimum pH for nickel sorption onto protonated rice bran biomass was 6.0, with a specific uptake value of 102 $mg.g^{-1}$. Hanif *et al.* (2007) also selected pH 6 for biosorption of nickel onto *Cassia fistula* (Golden Shower) biomass and reported specific uptake values as high as 166 $mg.g^{-1}$.

An attempt was made to correct for the interference of the nickel originating from the biomass, however it was found that the effects varied depending on the contact time of biomass and metal solution, initial pH and initial metal concentration.

Biosorption of gold

The solution with an initial concentration of 100 $mg.l^{-1}$ (508 µM) resulted in the greatest specific uptake of gold ions at pH 3.0, 31.5 $mg.l^{-1}$ (160 µM), corroborating results obtained by Niu and Volesky (2003). The authors reported a similar value for specific uptake of gold by waste crab shells and an optimum pH of 3.5. Pethkar and Paknikar (1998) also reported optimum pH values of 2 to 4, using biomass beads of the saprophytic fungus *Cladosporium cladosporioides*. In this study complete removal of gold from an initial concentration of 100 $mg.l^{-1}$ was achieved, resulting in specific uptake of 100 $mg.g^{-1}$. The increase in pH of the solution during sorption would have resulted in deprotonation of functional groups on the biomass surface, which reduced the number

of positively charged groups available for gold anion binding (Naja *et al.*, 2005) and hence reduced the specific uptake of the gold ions from solution.

Increased specific uptake values have been described by Ishikawa *et al.* (2002). The authors determined that gold I and gold III had specific uptake values of 147 $mg.g^{-1}$ and 618 $mg.g^{-1}$ respectively when eggshell membranes were used as a sorbent. Specific metal uptake values as high as 8000 $mg.g^{-1}$ have been reported for novel sorbents specifically designed for gold recovery (Ogata and Nakano, 2005). Although uptake of gold ions by biosorbents such as *C*. *cladosporioides* beads and eggshell membrane have the ability to sequester a significantly high concentration of ions from solution, the design of novel gold-binding products makes it unlikely that these biosorbents could be utilised cost-effectively in a real-world situation. It is even less likely that biosorption by *B. coddii* would be a feasible recovery method, particularly for gold concentrations higher than the µM range.

Biosorption of platinum

The highest specific uptake achieved for platinum ions with an initial concentration of 100 $mg.l^{-1}$ (513 µM) was 72 $mg.g^{-1}$ (370 $\mu M.g^{-1}$) at pH 2.0. The optimum pH for platinum sorption has previously been shown to be between 2.0 and 2.5 (Guibal *et al.*, 1999; Godlewska-Zyłkiewicz, 2003). Godlewska-Zyłkiewicz (2003) described specific platinum uptake values of 59 $mg.g^{-1}$ by yeast biomass, while the maximum uptake demonstrated by Ma *et al.* (2006) with bayberry tannin immobilized collagen fibre membrane was only 45 $mg.g^{-1}$. Sorption of platinum ions by *B. coddii* was more effective than sorption by yeast biomass and bayberry tannin immobilized collagen fibre membrane, however, Guibal *et al.* (1999) reported specific uptake values for platinum as high 346 $mg.g^{-1}$ after sorption by chitosan flakes. There is a lack of information in the literature regarding the biosorption of precious metals and few researchers publish their findings. Although *B. coddii* may appear more attractive than some of the biomass previously used, the presence of specific uptake values as high as 346 $mg.g^{-1}$ by chitosan flakes suggests there are biomass types more suitable for platinum removal from solution. In addition, results obtained for platinum sorption by *B. coddii* were not sufficiently reproducible to guarantee a specific uptake as high as 72 $mg.g^{-1}$ with every attempt to recover the metal from aqueous solution.

pH change after sorption

In all cases sorption of metals from solution resulted in a pH change of the solution. There did not appear to be a relationship between specific uptake of any of the metals and the change in pH and in most instances the change was less than half a pH unit; slight pH increases may have been attributed to bicarbonate production by the biomass as carbohydrates were broken down (Mack, 2005) while a decrease may have been caused by a release of protons from the biomass.

An exception to this trend was the increase of the 100 $mg.l^{-1}$ nickel solution with an initial pH of 4.0 to approximately pH 6 after sorption. Similarly, the pH of nickel solutions with initial pH values of 5.0 and 5.5 were also increased to approximately 6 (Appendix A, Figure A1). The increase in pH may be attributed to the release of nickel ions from the biomass; if the nickel within the leaves was bound in an ion exchange fashion, the release of nickel into solution may have resulted in the removal of protons from solution, hence the pH would increase. This occurred regardless of the metal solution used and would have had an influence in the net pH change of each metal solution. Sorption experiments were undertaken at ambient temperature with no temperature change occurring during or after the 30 min and 1 hour contact time, so it is unlikely that the slight pH changes observed after sorption are as a result of temperature.

4.7 Conclusions

Although it was only possible to speculate as to the presence of carboxyl functional groups on the *B. coddii* biomass surface, it was feasible to ascertain that there was very little difference in metal ion binding capacity of the two larger particle sizes (1000 µm and 20 mm). Based on this, and the optimum particle size for scale up operations, 1000 µm particle was selected for further biosorption studies.

Sorption of copper ions by *B. coddii* cannot be favourably compared to specific uptake by other biomass such as *Sphagnum* moss and *Spirulina* sp. However, the optimum sorption pH of 5.5 was found to be within the range cited in literature.

The presence of nickel in the leaves of the *B. coddii* resulted in more metal being detected after sorption than in the original solution, causing it to be a poor biosorbent for nickel. It did appear that optimum nickel ion removal would occur at pH 4, corroborating the work of previous authors.

Removal of gold ions from solution by *B. coddii* resulted in specific uptake values similar to those described in the literature with other biomass types. However, the development of gold-specific novel biosorbents with enhanced gold-binding capacity out competed results obtained with plain biomass.

Platinum sorption by *B. coddii* compared favourably to other specific metal uptake values reported in literature, but it is considerably outperformed by the use of chitosan flakes as the sorbent. In addition, there is little published information regarding the biosorption of platinum and so the presence of sorbents with increased binding capacity is unknown.

The pH change during biosorption was too slight to have a direct effect on the specific uptake and change was erratic and unpredictable and most likely attributed to nickel release into solution or bicarbonate production.

Biosorption of metals by *B. coddii* harvested from areas of phytoremediation resulted in the release of nickel ions deposited in the leaves into solution. Depending on initial pH between 5 and 10 $mg.l^{-1}$ of nickel was released into solution after all sorption experiments. In addition, other types of biomass generated higher specific metal uptake values with three of the metals investigated than *B. coddii* biomass. Due to poor comparability and the release of nickel into solution, it was decided to discontinue biosorption studies with *B. coddii*.

CHAPTER 5

SELECTION AND IDENTIFICATION OF FUNGAL SPECIES FOR WASTEWATER TREATMENT AND BIOACCUMULATION OF COPPER, NICKEL, GOLD AND PLATINUM

5.1 Introduction

Fungal identification is of great importance in clinical, pathological, biotechnological and environmental sectors for the diagnosis of disease, development of commercial products such as pharmaceuticals and enzymes and bioremediation of soil and water (Carlile *et al.*, 2001; Malik, 2004). Initially, morphology was the basis for the mycological classification of organisms, but this system has become less popular as more advanced molecular technologies have developed. Specifically, the phenotypic approach has been criticised for its lack of standardised terminology, its subjectivity and controversial grouping (Guarro *et al.*, 1999). In addition, the morphology of a fungal organism may be dependent on environmental factors such as availability of substrate, moisture conditions and temperature (Guarro *et al.*, 1999) resulting in misleading characteristics and false identification. A number of other features may be used to aid identification, such as the antigenic properties of the organism, protein composition, the production of low-molecular weight compounds, carbohydrate and cell wall composition and nutritional and physiological requirements. However, with the development of molecular techniques, the most reliable and accurate method of identification is based on the genetic sequence of sufficiently conserved regions of the fungal ribosomal DNA (Carlile *et al.*, 2001).

One of three sequences of rRNA is used during genetic identification of fungi; the 18S, 25S and internal transcribed spacers (ITS). Comparison of the 18S sequence is used to assess the relationships between large groups of organisms, however, variability of the sequence often results in insufficient information for complete identification and as a result large sections of the region need to be sequenced. The 25S variable domains allow for comparison from high taxonomic levels down to species level, while the ITS regions are more variable and are used to differentiate between species and demonstrate patterns of microevolution (Guarro *et al.*, 1999).

Identification of the organism of interest is essential for process optimisation and strain development in order to attain maximum efficiency for metal uptake and bioremediation of wastewaters (Malik, 2004).

5.2 Hypothesis

Fungal isolates found growing in copper, nickel, gold and platinum metal solutions at an acidic pH will have the ability to remove metal ions from solution and possibly tolerate wastewaters from precious metal refining activities containing the various metals.

5.3 Aims and objectives

The aims of this chapter were to screen each fungal isolate found colonising the metal solutions for its ability to remove the metal ions from solution and tolerate precious metal refining wastewaters; and to identify the isolate/isolates capable of metal uptake. In order to achieve this, the following objectives were set:

- Isolate and purify each of the colonising fungi through successive subculturing.
- Assess the ability of each fungal isolate to tolerate precious metal refining wastewaters by monitoring their growth in the wastewaters.
- Determine copper, nickel, gold and platinum uptake by monitoring metal ions remaining in solution over a period of time through AAS.
- Identify the isolate/isolates which have the ability to tolerate precious metal refining wastewaters and remove metal ions from solution through amplification and sequencing of the ITS region on the fungal ribosomal RNA.

5.4 Materials and methodology

Unless otherwise stated, all growth media and reagents were reagent grade Biolab and Saarchem brand respectively, supplied by Merck Chemicals (Pty) Ltd (South Africa) and used as received. All glassware was acid washed in 5 % nitric acid HNO_3 for a minimum of 5 hours, rinsed in deionised water overnight and dried before use.

5.4.1 Fungal isolation

Flasks being used for the biosorption of copper, nickel, gold and platinum by *Berkheya coddii* (Chapter 4) were left to stand for 48 hours without discarding the metal solutions. Fungal colonisation of each of the metal solutions was observed after this period and the colonising organisms subcultured on malt extract agar (20 $g.l^{-1}$ malt extract, 10 $g.l^{-1}$ glucose, 2 $g.l^{-1}$ yeast extract and 12 $g.l^{-1}$ agar bacteriological) at 28 ℃. Three morphologically different fungal colonies were selected and individually subcultured until a pure strain of each organism was obtained. Fungal cultures were maintained on malt extract agar and liquid stock cultures grown in malt extract broth (20 $g.l^{-1}$ malt extract, 10 $g.l^{-1}$ glucose, 2 $g.l^{-1}$ yeast extract).

5.4.2 Fungal strain selection

Three previously isolated and purified strains were screened for their ability to grow in two different precious metal refinery wastewaters (identified by source, Dam 2 and Dam 4) supplied by Anglo Platinum precious metal refinery (Rustenburg, South Africa). In addition the isolates were assessed for their ability to remove copper, nickel, gold and platinum ions from single metal synthetic solutions (Section 4.4.2).

Each wastewater was diluted to 25 % (v/v) with autoclaved malt extract broth (to yield a final concentration of 20 $g.l^{-1}$ malt extract, 10 $g.l^{-1}$ glucose, 2 $g.l^{-1}$ yeast extract) and further sterilised under UV light for 1 hour. Autoclaving the wastewater was avoided as the high pressure and temperature would have resulted in the loss of volatile organic molecules present in the wastewater. Three millilitres of each liquid fungal stock culture (late log phase) were used to inoculate 100 ml of each 25 % (v/v) wastewater solution in triplicate. The pH of the media was left unadjusted to prevent the occurrence of unknown chemical reactions taking place within the wastewater and to simulate conditions found in industrial wastewaters. Flasks were incubated at 28 ℃ for five days and aeration maintained by shak ing at 150 rpm on a bench top Orbital Shaker. After five days, the biomass was harvested by filtration through pre-dried and weighed Whatman no. 1 filters and dried at 80 ℃ for 24 hours in or der to ascertain the tolerance of the fungal isolates as a function of biomass dry mass.

Each metal solution was individually added to sterile malt extract broth in order to obtain a final metal concentration of 50 $mg.l^{-1}$. The pH was left unadjusted to simulate real world wastewaters. Three millilitre aliquots of each liquid fungal stock culture were used to inoculate the metal solutions in triplicate; flasks were incubated as described previously. Metal ions remaining in solution were determined daily for three days by flame AAS. Sample pH was adjusted to below 2 with 1.0 M HNO_3 and base and precious metals were filtered through 0.45 µm pore size nylon and cellulose acetate filters (Whatman), respectively.

One fungal isolate was selected for further studies based on its ability to tolerate each wastewater and remove metal ions from solution.

5.4.3 Fungal identification

Genomic extraction

The genomic extraction was carried out as described by Bond *et al.* (2000). Hyphal strands obtained from a malt extract agar plate were vortexed in phosphate buffered saline (PBS) (Appendix B, Table B1). Five hundred microlitres of sample were centrifuged (Biofuge pico, Heraeus Instruments, Germany) at maximum speed (10 000 *g*) for 1 minute and the supernatant

discarded. The pellet was re-suspended in 250 µl of 50 % (v/v) glycerol and 250 µl Buffer A (Appendix B, Table B1). The sample was then centrifuged at maximum speed again for 1 minute and the supernatant discarded. The pellet was re-suspended in 250 µl of Buffer A. Fifteen microlitres of Lysozyme (50 $mg.l^{-1}$) were added to the re-suspended pellet and incubated at 37 ℃ for one hour, after which 15 µl Proteinase K (50 $\mu g.ml^{-1}$) (Roche, USA) was added, and incubated at 50 ℃ for another hour. Four freeze-thaw cycles were performed with liquid nitrogen and six glass beads were added to the Eppendorf tube after the final cycle before vortexing the sample.

The sample was then transferred to a fresh Eppendorf tube to which 250 µl of 10 % sodium dodecyl sulphate (SDS) (w/v), 250 µl phenol and 250 µl chloroform:isoamyl alcohol (24:1) were added. The mixture was vortexed and centrifuged at maximum speed for 2 minutes. The aqueous layer was transferred to a new Eppendorf tube to which 500 µl chloroform:isoamyl alcohol were added. The sample was once again vortexed and centrifuged at maximum speed for 2 minutes and the aqueous layer transferred to a new Eppendorf tube. Fifty microlitres of 3.0 M sodium acetate were added to the tube, made up to a final volume of 1 ml with 96 % (v/v) ethanol and stored at -20 ℃ overnight. The sample was centrifuged at maximum speed for 25 minutes at 4 ℃ and the supernatant poured off. The pellet was then re-suspended in 50 µl sterile deionised water and underwent a polymerase chain reaction (PCR) to amplify the ITS region of the fungal rRNA.

Polymerase chain reaction

Twenty five microlitre reactions were made by mixing Master mix 1 and Master mix 2 (Promega, USA) (Appendix B, Table B2) and adding 2 µl of genomic DNA. The universal primers used to amplify the ITS region were ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), obtained from Promega (USA). The PCR reaction parameters were 94 ℃ for 2.5 minutes and 40 cycles of 94 ℃ f or 15 seconds, 53 ℃ for 30 seconds, 72 ℃ for 90 seconds and 72 ℃ 10 minutes.

The efficiency of the PCR was determined electrophoretically (Biorad powerpac 200, USA) in an ethidium bromide stained 1.0 % (w/v) agarose gel (Hispanagar, Whitehead Scientific, South Africa) at a constant voltage of 80 V for 1 hour in 1 × Tris-acetate-EDTA (TAE) buffer and visualised with a chemiluminescence and fluorescence documentation system (UVIpro chemi, UK) (Appendix B, Figure B1).

Ligation, transformation and sequencing

The PCR products were ligated into the pGEM-T Easy vector using the pGEM-T Easy vector system kit (Promega, USA) (Appendix B, Table B3). The transformation was carried out on ice. Two microlitres of the ligation reaction were added to 50 µl of competent cells, mixed gently and

left on ice for 20 minutes. The cells were then heat shocked at 42 ℃ for 45 seconds and cooled on ice for 5 minutes. One millilitre of SOC broth was added and the media incubated at 37 ℃ for one hour. Aeration was maintained by shaking on a bench top Orbital Shaker at 150 rpm. Cells were then spread plated onto MacConkey agar containing 100 $mg.l^{-1}$ ampicillin and incubated at 37 ℃ overnight. Light pink cells contained the ITS region insert and were selected for sequencing. Sequencing reactions were carried out by Inqaba biotec (Pretoria, South Africa). A BLAST (Basic logic alignment and search tool) search of the sequence obtained by Inqaba biotec was carried out to determine the species of the fungal isolate.

5.4.4 Fungal morphology

Tape mount slides of the selected fungal isolate were made to determine the morphology of the fungus. Lactophenol blue or methalene blue were used to visualise the hyphae and reproductive structures of the fungal isolate and the samples examined under a light microscope (Olympus U-CMAD3, Japan). Images were captured using a digital camera (Olympus C-4040, Japan).

Fungal samples used for scanning electron microscopy (SEM) were grown in malt extract broth at 28 ℃ for 3 days, by which stage the culture had entered stationary phase. Small volumes were removed and placed in Eppendorf tubes and centrifuged at maximum speed for 2 minutes. The supernatant was discarded and cold buffered fixative (2.5 % (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7) added to the pellet, samples were allowed to fix at 4 ℃ for 12 hours. Two 15 minute wash steps with cold 0.1 M sodium phosphate buffer, pH 7, were performed before dehydration with a series of increasing ethanol concentrations (30, 50, 70, 80, 90, 100 % (v/v)) with two changes of 100 % ethanol. The specimens were then dried in a critical point drier (Polaron, England), mounted on brass stubs, coated in gold (Balzers Union, Principality of Liechtenstein) and observed under a scanning electron microscope (Tescan Vega LMU, Czech Republic).

Certain fungal species produce pigments, the colour of which may be dependent on pH. The colour change may be used as a diagnostic tool in identification of the species. A NaOH spot test was carried out to determine if pigments produced by the fungal isolate changed colour at a high pH. Growing mycelia were exposed to 0.5 M NaOH and the colour change of the pigment observed over time.

5.5 Results

5.5.1 Fungal isolation

Three morphologically different fungal strains were isolated and subcultured to pure strains on malt extract agar, hereafter referred to as the white, pink or grey fungal isolate.

5.5.2 Fungal strain selection

The abilities of the three fungal isolates to grow in two precious metal refinery wastewaters (Dam 2 and Dam 4) were assessed. The dry weight of each isolate was determined after growth in 25 % (v/v) wastewater and malt extract for five days and these values were used as a means to determine tolerance to the wastewaters as illustrated in Figure 5.1.

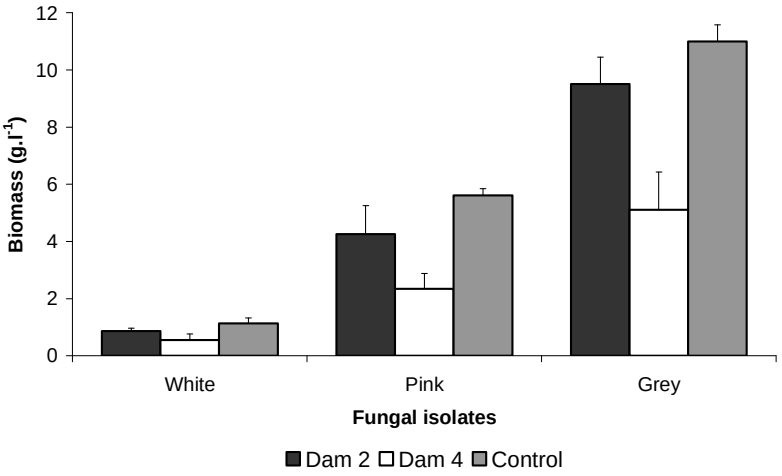

Figure 5.1 Dry weight of fungal biomass grown at 28 ℃ for 5 days in 25 % wastewater/malt extract broth (v/v) as an indication of tolerance toward the two wastewaters. Error bars represent standard deviation (n=3).

As can be seen in Figure 5.1, the white fungal isolate generated the least amount of biomass during the growth period. The grey isolate grew faster in both wastewaters than either of the other two fungi. In all cases, wastewater from Dam 4 had a greater inhibitory effect on fungal growth than wastewater from Dam 2.

The removal of copper, nickel, gold and platinum ions by each of the fungal isolates was monitored over a period of three days. The metal uptake ability of the white isolate is shown in Figure 5.2. The highest concentration of metal ions removed from solution was found to be nickel. Maximum nickel removal was approximately 9 $mg.l^{-1}$, however, the uptake of each metal was inferior when compared to the other isolates.

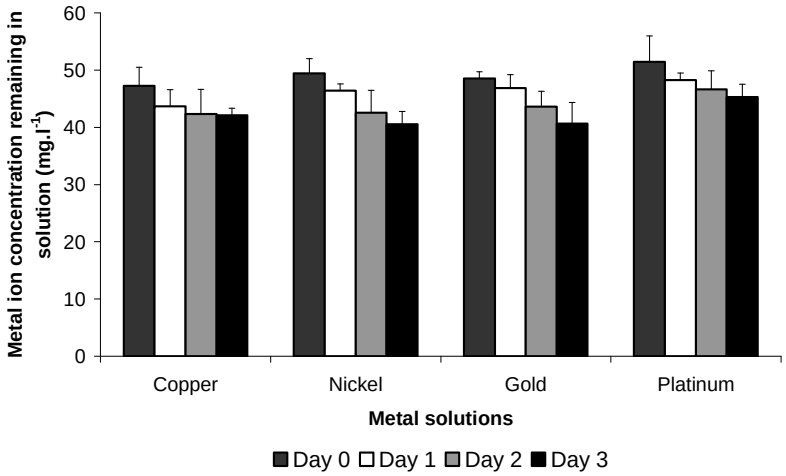

Figure 5.2 Copper, nickel, gold and platinum ion removal from solution with an initial metal concentration of 50 mg.l^{-1} by the white fungal isolate over a period of 3 days grown at 28 ℃ in malt extract broth. Error bars represent standard deviation (n=3).

As with the white isolate, the pink fungal isolate removed more nickel ions from solution than any other metal. The nickel concentration was reduced from 49.7 to 29.0 mg.l^{-1} during the three day period. In addition, gold removal was notable as shown in Figure 5.3; the gold ion concentration in solution was 33.3 mg.l^{-1} on the last day of sampling, however only 8 and 10 mg.l^{-1} of copper and platinum were removed from solution respectively.

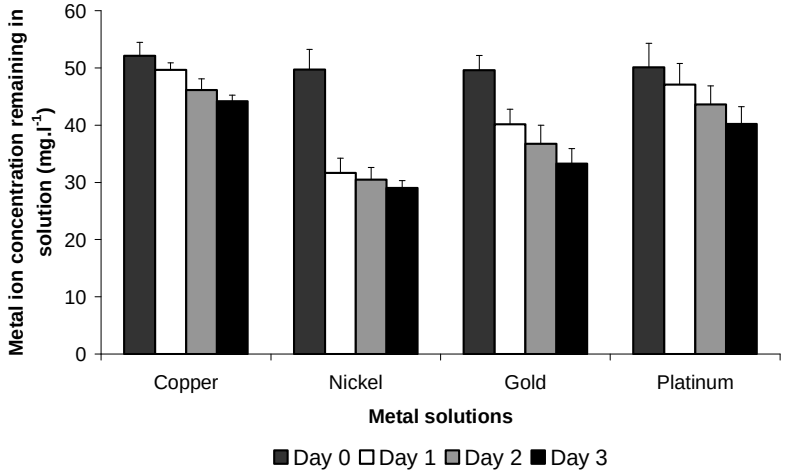

Figure 5.3 Copper, nickel, gold and platinum ion removal from solution with an initial metal concentration of 50 mg.l^{-1} by the pink fungal isolate over a period of 3 days grown in malt extract broth. Error bars represent standard deviation (n=3).

In comparison, the grey isolate grew extremely rapidly and removed 35 and 39 mg.l^{-1} of gold and platinum ions from solution respectively. In contrast to the other two fungal isolates, the grey isolate was the least efficient at removing nickel from solution; only 7 mg.l^{-1} was removed from

solution during the three day period. Figure 5.4 shows the superior precious metal removal compared to the two base metals investigated.

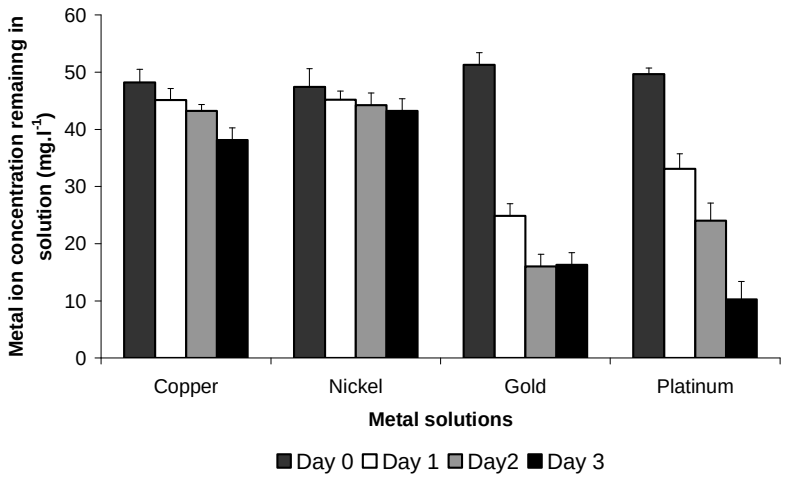

Figure 5.4 Copper, nickel, gold and platinum ion removal from solution with an initial metal concentration of 50 mg.l^{-1} by the grey fungal isolate over a period of 3 days grown in malt extract broth. Error bars represent standard deviation (n=3).

Based on the ability of the grey isolate to remove the most gold and platinum ions from solution (Figure 5.4), and the superior growth in both precious metal wastewaters (Figure 5.1), it was decided that further studies would be undertaken with the grey isolate alone. Hence it was the only isolate of the three to be identified.

5.5.3 Fungal strain identification

A BLAST search (NCBI) was performed to establish sequence homology of the grey isolate with other organisms in the GenBank database, and to determine the possible genus and species name of the isolate. The BLAST search generated sequence homology with a large number of uncultured soil fungi and an unknown species in the broad *Ascomycota* phylum (Appendix C); however, the only homologues listed to the species level were those in the *Phoma* genus. Table 5.1 lists the four most informative hits obtained by the BLAST search.

Table 5.1 Comparison of ITS ribosomal RNA of the grey isolate with known organisms in the GenBank database.

Accession code	Organism	Percentage coverage	Percentage homology
EF060476.1	*Ascomycota* sp. LM107	75	99
EF060576.1	*Ascomycota* sp. LM243	75	98
AY337712.1	*Phoma herbarum*	75	96
AY183371.1	*Phoma glomerata*	74	96

Based on the results obtained by the BLAST search the isolate was most likely to be a *Phoma* species within the *Ascomycota* phylum; this was further confirmed through evaluation of morphological characteristics.

5.5.4 Fungal morphology

Macromorphology

Macroscopic evaluation of the isolate resulted in observations of similar features to those found by do Amaral *et al.* (2003) as depicted in Figure 5.5. Cultures grown on malt extract varied in colour depending on age and growth phase. Initially the mycelia were white and woolly, gradually turning grey as the culture aged. When nutrient limitations occurred, aerial mycelia emerged from which droplets of an oily substance were secreted, the colour of which varied from clear to burned sienna (Figure 5.5A). At this point the isolate produced a pink to red pigment, the colour intensifying with culture age. These pigments were also produced in liquid culture, the colour and intensity depending on pH (strongly acidic and alkaline) and type of carbon substrate. The pigments produced ranged from yellow to red, green and black.

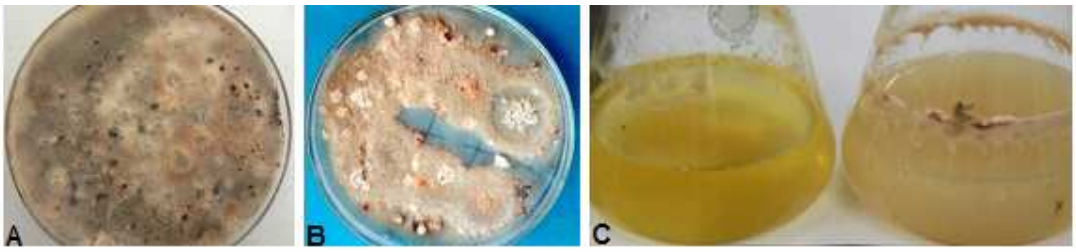

Figure 5.5 Macroscopic features of the fungal isolate (A) and those of *Phoma sorghina* (do Amaral *et al.*, 2003) (B) grown on malt extract agar and potato dextrose agar respectively. Pigments produced after growth in minimal media spiked with 40 $g.l^{-1}$ of nickel (left) and a control (right) at pH 2 for 72 hours (C).

Various species of *Phoma* have been reported to produce a pigmented antimicrobial compound, the colour of which is dependent on pH. A NaOH spot test was carried out by exposing growing mycelia to NaOH to increase the pH, and the colour development monitored. In Figure 5.6 it is possible to see the colour development of the pigment over time upon the application of NaOH, confirming the probability that the isolate was a *Phoma* species as this colour change would not be observed in many *Ascomycetes*.

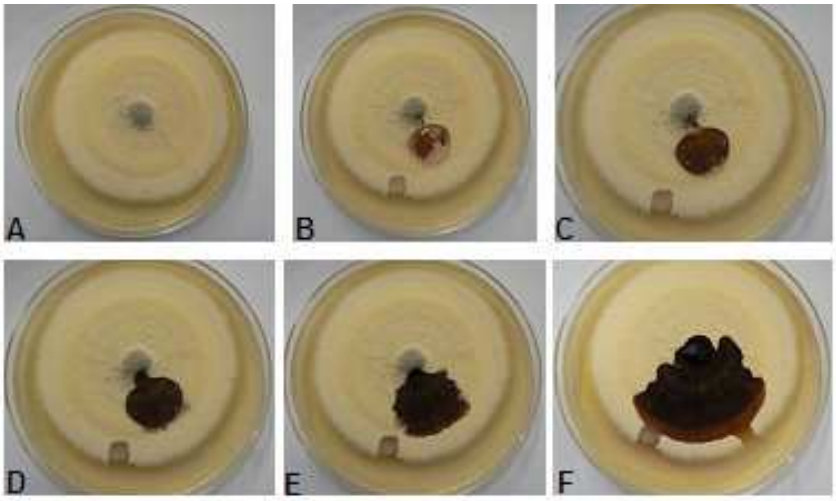

Figure 5.6 Pigmentation of the grey fungal isolate before exposure to 0.5 M NaOH (A), upon application of NaOH (B), 10 seconds (C), 1 minute (D), 5 minutes (E) and 10 minutes (F) after contact with NaOH.

Micromorphology

In addition to the similarities observed on a macroscopic level, in stationary phase the isolate produced reproductive structures identified as either pycnidia (all *Phoma* species as described by Rai (2000)) or perithecia (a number of *Ascomycota* species (Carlile *et al.*, 2001)). Figure 5.7 is a diagrammatic illustration of the structure of the pycnidium and the perithecium. These structures were examined further in order to determine whether they were pycnidia or perithecia.

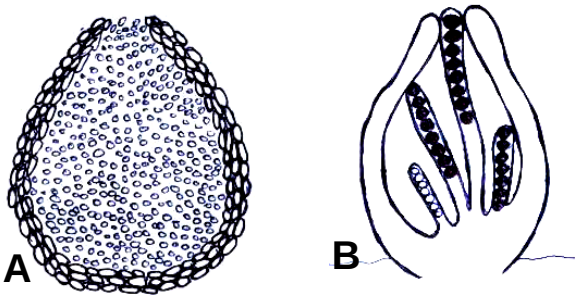

Figure 5.7 Schematic illustration of the structure of a pycnidium (A) and a perithecium (B) (Adapted from Carlile *et al.*, 2001 and Boerema *et al.*, 2004).

The reproductive structures shown in Figure 5.8A confirmed the presence of pycnidia filled with conidia indicative of a *Phoma* species rather than an *Ascomycota* species. The shape of the two pycnidia and their ostioles (opening at the neck of the structure), including conidia being released from the structure, are visible. Conidia are ellipsoidal and appear to be found in cohesive masses (Figure 5.8B).

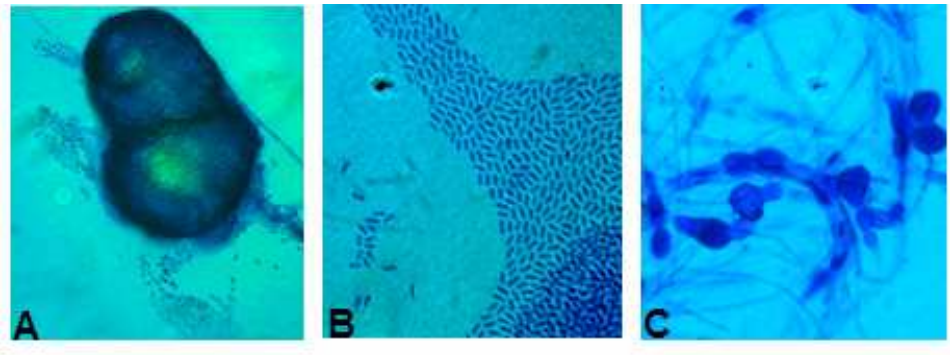

Figure 5.8 Pycnidia produced by the fungal isolate with the ostiole and spores clearly visible (400 ×) (A), spores released from pycnidia (1000 ×) (B) and chains of chlamydospores produced along the aseptate hyphae (1000 ×) (C). Cultures were grown on malt extract agar for approximately 14 days.

The reproductive structures seen in Figure 5.8C are those of chlamydospores. Terminal and intercalary chlamydospores growing in chains were observed, which can be more clearly viewed by SEM (Figure 5.9); here they can be seen developing in chains along the hyphae.

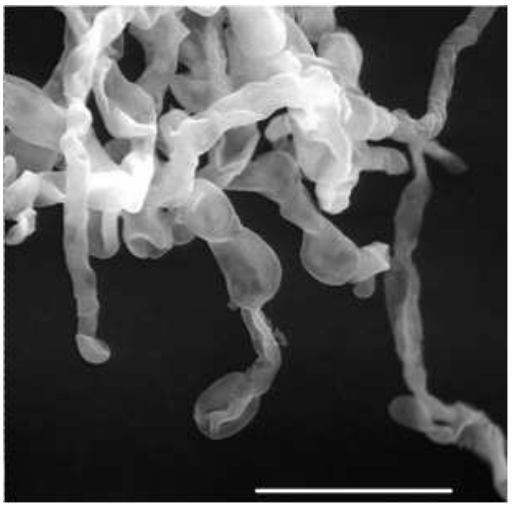

Figure 5.9 Terminal and intercalary chlamydospore morphology of the *Phoma* sp. grown in malt extract broth for 3 days. Bar indicates 20 µm.

5.6 Discussion

Fungi are ubiquitous chemoheterotrophic organisms and are important as pathogens, decomposers, animal and plant symbionts and spoilage organisms of both natural and manufactured products (Gadd, 2007). Fungi have been shown to be effective remediators of a range of xenobiotic compounds such as polyaromatic hydrocarbons and the ability of fungi to remove heavy metals from the environment has been demonstrated (Valix *et al.*, 2001; Malik, 2004).

5.6.1 Fungal isolation

Organisms growing in the metal solutions were subcultured until three distinct fungal species were identified. Based on their ability to grow in the metal solutions at an extreme pH, it was proposed that the white, pink and grey isolates may have the ability to accumulate metals and possibly tolerate two wastewaters generated through precious metal refining.

One of the first observations made was that the growth of the white isolate on malt extract was sluggish. The slow growth implied that the white isolate would be inferior as a bioremediation tool, as the substrate utilisation and metabolically linked metal uptake would not occur as quickly as it might in the other two isolates (Congeevaram *et al.*, 2007). It was nevertheless investigated for wastewater tolerance (Figure 5.1) and metal ion removal from solution (Figure 5.2).

The grey isolate grew faster than either of the other two isolates; however as the culture aged its morphology changed from light grey to pink. The pink colouration produced by the grey isolate was a different shade and intensity to the pink isolate. In addition, the pink fungal isolate was coloured from the beginning of growth and on the same substrate and the morphology of the colony was significantly different to assume the isolates were different species. It was thought that the grey isolate may be two species of fungi growing symbiotically, but further attempts to separate the species were unsuccessful and they were treated as a single species during the screening process. Fungal morphology is dependent on the culture age, the environment in which the organism finds itself and the substrates available to that organism (Carlile *et al.*, 2001). However, in some instances changes in morphology may be indicative of certain fungal genera; for example, the presence of coloured pigments is used to identify and differentiate between species in the *Phoma* genus (Boerema *et al.*, 2004).

5.6.2 Fungal strain selection

Bioremediation of the precious metal refinery wastewaters and removal of the metals of interest were the main features of this particular study, therefore logic dictated that the fungal isolate with the greatest tolerance to the wastewater and the highest metal removal capability would be selected for all further investigations.

The extremely slow growth of the white isolate resulted in less than 1 $g.l^{-1}$ of biomass being produced in a five day period in 25 % wastewater/malt extract broth (v/v). In comparison, the grey isolate generated 9.5 and 5.1 $g.l^{-1}$ of biomass in wastewater from Dam 2 and 4, respectively, during the same time period. Its enhanced growth suggested that it may have been able to tolerate higher concentrations of the wastewaters than the other two isolates, an important feature when considering potential reactor and scale up developments for bioremediation of the two dams as

faster acclimatisation would result in increased long term tolerance to the wastewaters (Malik, 2004).

In each case, the growth in Dam 2 was similar to that of the control; the wastewater only slightly inhibited biomass generation. However, in the presence of wastewater from Dam 4, biomass growth was approximately half of that of the control, indicating that Dam 4 had a greater toxic effect than Dam 2. Information regarding the composition of each wastewater was obtained from Anglo Platinum which showed that Dam 2 included predominantly sodium and ammonium chlorides and hydroxides, while Dam 4 included a range of organic solvents, a number of halogens, various acids and sodium and ammonium compounds, rendering Dam 4 a more complex wastewater containing an increased number of harmful compounds and chemicals. Observations regarding the slower growth in Dam 4 than in Dam 2 concur with the information provided by Anglo Platinum as to the contents of the wastewaters.

All three fungal isolates were grown in malt extract spiked with 50 $mg.l^{-1}$ of each metal ion for 72 hours and their metal removal capabilities compared. Both the white and pink isolates removed higher concentrations of nickel from solution than any other metal and a higher concentration than the grey isolate; 9, 20 and 4 $mg.l^{-1}$ were removed by the white, pink and grey fungi, respectively. Removal of all metal ions from solution by the white isolate was inferior to the uptake of the other two isolates (Appendix B, Figure B2). The poor metal uptake ability of the white isolate was most likely attributable to its slow growth, and this combined with its poor growth in low concentrations of wastewater from Dam 2 and 4 excluded the white isolate from further investigations.

The pink isolate exhibited the highest nickel removal capacity, and while these preliminary results suggested nickel uptake may be comparable to those found in other fungal studies (Dönmez and Aksu, 2000; Congeevaram *et al.*, 2007), the enhanced removal capacity of the precious metals by the grey isolate (Appendix B, Figure B2) represented greater potential financial benefits. At the time of writing, the prices of gold and platinum were $809.90 and $1462 per Troy ounce* (173.6 ZAR and 313.4 ZAR per gram) (www.thebulliondesk.com), while copper and nickel traded at $3.04 and $11.7 per pound (44.7 ZAR and 172 ZAR per kilogram), respectively (www.basemetals.com). The metal uptake ability in conjunction with superior growth in the dilute wastewaters resulted in further studies being carried out with the grey isolate alone, hence it was the only fungus selected for identification.

* Metal is traded in Troy ounces. One Troy ounce = 31.1 g.

5.6.3 Fungal strain identification

The PCR amplification of the ITS region on the ribosomal RNA generated a sequence of 756 base pairs (Appendix B, Figures B2 and B3). When compared to sequences in the GenBank database the vast majority of hits generated corresponded to uncultured soil fungi and various *Ascomycota* species (Appendix C). These results provided insufficient information on the genus and species level for an accurate, conclusive identification of the fungal isolate. However, in addition to the broader results, a number of hits were listed as *Phoma* species, in particular *Phoma herbarum* and *Phoma glomerata*. Each had a 96 % homology with the ITS region of the unknown isolate and greater than 74 % coverage of the sequence. Based on these results, it was concluded that it was highly probable that the grey isolate was a *Phoma* species, although the identification to species level was not definite. Further investigation of the morphological characteristics was undertaken to differentiate between the species.

5.6.4 Fungal morphology

Flask-shaped reproductive structures were observed in cultures in late stationary phase. All *Phoma* species produce pycnidia (Rai, 2000), while perithecia are produced by certain *Ascomycetes* such as *Aspergillus* and *Penicillium* species (Carlile *et al.*, 2001) in stationary phase. The fundamental difference between the pycnidium and the perithecium is that the pycnidia are produced during asexual reproduction, while the perithecia develop during sexual stages of reproduction. In addition, organisms which produce pycnidia have no known sexual reproductive stage (Ingold and Hudson, 1993; Carlile *et al.*, 2001). Conidiophores are produced within the pycnidium and once the conidia have matured, they are released through ostioles into the environment in mucilaginous masses (Boerema *et al*., 2004). The perithecium contains asci, which in turn each contain eight ascospores that undergo meiosis. In contrast to the pycnidium, the asci elongate through the neck of the perithecium to release their spores (Carlile *et al.*, 2001). Tape mount slides of the cultures were made to determine whether the flask-shaped structures were pycnidia or perithecia. In Figure 5.8A it can be seen that the structures were filled with conidia, rather than elongating asci. In addition, chlamydospores (Figure 5.8C) were observed, corroborating the results obtained from the BLAST search and confirming that the grey isolate was most probably a *Phoma* species.

Due to the superior percentage coverage of the ITS sequence and increased number of hits generated by the BLAST search (Appendix C) when compared to other species level hits, it was originally assumed that there was an increased probability that the grey isolate was *P. herbarum*. Boerema *et al.* (2004) described some of the *in vitro* characteristics of *P. herbarum* as colonies producing a reddish pigment on malt extract agar which turns slightly blue as the culture ages. The colour of the pigment changes to blue upon the addition of a drop of NaOH and the culture may have subglobose to elongated pycnidia which are non-papillate and may be covered by loose

strands of hyphae. Rai (2000) describes the pycnidia as being globose and flask shaped with a short neck, while the conidia develop an ovoid shape on malt extract agar.

The distribution of *P. herbarum* has been recorded worldwide with an extensive host range. *P. herbarum* has been isolated from dead plant and animal material (including human), nutritional and inorganic materials such as asbestos, butter, cement, oil-paint, rubber and soil to name a few. It has also been commonly isolated from dead seed coats, which had resulted in it being confused with other specific seed-borne *Phoma* species pathogens (Boerema *et al.*, 2004).

Cultures of *P. glomerata* growing in malt extract are fast growing and vary greatly in morphology and colour. They may or may not have aerial mycelia and appear woolly. Colours vary between buff, olivaceous and green and addition of NaOH results in a burned sienna colour formation (Rai, 2000; Boerema *et al.*, 2004). Rai (2000) reported that on various media other than malt extract the colonies had a buff-pink to red colour. Pycnidia are usually subglobose and papillate and are usually solitary, but may coalesce. Conidia vary in shape and size, but are most commonly found to be ellipsoidal on malt extract (Rai, 2000; Boerema *et al.*, 2004). One of the most stable morphological diagnostic features of this species is the production of *Alternaria*-like chains of chlamydospores (Rai, 2000). Chlamydospores may be highly variable in shape and size and are generally multicellular-dictyosporus found in branched or unbranched chains. They may be terminal, but normally occur as intercalary chains (Baxter *et al.*, 1994; Boerema *et al.*, 2004).

Phoma glomerata is a ubiquitous soil fungus, which may suggest that the uncultured soil fungi listed in the BLAST search may be *P. glomerata.* The species is predominantly found in temperate and subtropic regions and has been isolated from various plants; it has approximately a hundred host plant genera, as well as animal tissue (including human) and inorganic material. It is considered an opportunistic pathogen or secondary invader occurring commonly on dead seed coats and found in association with symptoms of leaf spot, blight and fruit rot. As with *P. herbarum*, *P. glomerata* has been isolated from chemical products such as paint, wood and wool fibres (Boerema *et al.*, 2004).

Macromorphology

The development of colour from buff to grey to pink on malt extract as the isolate aged implied that it was more likely to be *P. glomerata* than *P. herbarum*, since production of olivaceous, green or blue pigments did not occur at any stage of growth. While the formation of the pink pigment by *P. glomerata* was not described on malt extract by either Rai (2000) or Boerema *et al.* (2004), Rai (2000) reported the formation of pink pigments in media supplemented with glucose. Malt extract media described by both authors did not contain glucose, while the media used in this study

contained 20 $g.l^{-1}$ of glucose. Production of the pink pigment may therefore be related to the carbon substrate used and substrate availability.

One of the diagnostic characteristics of *P. herbarum* is the blue colour change observed during the NaOH spot test. In contrast, the NaOH spot test carried out on the isolate resulted in the colour changing from clear to burned sienna, a phenomenon described by Rai (2000) as being a feature of *P. glomerata*. In addition, aerial mycelia were clearly visible as the culture aged; *P. herbarium* does not produce aerial mycelia while *P. glomerata* does (Boerema *et al.*, 2004). On a macroscopic level, comparison of morphological characteristics of the grey isolate implies it was more likely *P. glomerata* than *P. herbarum* as described by Rai (2000) and Boerema *et al.* (2004).

Micromorphology

On a microscopic level the structure of the pycnidia and conidia of the grey isolate had more in common with those of *P. glomerata* than *P. herbarum* (Rai, 2000; Boerema *et al.*, 2004). In Figure 5.8A the papilla is visible on the right hand side of one of the pycnidia and no associated hyphae are observable, a feature associated with *P. glomerata* (Boerema *et al.*, 2004). Rai (2000) describes the conidia in *P. herbarum* cultures grown on malt extract as having an ovoid shape, and the conidia released from the pycnidia of this isolate are clearly ellipsoidal (Figure 5.8B). Based on these two characteristics, it can be concluded that the grey isolate was *P. glomerata* rather than *P. herbarum*. The evidence which unequivocally supported this assessment of the phenotypic characteristics exhibited by the grey isolate was the observation of *Alternaria*-like chlamydospores growing in branched chains (Figures 5.8C and 5.9). Both authors confirm that *P. herbarum* does not produce these reproductive structures, while *P. glomerata* does.

Based on the macro- and microscopic features described by Rai (2000) and Boerema *et al.* (2004) it is most likely that the grey isolate is *P. glomerata* and may be identified as such.

5.7 Conclusions

The growth of the white isolate in both wastewaters was inferior to the other two isolates, the grey isolate demonstrated the greatest biomass generation over the five day growth period. It was observed that Dam 4 exerted the greatest growth inhibition on all three fungal isolates.

The metal uptake capacity of the white isolate was particularly poor when compared to the other two fungal isolates, which could be attributed to its slow growth. The pink isolate possessed the ability to remove more nickel ions from solution than the other two isolates, but in contrast the grey isolate demonstrated a superior removal capability of the precious metals.

Based on financial considerations of the recovery of precious metals from solution and the enhanced ability to grow in wastewater from Dam 2 and Dam 4, further studies were carried out with the grey isolate alone and it was the only isolate selected for identification.

Amplification and sequencing of the ITS region of the grey isolate confirmed that the genus was most likely *Phoma*. Two possible species were returned by the BLAST search; *P. herbarum* and *P. glomerata* and a comparison of the morphological characteristics of the two species with the grey isolate needed to be undertaken.

Most of the morphological characteristics such as colour of the culture and shape of pycnidia and conidia suggested the grey isolate was more likely to be *P. glomerata* than *P. herbarum*. Furthermore, the presence of reproductive structures known as chlamydospores confirmed that the grey isolate was *P. glomerata*, as *P. herbarum* does not produce these structures.

The grey isolate was therefore identified as *P. glomerata*.

CHAPTER 6

PRECIOUS METAL REFINERY WASTEWATER TREATMENT BY *PHOMA GLOMERATA* AND REACTOR DESIGN

6.1 Introduction

The use of biological systems in the treatment of industrial wastewaters has become more and more recognised as improved technologies are developed. The utilisation of fungi in an industrial setting is gaining popularity and currently fungi are used in a variety of treatment technologies. Probably the most popular are white rot fungi, as they express ligninolytic enzymes which have the capability of breaking down a wide range of xenobiotic compounds including organic molecules and dyes (Kasinath *et al.*, 2003; Ehlers and Rose, 2005; Hai *et al.*, 2006), however other fungal species are being used to recover metals (Kapoor and Virahaghavan, 1997) and remove nitrogenous substances from solution (Hwang *et al.*, 2007). Fungi are largely an untapped resource with regard to wastewater treatment and new applications for their use are continuously being discovered (Gadd, 2000).

6.2 Hypothesis

Wastewater housed in Dams (reservoirs) 2 and 4 at Anglo Platinum precious metal refinery (Rustenburg, South Africa) contain metal ions and high concentrations of various salts and organic compounds. The toxicity of the metal ions will inhibit the treatment of the wastewater by conventional biological methods; therefore the metal must be removed before further treatment can be undertaken. *Phoma glomerata* has been shown to survive and grow in both wastewaters, in conjunction with its metal uptake capability. A fungal bioreactor may be a suitable pre-treatment step before further wastewater treatment is undertaken.

6.3 Aims and objectives

The aims of this chapter were to ascertain whether *P. glomerata* could grow in wastewaters from Dams 2 and 4 in a continuously fed bioreactor and to assess the quality of the pre-treated wastewaters. In order to achieve these aims the following objectives were set:

- Determine the optimal nutrient regime for *P. glomerata* grown in wastewater through comparing the effect of different carbon and nitrogen sources on biomass dry weight.
- Determine whether nutrient supplementation was needed in order for *P. glomerata* to grow in the two wastewaters based on biomass dry weight

- Monitor the change in ammonium, calcium, chloride and sodium ion concentration in the wastewaters during fed batch treatment with *P. glomerata* through the use of spectrophotometer kits
- Design and run a fungal packed bioreactor to determine if *P. glomerata* may be used as a pre-treatment step in biological treatment of wastewater from Dams 2 and 4; and monitor the concentrations of COD, ammonium, calcium, chloride and sodium ions while doing so.

6.4 Materials and methodology

Unless otherwise stated, all growth media, reagents (reagent grade) and test kits were Biolab or Saarchem brand, supplied by Merck Chemicals (Pty) Ltd (South Africa) and used as received.

6.4.1 Determination of optimum carbon and nitrogen sources for P. glomerata *growth in precious metal refinery wastewaters*

Stock solutions of *P. glomerata* were grown in malt extract broth (Section 5.4.1) and 3 ml of log phase culture were inoculated into minimal medium (Stajić *et al.*, 2006). The minimal medium was supplemented with 15 % (w/v) of carbon or 3.5 % (w/v) of nitrogen to determine the optimum carbon and nitrogen source for fungal growth and whether the wastewaters from Dam 2 and 4 were being utilised as a carbon or nitrogen source by *P. glomerata*. The carbon sources used were glucose, sucrose and mannitol, while the nitrogen sources investigated were yeast extract, ammonium nitrate and urea. Wastewater from each dam was added separately to the media to generate a final concentration of 25 % (v/v), then media were sterilised under UV light for 1 hour before being inoculated with *P. glomerata*. Minimal medium was prepared in such a way as to maintain the initial concentrations of the trace elements, carbon and nitrogen after addition of the wastewater. All investigations were carried out in Erlenmeyer flasks in triplicate, at 28 ℃ with aeration maintained by shaking at 150 rpm on a bench top Orbital shaker. After five days, biomass was harvested by filtering through pre-dried and weighed Whatman no. 1 filters and dried for 24 hours at 80 ℃ in order to ascertain the fungal dry weight. The dry weight of each culture was used as an indication of optimal carbon and nitrogen sources.

Combinations of carbon and nitrogen sources

All possible pairwise combinations of carbon and nitrogen sources were also investigated. Minimal medium was supplemented with each wastewater (as described above) and pairs of carbon and nitrogen sources. Three millilitres of *P. glomerata* stock culture were used to inoculate each medium (in triplicate). Cultures were grown and harvested as indicated above. Once the carbon and nitrogen combination generating the maximum fungal growth was ascertained, the optimum ratio of carbon to nitrogen was determined. A minimal medium and wastewater mixture was

supplemented with three concentrations of carbon (5, 10 and 20 mg.l^{-1}) and five concentrations of nitrogen, in triplicate and sterilized under UV light for 1 hour. Nitrogen concentrations were added as ratios of the carbon concentrations used (C:N of 10:1, 20:1, 40:1, 80:1, 160:1). Three millilitres of *P. glomerata* stock solution were used to inoculate each flask and growth maintained as described above. After five days the biomass was harvested as mentioned previously to determine the optimum carbon and nitrogen ratio based on the growth of the culture. Lastly, the effect of increased concentrations of wastewaters on *P. glomerata* growth was determined. Minimal medium with the optimum carbon and nitrogen combination at the most productive ratio was supplemented with 25, 50, 75 and 100 % (v/v) of each wastewater before sterilization under UV light for 1 hour. Biomass was inoculated, grown and harvested after 5 days as described above.

6.4.2 Pre-treated wastewater analysis

Batch test

Batch analysis of the treatment of wastewaters from Dam 2 and Dam 4 by *P. glomerata* was carried out in 500 ml Erlenmeyer flasks. Optimal minimal medium, determined from the previous investigation, was supplemented with 25 % (v/v) wastewater and sterilised with UV light. Three millilitres of *P. glomerata* stock culture were used to inoculate the minimal medium and wastewater in triplicate and growth conditions maintained as described above for six days. The concentrations of ammonia, calcium, chloride and sodium in the bulk liquid were determined daily with the use of spectrophotometric kits (Spectroquant, Merck) based on standard methods 4500-NH_3 D (ammonium), 3500-Ca B (calcium) 4500-Cl^- E (chloride), adapted from 4500-Cl^- E (sodium) (APHA *et al.*, 1998).

Reactor operation

Three reactor designs were considered for wastewater treatment by *P. glomerata*: a membrane bioreactor, fluidised bed reactor and packed bed reactor. The membrane bioreactor was deemed inappropriate as sophisticated chemical and physical backwashing systems would be required to prevent fouling of the membrane due to the fungal hyphae attachment and EPS production (Hai *et al.*, 2006; 2007). The fluidised bed reactor was not utilised as the *P. glomerata* biomass could not adhere to the surface of the Kaldnes K1 selected as the support. The bioballs chosen as support material for the packed bed reactor allowed for sufficient entrapment and subsequent growth of the biomass without significantly reducing the volume of the reactor. Each bioball had a volume of 3 ml, while maintaining a high surface area.

Two identical laboratory-scale bioreactors made from Perspex with working volumes of 9 l were constructed and packed with bioball filter packing material (Coralife, South Africa) (Figure 6.1). Air was introduced in the bottom of each reactor through a perforated Perspex tube, the packing

material was separated from the air sparger by a perforated Perspex plate which ensured even air distribution. An additional perforated plate was fitted directly above the bioballs to ensure that they remained submerged after the addition of the reactor seed. Influent was introduced at the bottom of the reactor and effluent collected from the top. The packed volume and total void volume were 0.73 l and 8.27 l respectively. The reactors were operated without sterilization in continuous mode, at ambient temperature, with a hydraulic retention time (HRT) determined from the batch analysis (4 days). Each reactor was inoculated with equal volumes of *P. glomerata* stock solution and operated in batch mode for five days until equal colonisation of the packing material was observed.

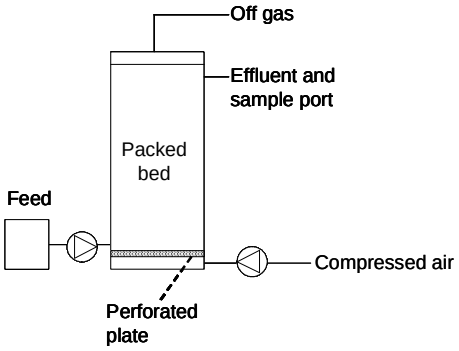

Figure 6.1 Schematic representation of one packed bed reactor.

One reactor was then fed continuously with wastewater from Dam 2 and the other with wastewater from Dam 4. The reactor feed was added to the reactor unsterilised with the feed tank being topped up every eight days from eight 25 l drums containing each wastewater. The influent and effluent concentrations of ammonia, chloride, sodium, calcium and COD were monitored twice during each HRT through the use of spectrophotometric kits described above (standard method equivalent COD test; 5220-COD D (APHA *et al.*, 1998)), in addition the change in pH was monitored with a pH meter. Influent salt concentrations varied depending on the storage drum used. Table 6.1 lists the minimum and maximum concentration of each analyte in the influent.

Table 6.1 Maximum and minimum ammonia, calcium, chloride, sodium and COD concentrations of the Dam 2 and Dam 4 wastewater influent.

Wastewater		Analyte concentration ($g.l^{-1}$)				
		Ammonium	**Calcium**	**Chloride**	**Sodium**	**COD**
Dam 2	**max**	1.16	52.50	115.20	62.00	128.00
	min	0.45	16.80	37.60	26.20	89.00
Dam 4	**max**	0.58	41.20	158.40	151.00	187.00
	min	0.27	28.20	97.60	73.20	84.00

Figure 6.2 depicts the two packed bed reactors on the left, and packing material on the right hand side of the image.

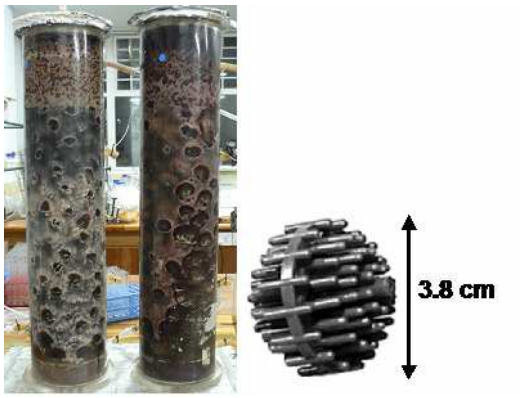

Figure 6.2 Packed bed reactors for wastewaters from Dams 2 and 4 (left) packed with bioballs (right) and inoculated with *P. glomerata*.

6.5 Results

6.5.1 Determination of optimum carbon and nitrogen sources for P. glomerata *growth in precious metal refinery wastewaters*

Each wastewater was assessed for its ability to act as a carbon and nitrogen source by eliminating either component from the minimal medium. Figure 6.3 illustrates the growth of *P. glomerata* in each type of medium and wastewater when compared to wastewater supplemented with the complex malt extract medium. From the difference in fungal mass at the end of the analysis period, it appeared that Dam 2 acted predominantly as a nitrogen source, while Dam 4 was observed to provide the carbon requirements for growth.

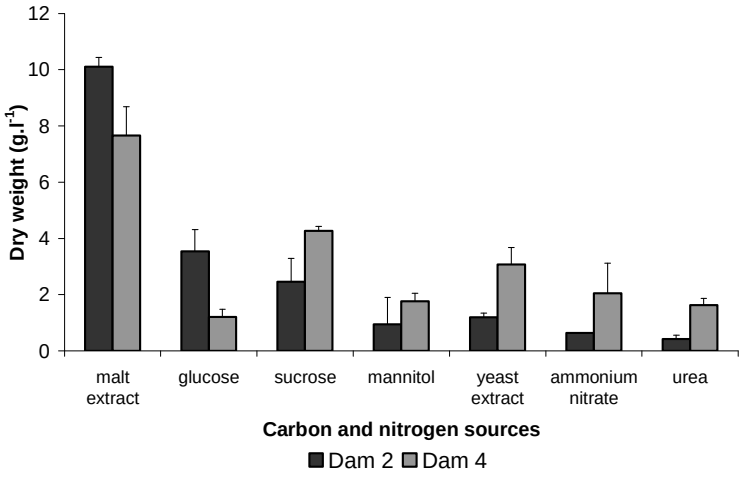

Figure 6.3 Growth of *P. glomerata* in 25 % (v/v) wastewater from Dam 2 and Dam 4 in complex medium (malt extract) and minimal medium supplemented with various carbon and nitrogen sources. Error bars indicate standard deviation (n=3).

The preferred carbon source in Dam 2 wastewater was glucose, and sucrose supplementation resulted in more growth in wastewater from Dam 4. In both cases, yeast extract was the optimum nitrogen source for *P. glomerata* growth.

The combinations of all the carbon and nitrogen sources were assessed to determine if certain combinations of carbon and nitrogen sources resulted in better growth. Table 6.2 lists the mass of the *P. glomerata* after the growth period in wastewater and minimal medium supplemented with combinations of the three carbon and nitrogen sources. The highest biomass growth was observed in medium and Dam 2 wastewater supplemented with sucrose and yeast extract, at a final concentration of 10.90 $g.l^{-1}$, this biomass concentration was comparable to that of *P. glomerata* in wastewater and complex malt extract broth (10.11 $g.l^{-1}$).

Table 6.2 *P. glomerata* biomass generation in 25 % (v/v) wastewaters from Dams 2 and 4 in minimal medium supplemented with different combinations of carbon and nitrogen sources.

Combination of carbon and nitrogen souces	**Fungal growth ($g.l^{-1}$)** mean values ± standard deviation (n=3)	
	Dam 2	**Dam 4**
malt extract	10.11 ± 0.32	7.66 ± 1.03
glucose and yeast extract	10.47 ± 1.07	5.24 ± 0.35
sucrose and yeast extract	10.90 ± 1.42	5.62 ± 1.90
mannitol and yeast extract	3.33 ± 1.13	4.63 ± 0.52
glucose and ammonium nitrate	4.29 ± 0.92	1.98 ± 1.05
sucrose and ammoinium nitrate	2.29 ± 0.90	2.50 ± 0.37
mannitol and ammonium nitrate	3.49 ± 1.78	3.22 ± 0.76
glucose and urea	0.99 ± 0.32	1.65 ± 0.33
sucrose and urea	1.38 ± 0.09	1.79 ± 0.36
mannitol and urea	2.24 ± 2.05	1.70 ± 0.92

Similarly, growth of *P. glomerata* in wastewater from Dam 4 supplemented with sucrose and yeast extract resulted in the highest generation of biomass, 5.62 $g.l^{-1}$, however, this was lower than the biomass generated in malt extract broth (7.66 $g.l^{-1}$). Due to the enhanced growth in the combination of sucrose and yeast extract when compared to the other carbon and nitrogen

sources with both wastewaters, all further investigations were carried out in minimal medium supplemented with sucrose and yeast extract.

The most suitable carbon concentration and ratio of carbon to nitrogen in the wastewaters were also assessed. Figure 6.4 illustrates the biomass growth at different initial sucrose concentrations and yeast extract concentrations in wastewater from Dam 2.

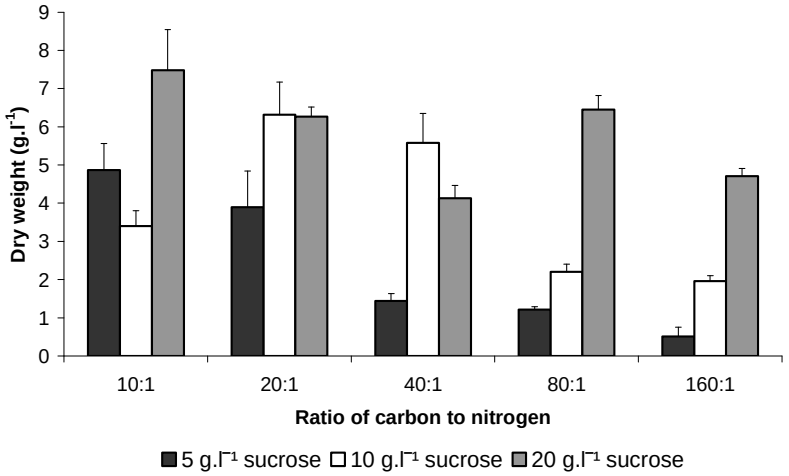

Figure 6.4 Biomass growth in 25 % (v/v) wastewater from Dam 2 supplemented with sucrose and yeast extract at various initial carbon and nitrogen concentrations. Error bars indicate standard deviation (n=3).

As anticipated, higher concentrations of sucrose allowed more growth to be achieved, and as the nitrogen concentration was decreased, *P. glomerata* growth appeared to be repressed. Similar observations were made with regard to the growth in wastewater from Dam 4 (Figure 6.5).

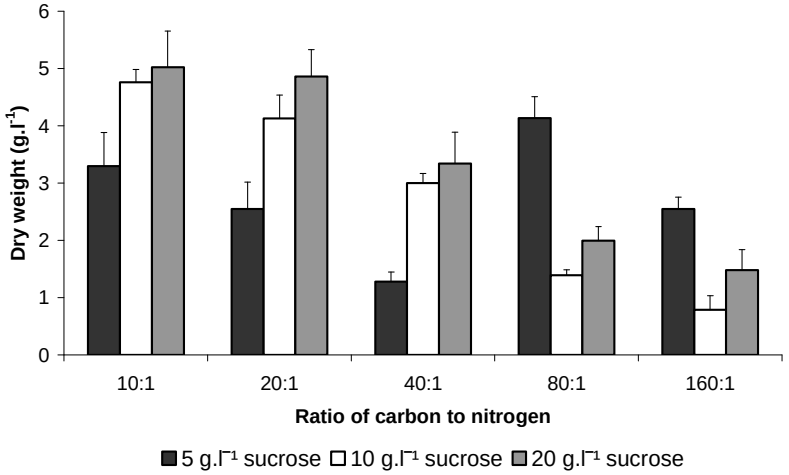

Figure 6.5 Biomass growth in 25 % (v/v) wastewater from Dam 4 supplemented with sucrose and yeast extract at various initial carbon and nitrogen concentrations. Error bars indicate standard deviation (n=3).

There appeared to be enough nitrogen present in the wastewater itself to overcome the nitrogen limitation imposed at low concentrations.

Figures 6.4 and 6.5 show that 20 $g.l^{-1}$ sucrose and a C:N ratio of 10:1 resulted in the highest biomass concentrations in both wastewaters. As 20 $mg.l^{-1}$ of sucrose resulted in the most growth of *P. glomerata*, further investigations were undertaken with media supplementation at this initial carbon concentration. In both wastewaters, higher concentrations of nitrogen resulted in improved growth and 2 $g.l^{-1}$ of yeast extract was therefore added to the minimal medium as the nitrogen source.

In addition to the determination of ideal media components, the effect of wastewater concentration on *P. glomerata* growth was investigated. Proportions from 25 to 100 % (v/v) were used to determine the maximum amount of wastewater that the fungal isolate could withstand. Figure 6.6 illustrates the growth of *P. glomerata* in minimal medium supplemented with sucrose and yeast extract at various wastewater concentrations from both dams.

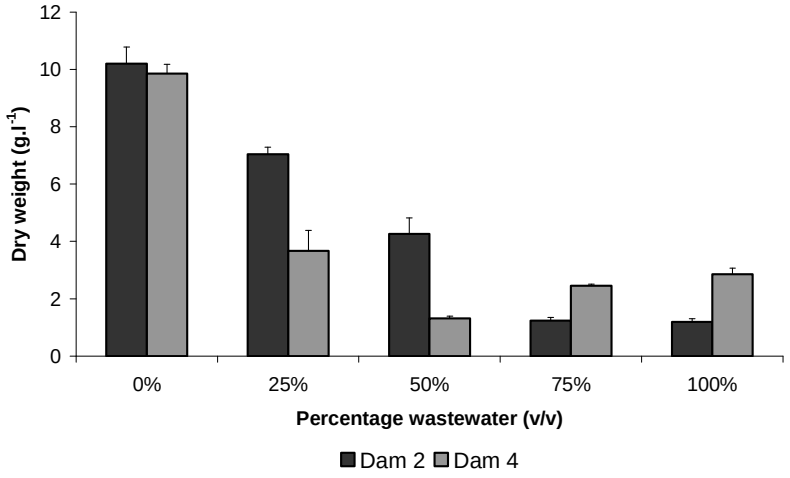

Figure 6.6 Growth of *P. glomerata* in minimal medium supplemented with sucrose and yeast extract and various concentrations of wastewater from Dams 2 and 4. Error bars indicate standard deviation (n=3).

Increasing the concentration of wastewater had a marked effect on fungal growth; however, growth was not completely inhibited by 100 % (v/v) wastewater.

6.5.2 Pre-treated wastewater analysis

Batch test

The batch test carried out in Erlenmeyer flasks revealed that after six days only the ammonium ion concentration had been significantly reduced (Table 6.3). In contrast, the concentrations of

calcium, chloride and sodium ions had removal efficiencies of less than 10 %, and almost no chloride ions were removed from Dam 4 wastewater.

Unfortunately, wastewaters from both dams contained compounds which interfered with metal analysis through ASS and inductively coupled plasma (ICP), and metal removal efficiencies could not be determined for either wastewater.

Table 6.3 Ammonia, calcium, chloride and sodium concentrations remaining in solution after batch flask treatment of 25 % (v/v) wastewaters from Dams 2 and 4 (supplemented with sucrose and yeast extract) by *P. glomerata* after six days (Mean values of triplicates. Standard deviation was 15 % of the mean.)

		Analyte ($g.l^{-1}$)			
	Day	**ammonium**	**calcium**	**chloride**	**sodium**
Dam 2	**0**	4.72	151.20	38.40	27.20
	6	0.01	139.60	36.70	26.90
	Removal efficency (%)	99.75	7.67	4.43	1.10
Dam 4	**0**	3.64	35.80	96.50	62.30
	6	0.02	34.40	96.10	60.30
	Removal efficency (%)	99.53	3.91	0.41	3.21

While the ion concentrations listed in Table 6.2 were those detected on the last day of the batch treatment, maximum ammonium ion removal had been achieved by the fourth day of the analysis and became the basis for determining the HRT to be used in the packed bed reactor as no further change in concentration was observed on days 5 and 6.

Reactor operation

Due to time constraints and lack of availablitity of wastewaters, only 10 HRTs could be undertaken. In contrast to the batch test, very little ammonia was removed from either of the wastewaters (Figure 6.7).

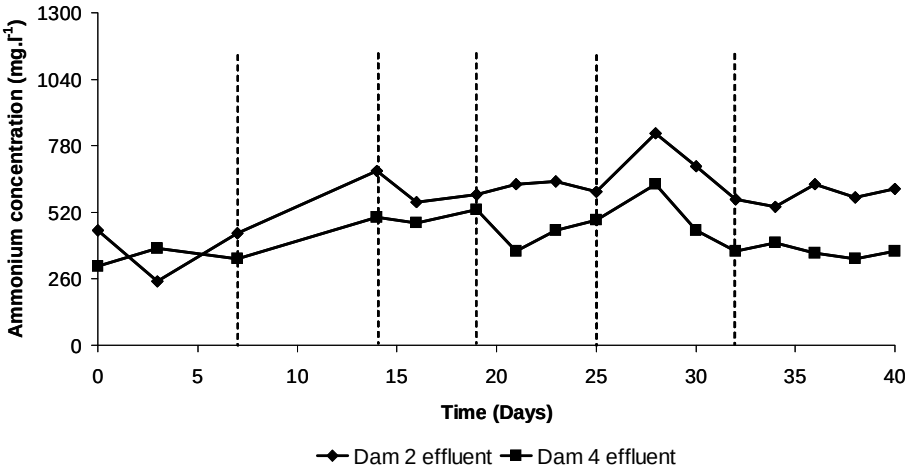

Figure 6.7 Mean ammonium concentration of wastewaters from Dams 2 and 4 before and after treatment by *P. glomerata*. Vertical lines indicate changes in influent concentration. Data points represent means of duplicate determinations. Standard deviation was less than 15 % of the mean.

The calcium concentration detected in the effluent of Dam 2 was lower than that detected in the influent, while the opposite was true for wastewater from Dam 4. Figure 6.8 depicts the erratic changes in calcium ion concentration over the 40 day period.

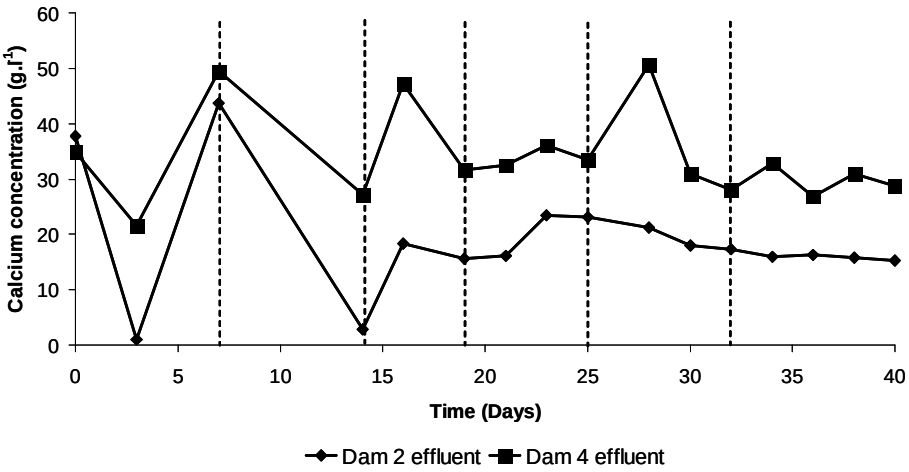

Figure 6.8 Mean calcium concentration of wastewaters from Dams 2 and 4 before and after treatment by *P. glomerata*. Vertical lines indicate changes in influent concentration. Data points represent the means of duplicate determinations. Standard deviation was less than 15 % of the mean.

The extreme variations in calcium concentrations were observed with wastewater from Dam 2 for the first few weeks but, by day 25 the effluent concentration appeared to have stabilised and a gradual decrease in calcium concentration was observed from day 30 to day 40. There was however a decrease in calcium concentration of the influent towards the end of the analysis period. Since *P. glomerata* could not have been generating calcium, it can be assumed that the influent

and effluent calcium concentrations were the same as each other, within the uncertainty of measurement of the test method used.

The chloride ion concentrations in both effluents (Figure 6.9) were erratic and the concentration detected in the effluent remained higher than the concentration of the influent. Once again an increase in chloride ion concentrations of both effluents was observed at day 16, when the influent concentration of Dam 2 wastewater changed from 115 to 145 $g.l^{-1}$ and Dam 4 wastewater changed from 150 to 184 $g.l^{-1}$, supporting the idea that effluent calcium concentration was dependent on influent concentration.

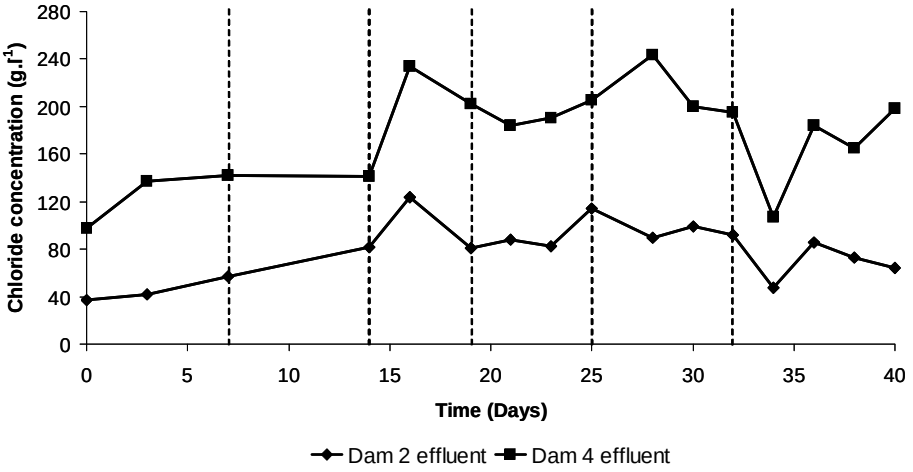

Figure 6.9 Mean chloride concentration of wastewaters from Dams 2 and 4 before and after treatment by *P. glomerata*. Vertical lines indicate changes in influent concentration. Data points represent means of duplicate determinations. Standard deviation was less than 15 % of the mean.

The removal of sodium did not occur in wastewater from Dam 2 at any point during the analysis (Table 6.3), however there was a reduction in sodium concentration in wastewater from Dam 4 within the first two weeks, after which sodium broke through in the effluent and concentrations became slightly erratic (Figure 6.10).

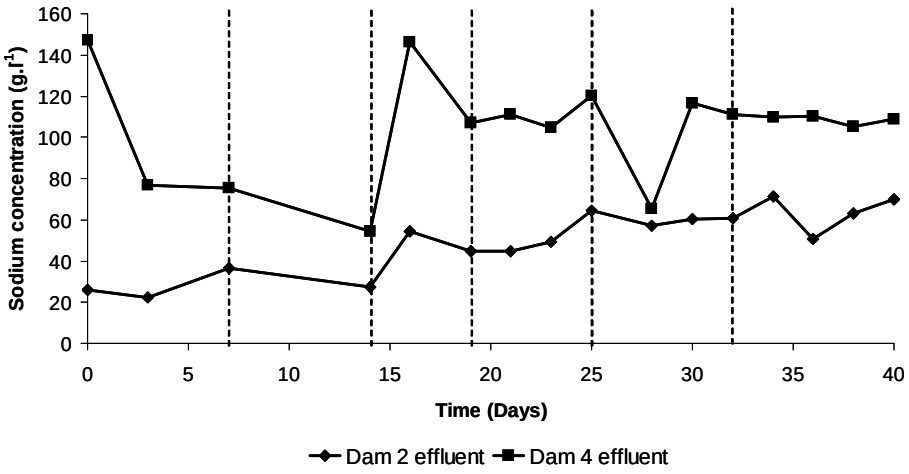

Figure 6.9 Mean sodium concentration of wastewaters from Dams 2 and 4 before and after treatment by *P. glomerata*. Vertical lines indicate changes in influent concentration. Data points represent the means of duplicate determinations. Standard deviation was less than 15 % of the mean.

The sodium in both wastewaters appears to pass through the reactors unused by *P. glomerata*. This was in contrast to the observations made with regard to the COD (Figure 6.10).

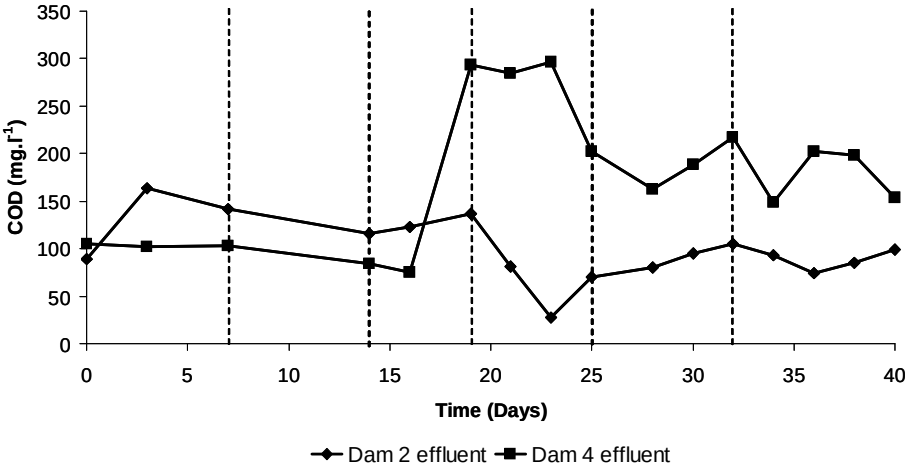

Figure 6.10 Mean COD concentration of wastewaters from Dams 2 and 4 before and after treatment by *P. glomerata*. Vertical lines indicate changes in influent concentration. Data points represent means of duplicate determinations. Standard deviation was less than 15 % of the mean.

While there was little COD removal in both reactors within the first two weeks of operation, from day 17 onwards there was a slight decrease in concentration of the effluent from both reactors when compared to the influents. A sharp increase in COD was observed in the influent of Dam 4 between days 16 and 19, corresponding with the use of wastewater from a different container in which the wastewaters were stored. Although the drums of wastewater used as influent were samples taken from the same point on the precious metal refinery site, at the same time, they were

found to be heterogeneous. Table 6.4 lists the concentration of each analyte in the influent and effluent, illustrating the heterogeneous nature of the wastewaters.

Table 6.4 Ammonium, calcium, chloride, sodium and COD concentrations of the influent and effluent and removal efficiency of the packed bed reactors on wastewaters from Dams 2 and 4.

Wastewater	Sample	Value	ammonium	calcium	chloride	sodium	COD
Dam 2	Influent ($g.l^{-1}$)	max	1.16	52.50	115.20	62.00	128.00
		min	0.45	16.80	37.60	26.20	89.00
	Effluent ($g.l^{-1}$)	max	0.83	43.60	124.00	71.40	164.00
		min	0.25	1.00	37.60	22.60	28.00
	Removal efficiency (%)	max	41.30	50.00	44.00	24.93	33.33
		min	12.31	-31.82	-41.54	-25.78	-49.47
Dam 4	Influent ($g.l^{-1}$)	max	0.58	41.20	158.40	151.00	187.00
		min	0.27	28.20	97.60	73.20	84.00
	Effluent ($g.l^{-1}$)	max	0.63	50.60	243.00	146.40	296.00
		min	0.31	27.20	97.60	54.40	75.00
	Removal efficiency (%)	max	0.01	27.27	6.38	40.94	35.26
		min	-53.13	-161.54	-39.992	-9.45	-37.34

Unfortunately, the removal of metal ions from the wastewater could not be monitored due to significant interference of components of the wastewater with the analytical techniques available. Despite this, metal removal from solution has been demonstrated (Chapter 5) and the growth of *P. glomerata* in both reactors at 100 % strength with no nutrient supplementation was observed. This point is discussed in the following section.

6.6 Discussion

6.6.1 Determination of optimum carbon and nitrogen sources for P. glomerata *growth in precious metal refinery wastewaters*

Monitoring the growth of *P. glomerata* biomass in the absence of a carbon or nitrogen source allowed for the determination of whether wastewater from Dams 2 and 4 were being utilised as a carbon or nitrogen source or both. In Figure 6.3 a considerable decrease in growth was observed when minimal medium with a single carbon or nitrogen source was compared to the complex medium, malt extract. This demonstrated that both a carbon and nitrogen source was required in both wastewaters to achieve maximum growth.

When each carbon and nitrogen source were compared, it was concluded that wastewater from Dam 2 was being utilised more as a nitrogen source than as a carbon source. This was based on the observation that more growth occurred in minimal medium supplemented with carbon than with nitrogen. In general, the opposite was true for wastewater from Dam 4, however, the most growth

was observed in media supplemented with sucrose, in opposition to the growth observed in media supplemented with glucose and mannitol (Table 6.2). The carbon and nitrogen sources resulting in the most growth for Dam 2 were glucose and yeast extract, while sucrose and yeast extract generated the most growth in Dam 4. (Figure 6.3)

The poor growth of *P. glomerata* in both wastewaters supplemented with mannitol may be due to its function within the cell. It has been shown to be involved in osmoregulation and regulating cytoplasmic pH, in addition it has been reported to interact with reactive oxygen species, regulate co-enzymes and facilitate carbohydrate storage (Vélëz *et al.*, 2007). These functions may prevent the molecule from being readily utilised as a carbon source by *P. glomerata* whereas sucrose and glucose are readily available. The improved fungal growth in the presence of yeast extract was probably due to the complex nature of the nitrogen source when compared to ammonia or urea. In addition to the nitrogen source, yeast extract contains other components of the yeast cell and this includes various carbohydrates, vitamins and other trace elements, many of which are known to be important microbial micronutrients. The least growth occurred in minimal medium supplemented with urea in both wastewaters. This is as a result of the fact that ammonia is used directly as a nitrogen source by microorganisms, while the nitrogen available in the form of urea needs to be reduced to ammonia before it can be utilised (Nwe and Stevens, 2004).

It was shown in Chapter 5 that wastewater from Dam 4 had a higher inhibitory effect on *P. glomerata* growth than wastewater from Dam 2 (Figure 5.1). This did not appear to be the case when comparing biomass growth in minimal medium supplemented with single carbon or nitrogen sources. The reason for this is most likely due to the nature of each wastewater. The components of Dam 2 were predominantly ammonium compounds and a number of chloride and sodium salts. In contrast Dam 4 contained high concentrations of organic molecules, ammonium, sodium and chloride ions. The presence of both ammonium ions and organic molecules would supplement the available carbon and nitrogen and result in increased growth in wastewater from Dam 4 when compared to Dam 2. This observation was confirmed when the growth of *P. glomerata* in combinations of carbon and nitrogen sources were compared. Once there was a carbon and a nitrogen source available the growth in wastewater from Dam 2 was almost double that recorded in Dam 4 wastewater with media supplemented with yeast extract and glucose or sucrose. Again, it was observed that mannitol, ammonia and urea additions generated the poorest *P. glomerata* growth for the reasons discussed above.

In contrast to the results obtained when minimal medium supplemented with single carbon or nitrogen sources were compared, the combination of sucrose and yeast extract resulted in the highest concentration of biomass after the analysis period in both wastewaters (Table 6.2). In

addition, when wastewater from Dam 2 and malt extract, and minimal medium and Dam 2 wastewater supplemented with the sucrose-yeast extract combination were compared, it was found that more biomass was obtained in the supplemented minimal medium than in the complex medium.

Based on the improved biomass growth in wastewaters from Dam 2 and 4 supplemented with sucrose and yeast extract it was decided that further investigations would be carried out in media supplemented with these carbon and nitrogen sources. The optimal carbon concentration for *P. glomerata* growth was assessed. In both Dams, the general trend indicated that as expected, higher concentrations of sucrose resulted in more biomass growth. In addition, the lower the yeast extract concentration, the less growth occurred. Gao *et al.* (2007) reported similar trends when reporting the optimal carbon concentration and carbon:nitrogen ratio for the growth of *Paecilomyces lilacinus* and *Metarhizium anisopliae*, however, the growth of a number of the other fungi investigated by the same authors was inhibited by increased carbon concentrations. This variation implies that carbon dosage and carbon:nitrogen ratios are specific for each fungal species. Results reported in this study were also affected by the carbon and nitrogen concentration of each wastewater.

Once the optimal nutrient regime for *P. glomerata* growing in each wastewater was established, the highest possible concentration of wastewater (supplemented with sucrose and yeast extract) which allowed biomass growth was determined through the comparison of biomass dry weight (Figure 6.6). It was observed that increasing the concentration of wastewater resulted in a decrease in *P. glomerata* growth. However, there seemed to be little difference in growth in wastewater concentrations of 75 and 100 % (v/v). This indicated that *P. glomerata* biomass could be used in a bioreactor to treat full strength wastewater from both dams, in addition, if the biomass were already established, it was probable that no nutrient supplementation would be required for *P. glomerata* survival and growth.

6.6.2 Pre-treated wastewater analysis

During the batch analysis of the wastewater treatment by *P. glomerata* it was observed that complete removal of ammonia occurred after four days in both wastewaters. In contrast the concentrations of calcium, chloride and sodium ions barely changed during the six day analysis period (Table 6.3). Unlike the in the batch study, *P. glomerata* was not able to decrease the ammonium concentration of the wastewaters in the packed bed reactor. Conversely, other fungi have been shown to reduce the ammonium concentration of several wastes. Eighty percent of the ammonia detected in landfill leachate was removed by *A. niger* (Ellouze *et al.*, 2007), while the same fungus removed all the ammonia present in a synthetic solution in a continuous fixed-slab

bioreactor (Hwang *et al.*, 2004). Unfortunately similar removal efficiencies were not observed in either of the packed bed reactors in this study and there was limited removal of calcium, chloride, sodium or COD from the wastewaters, indicating that *P. glomerata* would not be a suitable choice for complete treatment of the precious metal refining wastewaters. This result had been anticipated, as metal mining and refining wastewaters are notoriously recalcitrant in terms of treatment for the removal of ammonia (Koren *et al.*, 2000), dissolved sulphur and other salts (Hansford *et al.*, 2004) and low pH (Swalaha *et al.*, 2002). The objective of this section of the work was therefore not to achieve a complete treatment process in a single unit operation, but to evaluate the treated effluent in terms of downstream treatments and/or reuse options.

According to the South African Water Quality Guidelines, industrial water intended for reuse fall into four categories. The first category includes processes that require high quality water with strict specifications. Production of this quality of water requires specialised technology and as a consequence is generally costly. Water which falls into the first category is utilised in the pharmacy and petrochemical industry and in phase separation. The second category requires water of an intermediate standard between category 1 and domestic potable water, the specifications for category 2 water are slightly more stringent than for potable water; however, standard technology may be employed to ensure water quality criteria are met. Water in this category is used for humidification, gas cleaning, solution cooling and production dairy products and beverages. The third category includes domestic quality water, minimum treatment is required to obtain a sufficient water quality. Water in the third category may be used in the food industry, domestic use, surface washing, transport and dilution agent and for reaction vessel washing. In the fourth category, any water may be used provided it does not cause any operational problems such as corrosion or scaling. Water which falls into category 4 may be used for ash quenching, as a transport agent, in fire fighting and irrigation (Department of Water Affairs and Forestry, 1996).

Based on the DWAF guidelines, water containing salt concentrations above 200 $mg.l^{-1}$ would require extensive treatment before being reused in any process. The high concentrations of calcium contribute to the total hardness of the water and in excess may result in corrosion, scaling, precipitation and blockages, while the presence of chloride ions may cause severe corrosion and pitting of stainless steel, these effects are exacerbated by the presence of high concentrations of sodium ions in the solution. The high salt concentrations make the effluent unsuitable for reuse without further treatment. The COD concentration of each wastewater falls into the category of significant damage for all water applications in categories 1 to 3; however, wastewater with these COD concentrations (approximately 90 and 140 $mg.l^{-1}$ for Dams 2 and 4 respectively) may be used as process water and utility water in category 4 (Department of Water Affairs and Forestry, 1996).

This indicates that the salt concentration would be the focus for a downstream treatment step to generate reusable water. There are several methods available for treatment of wastewaters such as these. Chen *et al.* (2008) demonstrated that COD and ammonium could be removed from landfill leachate through the use of a moving-bed biofilm reactor with an anaerobic and aerobic component. Anaerobic microorganisms were responsible for removal of 92 % of the COD present in the leachate, while the aerobic component reduced the ammonium concentration by more than 97 %. In addition, immobilised white rot fungi have been shown to reduce phenol and chlorophenol concentrations due to the expression of lignin degrading enzymes (Elhers and Rose, 2005). Most investigations into the removal of chloride from wastewaters are carried out as chloride in the form of chlorophenols (Zhang and Nicell, 2000; Elhers and Rose, 2005; Majumder and Gupta, 2007) however, chloride ions may be liberated from wastewaters through electrolysis and release of chlorine gas at the anode, provided that the conductivity and chloride concentrations are high enough (Department of Water Affairs and Forestry, 1996). While there are few reports with regard to the removal of sodium ions from wastewaters, the removal of calcium from wastewater has been shown through the use of ureolytic microbiological carbonate precipitation (Hammes *et al.*, 2003). The authors reported 90 % calcium removal in semi-continuous reactors.

The poor wastewater treatment of *P. glomerata* would suggest that the biomass is unsuitable for remediation of precious metal refinery wastewaters; however, the fungal isolate was able to survive and grow in 100 % (v/v) wastewater. This, in conjunction with its ability to successfully remove metal ions from solution (Chapter 5), suggests that a packed bed reactor may be a suitable intermediate step in wastewater processing to alleviate metal toxicity to sensitive organisms in other processes.

Anglo Platinum supplied metal and salt concentrations detected in each wastewater in 2006. Table 6.5 lists the concentrations of each analyte monitored in this study.

Table 6.5 Concentrations of the analytes present in wastewaters from Dams 2 and 4 investigated in this study as supplied by Anglo Platinum. Readings were taken in 2006. NS indicates that data were not supplied

	Analyte	Analyte concentrations ($mg.l^{-1}$)	
		Dam 2	**Dam 4**
Salt	**ammonia**	14	824
	calcium	15940	32300
	chloride	42	137500
	sodium	35	54900
Metal	**copper**	107	13
	nickel	< 15	20
	gold	< 0.1	NS
	platinum	0.4	NS

The discrepancies between the concentrations supplied by Anglo Platinum and those detected in this study (Table 6.1) are most likely due to inconsistencies between analytic methods. Anglo Platinum determined the analyte concentrations with the use of inductively coupled plasma (ICP), while the concentrations in this study were determined spectrophotometrically.

The low metal concentrations reported to be in solution in the wastewater by Anglo Platinum are most likely as a result of metal precipitation. The guidelines laid out by DWAF state that metal concentrations in excess of 10 $mg.l^{-1}$ will have no significant effect on category 4 process water and based on the metal uptake by *P. glomerata* (Chapter 5) it is unlikely that these metals will remain in the wastewater and have any significant effect on reuse applications after treatment by immobilised fungal biomass.

6.7 Conclusions

Sucrose and yeast extract were shown to be the optimal nutrients for minimal medium and wastewater supplementation for the growth of *P. glomerata*.

The fungal isolate could grow in 100 % (v/v) wastewaters implying it would be an efficient pre-treatment option for the precious metal refinery wastewaters. It was found that the wastewaters were not altered enough by the *P. glomerata* biomass for reuse in any process without a further treatment step.

Although *P. glomerata* may not have remediated the water, based the on metal uptake capability of *P. glomerata* and its ability to grow in high concentrations of each wastewater, it is likely that *P. glomerata* may be utilised as an intermediate bioremediation step to reduce the metal toxicity for other biological processes. To do this, it is necessary to establish the toxicity threshold of refinery wastewater metals and the working limits of the process.

CHAPTER 7

BIOACCUMULATION OF COPPER AND NICKEL FROM SOLUTION BY *PHOMA GLOMERATA*

7.1 Introduction

Metals with a density above 5 $g.cm^{-3}$ are considered heavy metals, most are transition elements with d orbitals that are not completely filled. This characteristic provides heavy metals with the ability to form complex compounds and plays an important role in the functioning of trace elements in biochemical reactions. However, at high concentrations heavy metal ions form unspecific complexes within the cell, which has a toxic effect (Nies, 1999).

One of the most essential physiological heavy metals is copper (Pais and Jones, 1997). Copper ions are ordinarily involved in reactions with molecular oxygen and can undergo one redox reaction in the biological range (Kendrick *et al.*, 1992), -268 mV (Nies, 1999), to produce Cu^{+}. Only Cu^{+} and Cu^{2+} oxidation states are available for biological interactions (Kendrick *et al.*, 1992). Experiments undertaken to determine the toxicity of Cu^{+} and Cu^{2+} on phytoplankton showed that Cu^{2+} exerted a lethal effect at much lower concentrations than Cu^{+} (Crompton, 1997). Copper has a high bioaccumulative index and can accumulate in the food chain which poses a significant health risk to humans (Pais and Jones, 1997). The conductivity of copper makes it an important component of the electrical industry and mixed oxides have been shown to act as superconductors at temperatures below 50 °K. Its inertness is exploited for the production of water pipes and over 1000 different copper alloys are produced for various applications. In addition copper compounds are used in agriculture as fungicides (copper hydroxide) and insecticides (copper carbonate, -acetate, and -oxochloride) (Lee, 1991).

Another metal utilised in the production of alloys is nickel. It is added to steel to improve strength and resistance to chemical attack. Jet engines and gas turbines are made from the Nimonic series of alloys (75 % Ni with Al, Ti, Co and Cr) because of their ability to withstand high temperatures and stresses. Nickel alloys are also used in a variety of other applications such as production of imitation silver articles, Ni/Fe storage batteries and corrosive resistant coatings (Lee, 1991). Until 1975 nickel was thought to be a non-essential heavy metal and currently only four types of enzymes containing functionally important nickel are known (Kendrick *et al.*, 1992; Pais and Jones, 1997). This group of enzymes consists of ureases, hydrogenases, carbonyl dehydrogenase and methyl coenzyme M reductase cofactor F_{430} (Kendrick *et al.*, 1992) and is responsible for the catalysis of complex rearrangements (Nies, 1999).

Due to the extensive production of copper and nickel for use in industry generating large volumes of wastewater, and their potentially harmful effects on the environment, it is both commercially and environmentally advantageous to remove them from wastewaters produced during copper and nickel refining processes.

7.2 Hypothesis

At high concentrations copper and nickel will exert a toxic effect on *P. glomerata*; however, below the toxic threshold metal ions will be removed from solution and the extent of removal will be dependent on the initial metal ion concentration and pH.

7.3 Aims and objectives

The aims of this chapter included the determination of the toxicity threshold of *P. glomerata* for copper and nickel ions in solution and to identify the effects of initial metal ion concentration and pH on metal uptake and fungal growth. In order to achieve these aims, the following objectives were set:

- Measure the toxic effect of the metal ions as a function of respiration with the OxiTop® system.
- Determine the effect of initial metal concentration and pH on the uptake of metal by monitoring the metal ions remaining in solution through AAS.

7.4 Materials and methodology

Unless otherwise stated, all growth media and reagents (reagent grade) were Biolab or Saarchem brand respectively, supplied by Merck Chemicals (Pty) Ltd (South Africa) and used as received. All glassware was acid washed in 5 % HNO_3 for a minimum of 5 hours, rinsed in deionised water overnight and dried before use.

7.4.1 Copper and nickel toxicity

The toxicity of the copper and nickel ions (Section 4.4.2) was determined as a function of respiration in a closed system with the use of the OxiTop® method (WTW, Germany), a closed cell respirometer. A stock culture of *P. glomerata* was grown in malt extract broth at 28 ℃, with aeration maintained by shaking at 150 rpm on a bench top Orbital shaker, until mid log phase of growth. Three millilitres of the stock were used to inoculate 12 × 100 ml of minimal media (Section 6.5.1). In late log phase the cultures were transferred to 250 ml Schott bottles. Biomass and media were removed and metal ion solutions added to generate concentrations of 0, 10, 40, 75, 100 and 150 $mg.l^{-1}$ of metal with a final volume of 100 ml, in duplicate. Samples were fitted with pressure

sensors and incubated at 20 ℃ for 15 hours. Throug h the entrapment of carbon dioxide produced by the biomass on NaOH pellets, a decrease in pressure in the system could be observed and monitored as oxygen was removed from the gas phase. The pressure sensors within the device stored the data as the pressure was reduced over time and assuming ideality of the gas phase, pressure reduction was converted into specific oxygen uptake (SOU) using a derivation of the ideal gas law and Henry's law (Rudrum, 2005):

$$OUR = \left(-\frac{V_g}{V_1}\right) \times \left(\frac{1}{RT}\right) \times \left(\frac{dP_{O_2}}{dt}\right) \quad 7.1$$

Where:

V_g – Gas phase volume (l)

V_l – Liquid phase volume (l)

P_{O_2} – Partial pressure of oxygen (Pa)

T – Gas temperature (K)

t – Time (s)

R – Universal gas constant ($Pa.l.g^{-1}.K^{-1}$)

Specific oxygen uptake was calculated based on the unit mixed liquor suspended solids (MLSS) and expressed as $mgO_2.gMLSS^{-1}$. The SOU values for selected time points were selected and the SOUs for each metal at each concentration were represented as relative activity when compared to the control (0.00 $mg.l^{-1}$ metal).

$$RA = \left(\frac{SOU_{M(x)}}{SOU_C}\right) \quad 7.2$$

Where:

RA – Relative activity

$SOU_{M(X)}$ – SOU with metal concentration of x $mg.l^{-1}$

SOU_C – SOU control (0.00 $mg.l^{-1}$ metal concentration)

7.4.2 Copper and nickel uptake

Copper and nickel solutions were added to the double strength minimal medium selected in Section 6.5.1 for optimal growth of *P. glomerata*. The pH of the media was adjusted to 2, 4 and 6 (with 10.0 M NaOH) before making up to volume with the resultant metal concentrations being 25, 40 and 55 $mg.l^{-1}$ at each pH value in conical flasks, in triplicate. Metal solutions were then sterilised under UV light for an hour and inoculated with 3 ml of liquid fungal stock culture (Section 5.4.1). Controls included media alone, media and metal solution and media and *P. glomerata*. Cultures

were grown at 28 ℃ and aeration maintained by shak ing at 150 rpm on a bench top Orbital Shaker (Labcon).

Five millilitre samples were removed from each solution at predetermined time intervals for a period of 72 hours. An attempt was made to maintain homogeneity of the solution during sample extraction in order to generate a true representation of the ratio of media to biomass. Biomass and supernatant were then separated through centrifugation (5000 *g*) (Labofuge Ae, Heraeus sepatech, Germany). The pH and of the supernatant was measured with a pH meter before being filtered through 0.45 µm pore size nylon filters (Whatman), after which the pH was adjusted to below 2 with 1.0 M HNO_3. The concentration of metal ions remaining in solution was then determined by AAS. Biomass dry weight was determined as described in Section 5.4.2.

Results were expressed as units of dried cell mass (X_m: $g.l^{-1}$), bioaccumulated metal ion concentration at the end of growth (C_{acc}: $mg.l^{-1}$), specific metal uptake as a function of metal ions per unit of dry weight (q_m: $mg.g^{-1}$) and the percentage uptake efficiency defined as the concentration of bioaccumulated metal at the end of the analysis period divided by the initial metal concentration and multiplied by 100.

7.5 Results

7.5.1 Copper and nickel toxicity

Copper and nickel toxicity were determined as a function of *P. glomerata* respiration through the use of the OxiTop® system. Figure 7.1 illustrates the effect of increasing copper ions on the biomass respiration over a period of 14.5 hours represented as specific oxygen uptake (SOU, $mgO_2.gMLSS^{-1}$). It was anticipated that the higher concentrations of metal ions in solution would cause retardation of respiration and this was found to be the case at all concentrations, except at 10 $mg.l^{-1}$. An induction effect on respiration was observed at this low concentration.

The initial sharp increase in SOU was attributed to stabilisation of the pressure in the OxiTop® system before accurate pressure readings could be recorded.

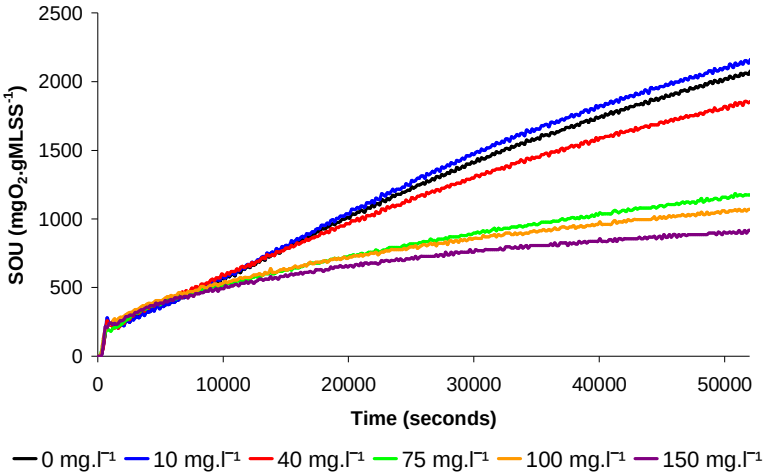

Figure 7.1 Specific oxygen uptake ($mgO_2.gMLSS^{-1}$) of *P. glomerata* exposed to increasing concentrations of copper ions over a period of 14.5 hours at 20 ℃.

Table 7.1 lists the relative activity of each copper concentration when compared to the control selected at three time intervals during incubation of *P. glomerata* with the copper ions.

Table 7.1 The mean relative activity of each copper concentration compared to the control at three time intervals during *P. glomerata* incubation with the copper ions at 20 ℃ (in duplic ate).

Initial metal concentration ($mg.l^{-1}$)	Relative activity $\left(\frac{SOU_{M(X)}}{SOU_C}\right)$		
	3 hours	8.5 hours	14 hours
0	1.00	1.00	1.00
10	1.01	1.04	1.04
40	1.03	0.92	0.90
75	0.90	0.63	0.57
100	0.92	0.59	0.51
150	0.85	0.53	0.44

The induction effect of 10 $mg.l^{-1}$ of copper was observed during the entire incubation period (values above 1). While 40 $mg.l^{-1}$ appeared to increase respiration initially, a toxic effect was observed after a few hours. There was very little difference in the toxic effect of copper at 75 and 100 $mg.l^{-1}$; however, a marked decrease in respiration at 150 $mg.l^{-1}$, when compared to the control, was observed from the beginning, indicating extreme acute toxicity.

In contrast, the presence of nickel in solution had no induction effect on the respiration of *P. glomerata*, and appeared to have a greater toxic effect than copper. Figure 7.2 illustrates the increased toxic effects of increasing nickel concentrations when compared to the control and to the corresponding copper concentrations.

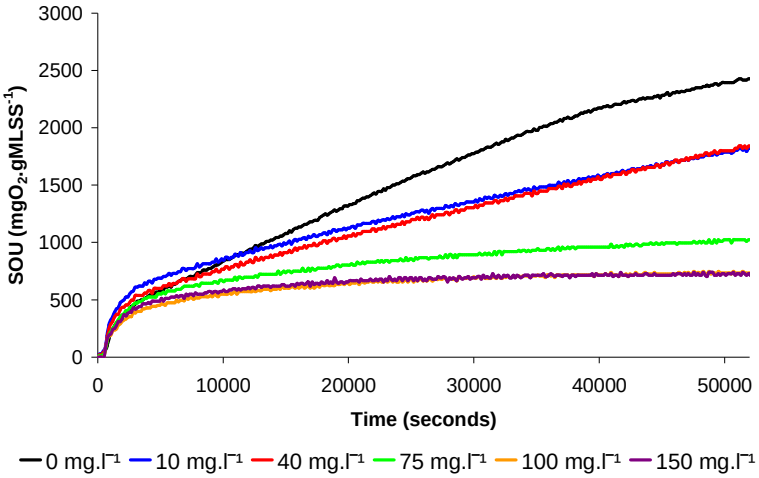

Figure 7.2 Specific oxygen uptake ($mgO_2.gMLSS^{-1}$) of *P. glomerata* exposed to increasing concentrations of nickel ions over a period of 14.5 hours at 20 ℃.

Initially an increase in respiration at 10 $mg.l^{-1}$ was observed, however, toward the end of the incubation period, there was little difference between the toxicity of nickel at 10 and 40 $mg.l^{-1}$. A marked decrease in SOU was observed at 75 $mg.l^{-1}$ when compared to 10 and 40 $mg.l^{-1}$; however the greatest toxic effect was recorded at 100 $mg.l^{-1}$ as there was no difference between respiration at 100 and at 150 $mg.l^{-1}$. Table 7.2 lists the relative activity of the nickel concentrations at 3, 8.5 and 14 hours during *P. glomerata* incubation.

Comparison of the relative activity of the copper and nickel ions resulted in confirmation of the increased nickel toxicity observed by the curves generated at each concentration in Figures 7.1 and 7.2. After 14 hours the relative activity of 150 $mg.l^{-1}$ of copper was determined to be 0.44, while a similar relative activity for nickel was recorded at a concentration of 75 $mg.l^{-1}$.

Table 7.2 The mean relative activity of each nickel concentration compared to the control at three time intervals during *P. glomerata* incubation with the nickel ions at 20 ℃ (in duplic ate).

Initial metal concentration ($mg.l^{-1}$)	Relative activity $\left(\frac{SOU_{M(X)}}{SOU_C}\right)$		
	3 hours	8.5 hours	14 hours
0	1.00	1.00	1.00
10	1.00	0.76	0.75
40	0.90	0.73	0.75
75	0.77	0.49	0.43
100	0.64	0.38	0.31
150	0.68	0.38	0.30

The mass of *P. glomerata* after the fifteen hours increased in the presence of both metal ions at all initial concentrations. However, since the effect of 10 and 40 $mg.l^{-1}$ of nickel (Table 7.2) on the

SOU of *P. glomerata* was so similar and 10 mg.l^{-1} of copper generated an induction effect, 25 and 40 mg.l^{-1} of initial ion concentrations were selected for further metal uptake investigations. The third initial metal ion concentration used was 55 mg.l^{-1}. This concentration was selected because the increased toxicity of nickel when compared to copper and the relative activity at 75 mg.l^{-1} of copper being almost half that at 40 mg.l^{-1} (Table 7.1) both observations suggested that concentrations closer to 70 mg.l^{-1} would not result in sufficient growth for metal uptake.

These three concentrations were then used to group the metal uptake analyses in order to make comparisons between data clear.

7.5.2 Copper and nickel uptake

Copper uptake

The maximum growth (X_m), bioaccumulated metal (C_{acc}), specific uptake (q_m) and highest uptake efficiency observed during the analysis period are reported in Table 7.3. It should be noted that the maximum values for each of these parameters did not occur at the same time, as toward the end of the analysis copper ions were released back into solution while the biomass continued to gain mass, resulting in an increase in biomass concentration (Appendix D, Figures D1 to D3) and a decrease in bioaccumulated metal (Appendix D, Figures D4 to D6), specific uptake (Appendix D, Figures D7 to D9) and uptake efficiency (Appendix D, Figures D10 to D12).

Table 7.3 Biomass concentration, bioaccumulated metal, specific uptake and uptake efficiency of *P. glomerata* in copper solutions at various initial metal ion concentrations and pH values, grouped into three concentration ranges.

Metal ion grouping (mg.l^{-1})	pH	Initial metal ion concentration (mg.l^{-1}) mean values ± standard deviation (n=3)	X_m (g.l^{-1})	C_{acc} (mg.l^{-1})	q_m (mg.g^{-1})	Uptake efficiency (%)
25	**2**	32.25 ± 0.37	8.01	15.93	17.79	49.38
	4	31.59 ± 2.53	7.23	31.59	8.58	100.00
	6	31.50 ± 1.77	7.87	31.5	8.11	100.00
40	**2**	43.28 ± 2.24	5.97	20.16	12.38	46.58
	4	43.25 ± 2.02	5.94	36.55	10.36	84.50
	6	44.57 ± 0.44	6.67	43.66	11.67	97.94
55	**2**	60.28 ± 0.85	8.79	29.86	17.03	49.54
	4	58.44 ± 5.35	5.69	46.48	12.76	78.47
	6	54.80 ± 3.37	4.89	46.48	12.91	89.05

Within each initial metal ion concentration group (25, 40 and 55 mg.l^{-1}), an increase in bioaccumulated metal and uptake efficiency with increasing pH was observed. With initial copper concentrations of 31.50 mg.l^{-1} and 54.80 mg.l^{-1} (at pH 6 in the 25 mg.l^{-1} and 55 mg.l^{-1} group,

respectively) the total amount of bioaccumulated metal was 31.50 $mg.l^{-1}$ and 46.48 $mg.l^{-1}$ respectively. However, copper uptake efficiency was reduced by 20.95 % at the higher concentration.

The highest specific uptake value was calculated to be 17.97 $mg.g^{-1}$ at 25 $mg.l^{-1}$ initial copper concentration, pH 2, while the lowest was 8.11 $mg.g^{-1}$ at the same initial copper concentration at pH 6. In contrast, the uptake efficiencies in each case were 49.38 and 100.00 % respectively. This reduction in specific uptake with increased uptake efficiency is due to the improved biomass growth at pH 6 when compared to pH 2. Specific uptake is related to the concentration of biomass within the system. If there is a larger biomass concentration, the specific uptake value will decrease, and vice versa. In a system in which the biomass concentration does not change, the specific uptake provides an ideal means with which to compare removal of different metals by different biomass; however, when the biomass concentration changes continuously (as with a living system), a lower specific uptake value does not correlate with the uptake efficiency or metal removal. In this case the highest specific uptake values were attained at pH 2, where the slowest initial biomass growth occurred (Appendix D, Figures D1 to D3). This results in the impression that the biomass is able to remove more ions from solution at pH 2 than pH 6, however half the concentration of copper ions accumulated at pH 6 were accumulated at pH 2.

No correlation between the concentration of biomass at the end of growth and the various initial metal concentrations and pH values (Table 7.3) was observed. However, pH had a distinct effect on the morphology of the fungi growing in the copper solutions; at pH 2 a yellow pigment was produced which turned red and grew darker with time. Even though biomass growing at pH 2 had a greater mass at the end of the analysis period, the volume of biomass was less than that growing at pH 4 and 6. In addition, the biomass at pH 2 grew in small, dense mycelial balls which settled when the flask was not being shaken, rather than the evenly distributed, loose mycelial strands more typical of the species.

A more accurate representation of the effect of pH and initial metal ion concentration on the fungal growth may be obtained by comparing the length (hours) of the lag phase and the fungal mass at the end of lag phase (Appendix D, Figures D1 to D3). Table 7.4 summarises the effects of initial metal ion concentration and pH on biomass growth during lag phase. The lag phase of the biomass growing in copper was found to be 12 hours irrespective of the initial copper concentration.

Table 7.4 The effect of initial copper ion concentration and pH on the biomass concentration of *P. glomerata* at the end of the lag growth phase.

Initial metal concentration ($mg.l^{-1}$)	Lag phase (hours)	Fungal mass ($g.l^{-1}$) mean values ± standard deviation (n=3)		
		pH		
		2	4	6
25	12	0.49 ± 0.05	0.56 ± 0.12	0.77 ± 0.08
40	12	1.07 ± 0.02	1.13 ± 0.21	1.36 ± 0.21
55	12	1.27 ± 0.10	1.33 ± 0.16	1.61 ± 0.23

Increasing the initial metal concentration resulted in increased fungal growth; at pH 2, 0.49 $g.l^{-1}$ of biomass was present in a solution containing 25 $mg.l^{-1}$ of copper and 1.27 $g.l^{-1}$ of biomass in 55 $mg.l^{-1}$ of copper. In addition, the higher the pH, the more growth was observed; 1.07 $g.l^{-1}$ vs. 1.36 $g.l^{-1}$ of biomass in 40 $mg.l^{-1}$ copper solutions at pH 2 and 6 respectively (Table 7.4).

Of the three initial metal concentrations, 40 $mg.l^{-1}$ was the only one which resulted in a lower biomass concentration after the analysis at pH 2 than pH 4 or 6 (5.79, 5.94 and 6.67 $g.l^{-1}$ respectively), in contrast to the trend observed at 25 and 55 $mg.l^{-1}$ (Table 7.3). Figure 7.3 illustrates the change in pH during the analysis period of the biomass growing in an initial metal concentration of 55 $mg.l^{-1}$. At approximately 40 hours the pH of the samples with an initial pH of 2 began to increase, a similar occurrence could also be observed with the initial metal ion concentration of 25 $mg.l^{-1}$ (Appendix D, Figure D13). This increase in pH corresponded directly with the point at which the biomass growth at pH 2 exceeded that at pH 4 and 6 (Appendix D, Figures D1 and D3).

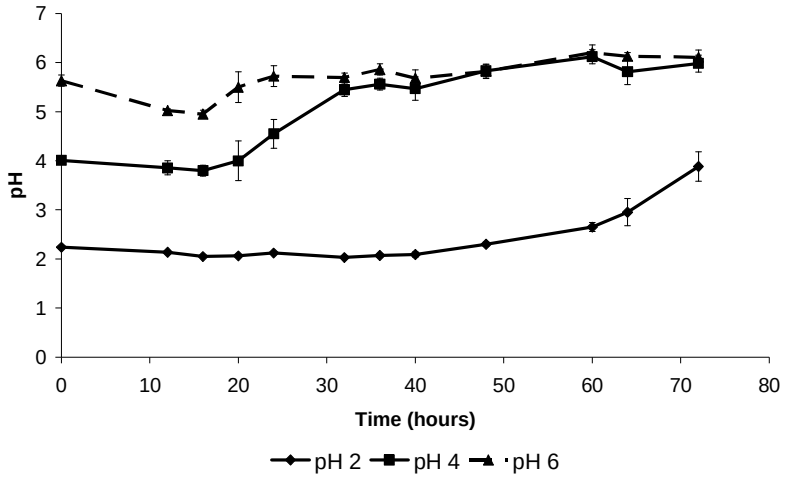

Figure 7.3 Change in pH over time due to the growth of *P. glomerata* in the copper solution containing an initial copper concentration of 55 $mg.l^{-1}$ at an initial pH of 2, 4, or 6. Error bars indicate standard deviation (n=3).

The pH of the 40 mg.l^{-1} copper solution with an initial pH of 2 did not change significantly (Appendix D, Figure D14) and the rate at which the biomass concentration increased remained below that of the control and the initial pH values of 4 and 6 (Appendix D, Figure D14).

At all initial copper ion concentrations the pH of the solutions with an initial pH of 4 and 6 decreased slightly in the first 12 hours as the biomass acclimatised and began to grow (Figure 7.3, Appendix D, Figures D13 and D14) a phenomenon which was also observed in the biomass control (Appendix D, Figure D15). Fungi have the intrinsic ability to acidify their environments (Zapotoczny *et al.*, 2007) due to H^+ efflux (Gadd, 2000), and the pH drop and subsequent increase can be used as an indication of the toxic effect of metal ions on biomass growth.

Nickel uptake

The removal of nickel from solution by *P. glomerata* was represented similarly to the bioaccumulation of copper ions, with maximum biomass concentration (Appendix D, Figures D16 to D18), bioaccumulated metal (Appendix D, Figures D19 to D21), specific uptake (Appendix D, Figures D22 to D24) and uptake efficiency (Appendix D, Figures D25 to D27) listed in Table 7.5.

Table 7.5 Biomass concentration, bioaccumulated metal, specific uptake and uptake efficiency of *P. glomerata* in nickel solutions at various initial metal ion concentrations and pH values, grouped into three concentration ranges.

Metal ion grouping (mg.l^{-1})	pH	Initial metal ion concentration (mg.l^{-1}) mean values ± standard deviation (n=3)	X_m (g.l^{-1})	C_{acc} (mg.l^{-1})	q_m (mg.g^{-1})	Uptake efficiency (%)
25	**2**	24.06 ± 0.85	4.29	6.26	21.76	24.02
	4	27.43 ± 2.11	4.78	10.97	23.78	39.98
	6	24.04 ± 1.55	6.19	12.51	7.56	40.98
40	**2**	38.28 ± 0.73	0.44	8.65	14.07	22.28
	4	41.09 ± 0.93	2.43	12.12	10.48	29.49
	6	39.09 ± 2.23	6.20	10.33	4.50	26.45
55	**2**	56.77 ± 2.33	0.65	8.32	24.14	17.29
	4	55.30 ± 2.22	1.26	7.20	13.15	13.01
	6	54.23 ± 2.39	2.31	12.64	16.36	23.30

The highest specific uptake of nickel recorded was 24.14 mg.g^{-1} at an initial concentration of 55 mg.l^{-1} and pH 2 however, in most cases less than 30 % of the nickel was removed from solution. Nickel removal at the different initial metal concentrations and pH values was erratic and appeared to show no definite trends. Figures D19 – D21 (Appendix D) illustrate the inconsistency of metal removal. Although there was a net removal of nickel from solution at each initial nickel concentration and pH, over the 72 hour time period nickel concentrations appeared to be in a permanent state of flux with most of the removal occurring within the first 12 hours.

The generation of biomass during the growth period at each initial nickel concentration appeared to be predominantly dependent on the initial pH of the solution with more biomass growth occurring at a higher pH. The biomass concentration at pH 2 in the group with an initial nickel concentration of 40 $mg.l^{-1}$ was 0.44 $mg.l^{-1}$, while at pH 6, 6.187 $mg.l^{-1}$ of biomass was generated. As illustrated previously in Section 7.5.1, the toxic effect of nickel increased with increasing metal ion concentrations and this resulted in an increased lag phase at higher initial concentrations of nickel (Table 7.6). The lag phase of the initial nickel concentration of 55 $mg.l^{-1}$ was as long as 52 hours, while 25 and 40 $mg.l^{-1}$ had considerably shorter lag phases of 28 and 32 hours respectively.

Table 7.6 The effect of initial nickel ion concentration and pH on the biomass concentration of *P. glomerata* at the end of the lag growth phase.

Initial metal concentration ($mg.l^{-1}$)	Lag phase (hours)	Fungal mass ($g.l^{-1}$) mean values ± standard deviation (n=3)		
		pH		
		2	4	6
25	28	0.47 ± 0.17	0.43 ± 0.01	1.85 ± 0.33
40	32	0.40 ± 0.20	0.71 ± 0.10	1.17 ± 0.34
55	52	0.60 ± 0.17	0.99 ± 0.25	1.98 ± 0.16

The characteristic drop in pH of the samples with an initial pH of 4 and 6 would have normally occurred between 20 and 40 hours of incubation. Samples with an initial nickel concentration of 25 $mg.l^{-1}$ and a pH of 6 were able to adjust the pH of the solution within the usual time frame mentioned above (Appendix D, Figure D28). The pH of the solutions with an initial nickel concentration of 40 and 55 $mg.l^{-1}$ was reduced at 20 hours, but was only increased towards the end of the analysis period (Figure 7.4, Appendix D, Figure D29). In addition, at these concentrations only samples with an initial pH of 6 generated the change in the pH of the solution and at 25 $mg.l^{-1}$ the change at an initial pH of 4 only occurred after 40 hours of incubation.

Similarly to samples growing at pH 2 in the copper solutions, the pH of the solutions with an initial pH of 2 did not change at any of the three nickel concentrations and in all cases the biomass growth at this pH was lower than that observed at pH 4 or 6 (Appendix D, Figures D16 to D18).

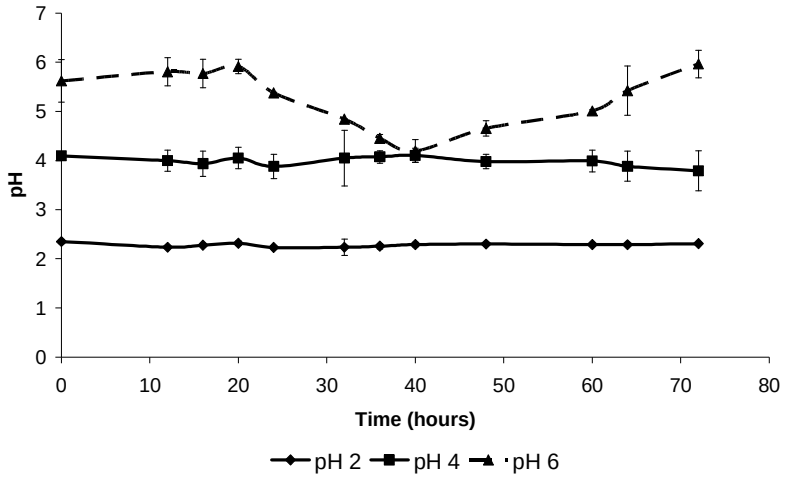

Figure 7.4 Change in pH over time due to the growth of *P. glomerata* in the metal nickel solution containing an initial nickel concentration of 55 $mg.l^{-1}$ at an initial pH of 2, 4, or 6. Error bars indicate standard deviation (n=3).

7.6 Discussion

7.6.1 Copper and nickel toxicity

Metal toxicity can fall into three categories: Type I – non-critical, Type II – potentially toxic and relatively available and Type III – potentially toxic, but relatively unavailable due to rarity or insolubility of the metal. Copper and nickel are both considered to have Type II toxicity as copper is essential for the function of a wide variety of enzymes, and nickel for a select few; however in increased concentrations both can become toxic to the cell (Gaylarde and Videla, 1995). The effect of metal ions on respiration of mixed microbial cultures has been documented (Clark *et al.*, 1999; 2000).

Oxygen limitation within the analysis time is the main drawback of using the OxiTop® system in determining metal toxicity as a function of respiration. Because of this, the analysis may only be carried out over short periods in order to avoid biomass death due to lack of oxygen, rather than due to the toxic effects of the metal (Rudrum, 2005). The data generated during this process provided information regarding the acute toxicity of the metal and did not take into account the long term effects of biomass acclimatisation or the continued toxic effects of metals at low concentrations experienced by the biomass. Nevertheless, the use of respiration as a method to compare the toxic effects of metals provides a useful benchmark for the comparison of different metal concentrations and different metal ions, especially when normalised against the control.

Direct comparison of the SOU for each metal ion and concentration does not provide an accurate representation of the toxicity as the age and concentration of the biomass differed slightly with

each metal investigated (SOU for the copper and nickel controls were 600 and 873 $mgO_2.gMLSS^{-1}$ respectively); therefore the relative SOU compared to its control was used to compare different concentrations of the same metal ion, and the different metal ions at the same concentration.

During the evaluation of copper toxicity on *P. glomerata* it was observed that at 10 $mg.l^{-1}$ of copper the SOU surpassed that of the control (Figure 7.1). This increase is due to the fact that copper is an essential trace element and as such will enhance metabolic activities at low concentrations (Pais and Jones, 1997). This has been demonstrated in biological wastewater treatment: copper additions of between 1 and 5 $mg.l^{-1}$ of $CuSO_4$ have been used to improve the treatment of textile wastewaters by activated sludge (Vandevivere *et al.*, 1998) which contains fungi and bacteria..

Within 3 hours, both 10 and 40 $mg.l^{-1}$ appeared to enhance the respiration of *P. glomerata*, while 75 and 100 $mg.l^{-1}$ of copper only created a slight decrease in relative activity of SOU; however by 14 hours, the relative activity at 40 $mg.l^{-1}$ had dropped below that of the control, and at 75 $mg.l^{-1}$ it had been halved. The decrease in SOU clearly demonstrated the toxic effect of increased copper ion concentrations on *P. glomerata*.

In contrast, nickel is not an essential trace element and no induction of respiration occurred at any of the nickel concentrations investigated (Figure 7.2). There was an obvious toxic effect of nickel on *P. glomerata*, even at concentrations as low as 10 $mg.l^{-1}$. Relative activity values calculated after 14 hours for 100 and 150 $mg.l^{-1}$ were a third of that of the control; 75 $mg.l^{-1}$ of nickel had a similar relative activity to 40 $mg.l^{-1}$ of copper, illustrating the increased toxicity of nickel when compared to copper.

The toxicity of the two cations was further substantiated when the bioaccumulation study was carried out. Increasing concentrations of metal ions decreased the biomass concentration, while the most favourable pH for growth of *P. glomerata* was pH 6 (Table 7.3 and 7.5). A similar trend was observed by Dönmez and Aksu (1999; 2000) and Dursun *et al.* (2003).
The toxic effect of copper is based on the production of hydroperoxide radicals (Suwalsky *et al.*, 1998; Nies, 1999), while nickel toxicity can be observed in proteins, cell homeostasis (Manini *et al.*, 2003) and on DNA, directly or indirectly, through the production of reactive oxygen species which interfere with DNA synthesis and repair (Lloyd and Phillips, 1999).

7.6.2 Copper and nickel uptake

While the specific uptake value (*q*) is a useful tool with which to compare results of various metals and biomass, the system is nevertheless slightly flawed. In Table 7.3, the highest specific uptake value recorded was 17.97 $mg.g^{-1}$ (25 $mg.l^{-1}$ copper, pH 2), while the concentration of

bioaccumulated metal was only 15.93 mg.l^{-1} with an uptake efficiency of less than 50 %. The lowest specific uptake (8.11 mg.g^{-1}) occurred at pH 6 at the same initial concentration; however 100 % of copper ions were removed from solution. In contrast, the highest specific uptake of nickel from solution was 24.14 mg.l^{-1} at an initial concentration of 55 mg.l^{-1} at pH 2, while only 8.32 mg.l^{-1} of nickel was bioaccumulated (Table 7.5). It can therefore be shown that however convenient it is to compare specific uptake values for different biomass and metals, if the biomass is growing the lowest specific uptake value may occur concurrently with the highest metal bioaccumulation. For this reason, specific uptake cannot be used as the sole gauge of the competence of metal bioaccumulation of a particular biomass.

Copper uptake

One of the first observations made when assessing the bioaccumulation of copper was that increased metal concentrations reduced the uptake efficiency. Uptake efficiencies of 100.00, 97.94 and 89.05 % were determined at pH 6 for 25, 40 and 55 mg.l^{-1} of copper respectively, however, the higher the metal concentration, the more copper was bioaccumulated at all initial pH values and metal concentrations (Table 7.3). Dursun *et al.* (2003) reported a similar trend in the copper concentration range of 25 to 75 mg.l^{-1} at pH 4.5 when assessing the bioaccumulative capability of *Aspergillus niger*. At concentrations higher than this an inhibitory effect on biomass growth resulted in a decrease in bioaccumulation. The authors reported uptake efficiencies of 57.20 and 51.80 % (14.30 and 25.90 mg.l^{-1} of bioaccumulated copper) at initial concentrations of 25 and 50 mg.l^{-1} respectively. In contrast the copper uptake by *P. glomerata* resulted in uptake efficiencies of 100.00 and 78.47 % at each initial metal concentration and total bioaccumulated copper concentrations of 31.59 and 46.48 mg.l^{-1} respectively. In addition, the specific uptake at pH 4 for initial concentrations of 25 and 55 mg.l^{-1} were 8.58 and 12.76 mg.g^{-1}, while the authors reported values of 4.9 and 10.4 mg.g^{-1} respectively (Dursun *et al.*, 2003). The enhanced copper removal by *P. glomerata* when compared to *A. niger* suggests it is more suitable for remediation of copper contaminated wastewaters.

The bioaccumulation of copper by *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* has also been reported (Dönmez and Aksu, 1999). Both organisms showed increased bioaccumulative abilities until the toxic threshold of copper was reached. When compared to *P. glomerata*, *S. cerevisiae* had a reduced ability to remove copper ions from solution. Maximum uptake efficiency and specific uptake at pH 4 for 25 and 50 mg.l^{-1} were reported to be 74.2 % and 2.04 mg.g^{-1} and 63.2 % and 3.93 mg.g^{-1}, respectively. On the other hand, *K. marxianus* had increased uptake efficiency, but reduced specific uptake at these concentrations (pH 4) when compared to *P. glomerata*. The reduced specific uptake was due to *K. marxianus* having enhanced growth compared to *P. glomerata*; 12.4 g.l^{-1} (25 mg.l^{-1} of copper) and 11.6 g.l^{-1} (50 mg.l^{-1} of copper)

and 7.23 and 5.59 g.l^{-1} respectively (Table 7.3). The authors also evaluated the copper ion removal capability of a *Candida* species and reported that at initial concentrations of 25 and 50 mg.l^{-1}, the *Candida* species was able to generate uptake efficiencies of 72.6 and 67.0 % respectively (Dönmez and Aksu, 1999). Although these values are lower than those reported for *P. glomerata*, the *Candida* species was able to generate 7.7 g.l^{-1} of biomass in an initial copper concentration of 708.2 mg.l^{-1} (with an uptake efficiency of 12.8 %). In a follow up paper, the authors showed that the *Candida* species could remove over 50 % of copper from solution in concentrations up to 578.7 mg.l^{-1} once adapted to the metal ion (Dönmez and Aksu, 2000). This suggests that if *P. glomerata* were to be acclimatised to the metal ions prior to determining its ability to remove copper from solution, it may have a greater metal uptake capability.

In addition to higher initial copper concentrations resulting in increased bioaccumulation, higher the initial pH correlated with higher uptake efficiency in *P. glomerata*. This may have been due to a number of reasons. At a higher pH, the biomass only experienced the stress of excess metal ions in solution, while at a low pH, the biomass had to acclimatise to the artificial environment, resulting in decreases in metabolic function and slower growth when compared to pH 4 and 6. As discussed in Chapters 2 and 4, the pH of the solution plays an important role in the availability of functional groups for cation binding in biosorptive reactions and in metal speciation (Akar and Tunali, 2006), which in turn will affect the bioaccumulation of the particular metal ion. At the lower pH, hydroxyl ions would compete for binding sites on the cell surface with the copper ions, resulting in a decreased removal of copper from solution. Dönmez and Aksu (1999; 2000) and Dursun *et al.* (2003) also reported an increase in copper removal with increased pH up to pH 5.

The inconsistency of the biomass growth and the final mass of *P. glomerata* in copper solutions at pH 2 was attributed to the generation of impermeable pigmented cell walls which have a higher density than those of cells growing under natural conditions (Gadd, 1993). A resultant effect of the retardation of the biomass growth was the inability of the culture to increase the pH after acidification of the media at 12 hours. In the copper solutions at pH 4 and 6 at all three initial metal concentrations, after 24 hours the pH of the solution had been increased, while higher nickel concentrations caused *P. glomerata* to take 50 hours to increase the pH.

Nickel uptake

Nickel ion removal from solution by *P. glomerata* was low when compared to the copper uptake. The chief reason for the reduction in metal uptake was probably the toxicity experienced by the biomass due to the presence of nickel ions (Section 7.5.1). A comparison of the biomass growth in any initial metal concentration at any pH showed that the concentration of nickel ions and pH had a greater effect on *P. glomerata* than the copper ions (Tables 7.3 and 7.5). Due to the poor growth in

the nickel solution (particularly at 55 $mg.l^{-1}$), it is most likely that initial nickel uptake was as a result of biosorption, rather than bioaccumulation. This is further supported by the fact that at all initial nickel concentrations the majority of the nickel removed from solution occurred within the first 12 hours. Only at the end of the analysis period, once the biomass had acclimatised and was able to grow, was there an increase in nickel uptake (Appendix D, Figures D19 – D21). This only occurred at initial concentrations of 25 and 40 $mg.l^{-1}$; further verifying the toxicity of increased nickel concentrations.

Whether the central mechanism of nickel uptake by *P. glomerata* was a function of bioaccumulation or biosorption, many authors have shown improved uptake capabilities by other biomass in both instances. Magyarosy *et al.* (2002) analysed the nickel bioaccumulation by *A. niger*. The authors report 99 % removal of nickel from solution at pH 3 with an initial concentration of 6.5 mM (±60 $mg.l^{-1}$). Similarly, at pH 5, Congeevaram *et al.* (2007), reported 90 % nickel ion removal from solution with an initial concentration of 50 $mg.l^{-1}$ by an *Aspergillus* species, while Dönmez and Aksu (2000) found that 71 % of nickel ions from a solution with an initial concentration of 67 $mg.l^{-1}$ (pH 4) were taken up by adapted *Candida* species. If a biosorption mechanism is considered, the highest specific uptake of nickel by *P. glomerata* was found to be less than 25 $mg.g^{-1}$ (Table 7.5), while a number of authors (Chapter 4) have reported specific uptake values of 102, 121 and 166 $mg.g^{-1}$ by protonated rice bran, *Sphagnum* moss and *Cassia fistula*, respectively (Zhang and Banks, 2006; Hanif *et al.*, 2007; Zafar *et al.*, 2007).

Metal uptake by Phoma *species*

Copper and nickel uptake have not been reported with any *Phoma* species, either as bioaccumulation or biosorption, however, one report of the bioaccumulation of cadmium (II) by *Phoma* species F2 isolated from blende soil is available (Yuan *et al.*, 2007). The authors report that 96 % of cadmium was removed from solution with an initial concentration of 163.8 $mg.l^{-1}$ of cadmium in four days. The concentration of metal in the study undertaken by Yuan *et al.* (2007) was almost triple that in the present study, however, the *Phoma* species was isolated from an environment in which cadmium was present. Acclimatisation of the microbe to the metal would have occurred, hence the increased tolerance to the metal ion (Dönmez and Aksu, 2000). In addition, different metals exert varying toxicities on different microbes and the effects of cadmium may not have been as toxic to *Phoma* as those of copper or nickel (Gadd, 1993). Further studies would have to be undertaken with acclimatised *P. glomerata* biomass before the long term uptake capacities of each metal could be compared.

7.7 Conclusions

Acute toxicity of copper and nickel to *P. glomerata* increased with increasing metal ion concentrations, except at 10 $mg.l^{-1}$ of copper, which induced metabolic activity in the fungal isolate.

Based on respiration, nickel was found to be more toxic than copper. This conclusion was corroborated when metal uptake studies were carried out on the two ions.

Copper removal from solution by *P. glomerata* was most effective at pH 4 and 6. Increasing the initial metal concentration resulted in a reduction in uptake efficiency, however, the concentration of bioaccumulated metal increased. Copper uptake by *P. glomerata* was found to be more efficient than a number of other biomass in literature.

Nickel removal from solution was poor, most likely due to the toxicity of the metal. It compared unfavourably with previous bioaccumulation and biosorption studies on the uptake of nickel ions from solution.

Improved metal ion uptake may be achieved if acclimatisation of *P. glomerata* to copper and nickel ions was undertaken.

Without undertaking acclimatisation of *P. glomerata* to the metal ions, treatment of wastewaters containing copper by *P. glomerata* would result in complete removal of the copper ions from solution. However, if the wastewater contained nickel ions, these would not be taken up significantly and would inhibit fungal growth, most likely hindering the bioaccumulation of copper ions.

CHAPTER 8

BIOACCUMULATION OF GOLD AND PLATINUM FROM SOLUTION BY *PHOMA GLOMERATA*

8.1 Introduction

Gold is a significant element and its discovery in the form of nuggets led to many gold rushes throughout history. In modern times it is used mainly as the international currency (gold bullion) and for the manufacture of jewellery. It also has applications in the electronics industry as corrosion-free electrical contacts (particularly in the computer industry) and thin layers may be used as insulators of prestigious buildings (Lee, 1991). Gold ions also have medical significance due to their ability to interact readily with proteins, particularly amino acids containing thiol groups. Gold thiolate compounds are used for the treatment of severe rheumatoid arthritis, however, the gold concentration in the patient's body needs to be monitored on a regular basis as the gold ions are accumulated in membrane-bound lysosomes within the cells (Kendrick *et al.*, 1992).

Platinum is a rare element (Lee, 1991) which is also used in the treatment of disease due to its ability to interact with protein molecules. Cis-dichlorodiammineplatinum(II) has powerful antitumour characteristics and is used for the treatment of sarcomas and leukaemia. As with gold, the concentration of platinum in the body must be carefully monitored as platinum ions are severely toxic to the kidneys and bone marrow (Kendrick *et al.*, 1992). The main uses for platinum include the production of jewellery, investment and industrial purposes and for the manufacture of catalytic converters in cars.

Complete metal recovery is not always possible during the extraction and purification of gold and platinum; as a result both metals are present in precious metal refinery wastewaters alongside the contaminating base metals. Both gold and platinum have significant commercial value (www.thebulliondesk.com) and it is economically viable to treat wastewaters containing these ions in order to recover them for further processing.

8.2 Hypothesis

At high concentrations gold and platinum will exert a toxic effect on *P. glomerata*; however, below the toxic threshold metal ions will be removed from solution and the extent of removal will be dependent on the initial metal ion concentration and pH.

8.3 Aims and objectives

The aims of this chapter included the determination of the toxicity threshold of *P. glomerata* for gold and platinum ions in solution and identification of the effects of initial metal ion concentration and pH on metal uptake and growth. The following objectives were set in order to achieve the aims:

- Measure the toxic effect of the metal ions as a function of respiration with the OxiTop® system.
- Determine the effect of initial metal concentration and pH on the uptake of metal by monitoring the metal ions remaining in solution through AAS.

8.4 Materials and methodology

All methodology used was as described in Chapter 7, Section 7.4. Only three changes were made to the protocol (Section 7.4.2); gold and platinum solutions were used instead of copper and nickel, 0.45 µm cellulose acetate membrane filters (Whatman) were used instead of nylon membrane filters and results were expressed as units of dry mass (X_m: $g.l^{-1}$) and metal remaining in solution (C_{rem}: $mg.l^{-1}$).

8.5 Results

8.5.1 Gold and platinum toxicity

As with copper and nickel (Chapter 7), gold and platinum toxicity was determined as a function of respiration. The presence of gold ions had a marked effect on the respiration of *P. glomerata* as shown in Figure 8.1.

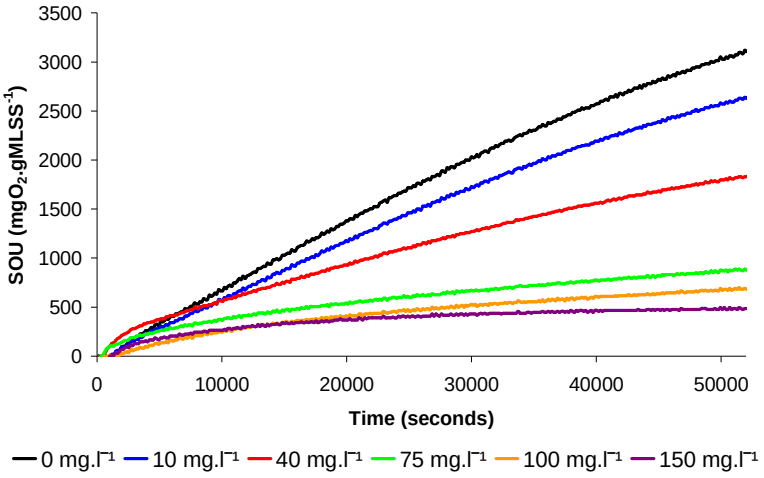

Figure 8.1 Specific oxygen uptake ($mgO_2.gMLSS^{-1}$) of *P. glomerata* exposed to increasing concentrations of gold ions over a period of 14.5 hours at 20 ℃.

There was a significant decrease in respiratory activity between the concentrations of 40 and 75 mg.l^{-1}, after 14 hours the relative SOU activity at 75 mg.l^{-1} was almost half that at 40 mg.l^{-1} and a third of the control. Table 8.1 lists the relative activity of each initial concentration at 3, 8.5 and 14 hours when *P. glomerata* was exposed to various concentrations of gold ions.

Table 8.1 The mean relative activity of each gold concentration compared to the control at three time intervals during incubation of *P. glomerata* with the gold ions at 20 ℃ (in duplicate).

Initial metal concentration (mg.l^{-1})	Relative SOU $\left(\frac{SOU_{M(x)}}{SOU_C}\right)$		
	3 hours	8.5 hours	14 hours
0	1.00	1.00	1.00
10	0.85	0.85	0.85
40	0.83	0.63	0.59
75	0.54	0.33	0.29
100	0.36	0.26	0.22
150	0.38	0.21	0.16

The relative activity of 10 mg.l^{-1} remained constant during the entire 14 hours and was only slightly less than the control, while 100 and 150 mg.l^{-1} had similar relative activities during the entire incubation period. No induction of metabolism or respiration took place at any initial gold ion concentration, in contrast, the respiration of *P. glomerata* was increased at every concentration of platinum except 150 mg.l^{-1} (Figure 8.2).

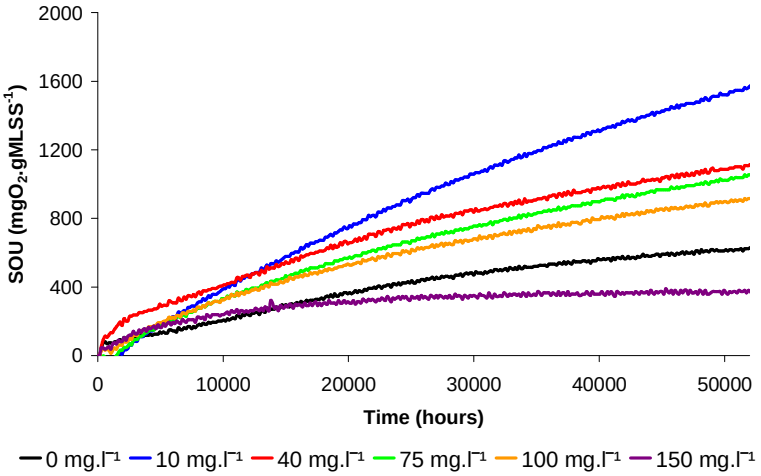

Figure 8.2 Specific oxygen uptake (mgO_2.gMLSS^{-1}) of *P. glomerata* exposed to increasing concentrations of platinum ions over a period of 14.5 hours at 20 ℃.

The induction effect of platinum was decreased as the metal ion concentrations increased; however, even at 100 mg.l^{-1} the relative activity was 1.5 times greater than the control. Ten milligrams per litre was the only platinum concentration to generate an increasing induction effect

during the 14 hours to generate a relative activity 2.5 times greater than the control. Table 8.2 lists the relative activities of each concentration over time.

Table 8.2 The mean relative activity of each platinum concentration compared to the control at three time intervals during incubation of *P. glomerata* with the platinum ions at 20 ℃ (in duplicate).

Initial metal concentration ($mg.l^{-1}$)	Relative SOU $\left(\frac{SOU_{M(X)}}{SOU_c}\right)$		
	3 hours	8.5 hours	14 hours
0	1.00	1.00	1.00
10	1.89	2.23	2.49
40	1.94	1.76	1.77
75	1.61	1.57	1.67
100	1.59	1.41	1.46
150	1.15	0.73	0.61

The *P. glomerata* biomass gained mass when exposed to both gold and platinum ions for 14 hours, suggesting that although the presence of gold ions significantly inhibits the respiration of *P. glomerata* it did not have a lethal effect at these concentrations on established biomass, as growth still occurred.

Similarly to the base metals (Chapter 7), 25, 40 and 55 $mg.l^{-1}$ were the concentrations selected for further metal ion uptake analyses. Due to the significant reduction in respiration of *P. glomerata* in 75 $mg.l^{-1}$ of gold ions when compared to 40 $mg.l^{-1}$, it was decided that concentrations higher than 55 $mg.l^{-1}$ may inhibit biomass growth and as a result metal uptake, as the biomass used in the study would not be an established culture in late log phase of growth.

8.5.2 Gold and platinuml uptake

The use of bioaccumulated metal ion concentration (C_{acc}), uptake efficiency (%) and specific uptake (q) was not possible with gold and platinum as a precipitate was formed in the medium before inoculation with *P. glomerata*. This resulted in the detection of lower metal concentration in solution than was actually present and the concentration of precipitated metal appeared to decrease with increased pH. Figure 8.3 shows the gold ions remaining in solution (C_{rem}) during incubation of *P. glomerata* in a solution with an initial gold concentration of 55 $mg.l^{-1}$, this was observed at three initial gold concentrations (Appendix E, Figure E1 and E2). The most notable feature on the figure is the increase in gold ions in solution compared to the original concentration between 12 and 16 hours at pH 6. This is followed by a rapid removal of ions and by 32 hours less than 5 $mg.l^{-1}$ of gold remained in solution.

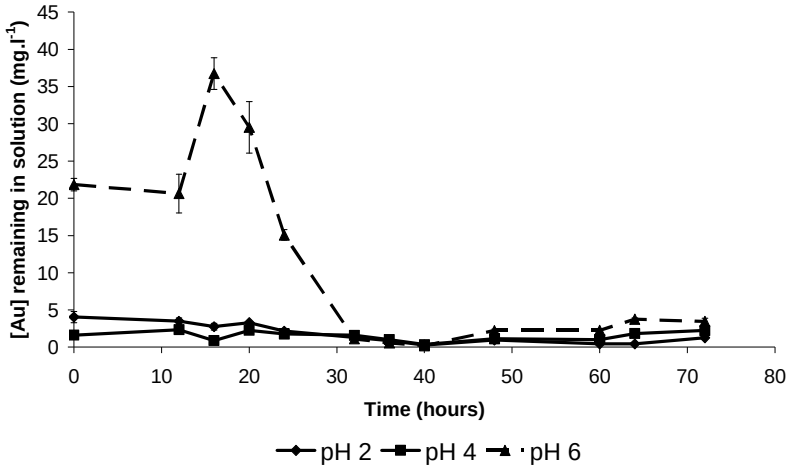

Figure 8.3 Concentration of gold ions remaining in solution during *P. glomerata* growth in gold solutions with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation and in some cases are too small to be visible.

A similar trend was observed with the platinum ions, however, the mobilisation of metal ions occurred between 12 and 28 hours and was visible at all initial pH values (Figure 8.4, Appendix E, Figures E3 and E4).

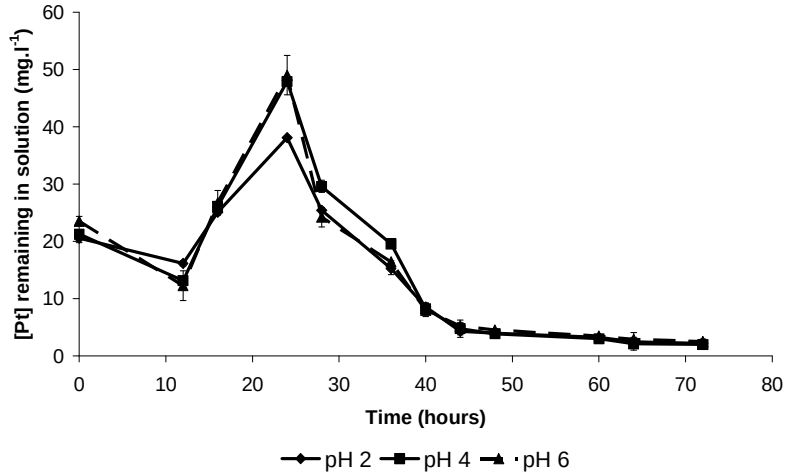

Figure 8.4 Concentration of platinum ions remaining in solution during *P. glomerata* growth in platinum solutions with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation and in some cases are too small to be visible.

There was a significant difference in the morphology of the biomass growing in the gold solutions when compared to platinum, copper and nickel. The mycelia grew in smooth mycelial balls rather than loose strands and it was possible to observe the purple-black coloured gold precipitate being removed from the media and deposited within the biomass. Figure 8.5 illustrates the morphology of *P. glomerata* in a gold solution and the deposition of gold within the mycelial ball.

Figure 8.5 Gold ion deposition within the mycelial balls of *P. glomerata* within 24 hours of incubation at 28 ℃ (pH 6), top: control with gold precipitate, b ottom: media cleared of precipitate and gold localised within the biomass.

Despite the fact that gold ions appeared to be more toxic to *P. glomerata* while platinum ions induced respiration, more biomass was generated in gold solutions at pH 4 and 6 than in platinum solutions, however, there did not appear to be usual inhibition of growth in platinum solutions with an initial pH of 2 as with gold, copper and nickel (Table 8.3, Appendix E, Figures E5 to E10). This indicated that pH played a role in the toxicity of metal ions exerted on *P. glomerata*.

Table 8.3 Mass of *P. glomerata* biomass grown in gold and platinum solutions with varying initial metal ion concentrations at pH 2, 4 and 6 for 72 hours at 28 ℃.

Metal	Initial metal ion concentration ($mg.l^{-1}$)	Fungal mass ($g.l^{-1}$) mean values ± standard deviation (n=3)		
		pH		
		2	4	6
gold	25	0.67 ± 0.11	4.57 ± 0.34	4.89 ± 0.20
	40	0.99 ± 0.10	4.53 ± 0.56	4.69 ± 0.22
	55	1.83 ± 0.28	4.31 ± 0.29	5.09 ± 0.21
platinum	25	3.78 ± 0.67	3.99 ± 0.24	3.93 ± 0.27
	40	3.65 ± 0.15	3.93 ± 0.27	3.97 ± 0.16
	55	3.55 ± 0.01	3.76 ± 0.26	3.95 ± 0.06

The similarity in growth between all initial platinum ion concentrations and pH values is illustrated in Table 8.3. The initial platinum concentration of 55 $mg.l^{-1}$ had only a slight inhibitory effect on biomass growth when compared to the other concentrations, while increasing the pH appeared to enhance growth minimally. In contrast, there was clear inhibition of the growth of *P. glomerata* in gold solutions with an initial pH of 2.

Change in pH of the solutions of gold and platinum due to the growth of *P. glomerata* was almost negligible (Appendix E, Figures E11 to E16). The period of metal mobilisation corresponded with

the lag phase of biomass growth, while the rapid removal of gold and platinum ions occurred as the *P. glomerata* biomass entered log phase of growth.

8.6 Discussion

8.6.1 Gold and platinum toxicity

A literature search on the fungal toxicity of gold and platinum revealed that there is a knowledge gap with regard to this subject. A number of authors report the phytotoxicity of platinum on grasses, vegetables and shrubs (Fargo and Mullen, 1979; Lustig *et al.*, 1996; Ek *et al*., 2004), while platinum and gold sensitivity has been reported in humans (Pais and Jones, 1997); however the toxicity of both metals on fungi has not been investigated. Both metal ions have been classified as Type II i.e. relatively available and potentially toxic (Gaylarde and Videla, 1995).

The data collected during the respirometry studies on all four metal ions revealed that gold was the most toxic to *P. glomerata* and concentrations higher than 40 $mg.l^{-1}$ severely inhibited metabolism (Figure 8.1). However, *P. glomerata* inocula in the gold solutions at initial pH values of 4 and 6 were able to generate more mass after the growth period than inocula growing in platinum solutions. This suppressed respiration with increased biomass growth may have been an indication of metabolic stimulation (Burgess *et al.*, 1999) as the toxic effects of gold are ameliorated to a certain extent by its speciation.

The speciation of gold at the initial pH values of 2, 4 and 6 varies (Nakajima, 2003). The author states that at pH 2 gold exists predominantly as $AuCl_4^-$, at pH 4 one or two chloride ions are replaced by hydroxide ions; $Au(OH)Cl_3^-$ or $Au(OH)_2Cl_2^-$ and at pH 6 all the chloride ions are replaced by hydroxide ions to produce $Au(OH)_4^-$, an insoluble salt. Gold chloride has been reported to be toxic to humans (Gold chloride Material Safety Data Sheet, 2006) most likely due to its bioavailability, while the toxicity of gold hydroxides has not been documented, similar toxic effects may apply to fungal biomass. The hydroxylation of the gold ions results in precipitation of the metal ions (Nakajima, 2003) and this may account for the reduced inhibition of growth at the higher pH values as the hydroxide precipitate is probably less reactive than the chloride species. In addition, due to their lower toxicity, gold precipitates may be more easily engulfed by growing mycelia, a mechanism already observed to remove precipitate films from glassware surfaces.

The increased gold ion precipitation at higher pH values is in direct contradiction to the observation made that the lower the pH, the higher the concentration of precipitated gold ions. The increased gold precipitation observed at the lower pH may be due to the components of the minimal media. The media contained 2 $g.l^{-1}$ of yeast extract, which would have contained any cellular components

of the yeast including amino acids, proteins, carbohydrates and lipids. As mentioned previously, gold and platinum readily interact with protein molecules (Kendrick *et al.*, 1992), the charge and solubility of which depend on the pH of the environment (Wilson and Walker, 2001). The carboxylate moieties on amino acids play an integral role in protein chemistry and stabilisation (Garret and Grisham, 1999). Three carboxylate groups occur in proteins, α, β and γ; each have a particular pK_a value, 2.3, 3.9 and 4.2 respectively (Garret and Grisham, 1999). At pH values below these values, the carboxylate groups are protonated and lose their charge (Volesky, 2003). The loss of charge on a protein reduces the stability of the molecule in solution (Wilson and Walker, 2001). At pH 2, all of the carboxylate groups would have been deprotonated resulting in denaturation and loss of structural integrity of the protein; as a result the hydrophobic centre of the protein would have been exposed to the aqueous media, causing it to precipitate. This precipitation was observed at all pH values, but became more pronounced at lower pH values as a higher concentration of carboxylate groups were protonated.

The precipitation of proteins was not observed in the base metal solutions (Chapter 7). There are two possible explanations for this. Firstly, the interaction of free Cu^{2+} and Ni^{2+} with the functional groups in the amino acids, particularly the carboxylate groups, would still have resulted in the occurrence of a charge on the protein (Lee, 1991), hence the molecule would have been stabilised by the metal binding (Gromiha, 2005). In contrast, the negatively charged precious metal complexes would not have interacted with the anionic carboxylate group. In addition, the increased affinity of the precious metals for protein molecules may have resulted in the agglomeration of the proteins if one metal ion interacted with more than one protein molecule. Secondly, the base metals were added to the media in HNO_3, while the precious metals were dissolved in HCl (Section 4.4.2) and the pH of the media adjusted with 10.0 M NaOH. In the precious metal solutions, the combination of HCl and NaOH would have resulted in the production of NaCl, which would not have occurred in the base metal solutions. The presence of NaCl would result in precipitation of the protein molecules as the NaCl competed for waters of solvation, compromising the solubility of the protein (Garret and Grisham, 1999).

The more unstable a protein molecule is in terms of charge, the greater the effect of the NaCl concentration, hence, in the gold solutions at the lower pH values, more protein-metal complexes were precipitated. The effect of the production of NaCl was noted when the precipitation of gold and platinum was compared. Less precipitation occurred in gold solutions of high pH; in contrast, the concentration of precipitated platinum was similar at all pH values. This may have been due to the fact that gold ions were added to the media in 1.0 M HCl, while platinum ions were dissolved in 0.5 M HCl, resulting in twice as much NaCl being formed in the gold solutions than the platinum

solutions. Whatever the mechanism of protein and metal precipitation protein-metal interactions are complex processes which are little understood at this time.

In contrast to the toxic effect of the gold ions, platinum stimulated metabolism at all concentrations except 150 $mg.l^{-1}$ (Figure 8.2), this is in opposition to reports that platinum inhibited cell division in *Escherichia coli* (Kendrick *et al.*, 1992). The induction may be as a result of the response of an organism to toxic compounds which are in low enough concentrations to not completely inhibit metabolism (Valix *et al.*, 2001). The authors described the growth of fungi exposed to toxins as having five distinct phases. The first is the lag phase after inoculation during which very little growth takes place, during the second stage rapid growth occurs, however it is still repressed when compared to the control. In the third stage a decline in growth occurs, before the growth rates of the test and control organisms are similar in the fourth stage (indicative of increased tolerance by the organism). Finally, in the fifth stage, growth is rapid and often exceeds that of the control.

Since the toxicity test was carried out on established biomass in late log phase, the progression through the five stages outlined above was probably more rapid than if the biomass was inoculated into fresh platinum solutions. In addition, the fact that only 150 $mg.l^{-1}$ of platinum resulted in suppression of *P. glomerata* metabolism suggests that platinum ions are not as toxic to the fungal isolate as gold, copper and nickel at low pH values.

The reduced toxicity of platinum may be as a result of the respective molar concentrations of the various metals i.e. 100 $mg.l^{-1}$ of copper is equivalent to 1.57 mM of metal, while 100 $mg.l^{-1}$ of platinum equates to 0.51 mM of metal. If the toxicity test was carried out with metal solutions with a higher pH, based on the biomass growth in the gold solutions at pH 4 and 6 and the fact that the molar concentrations of gold are about half those of copper and nickel, it may have been found that the gold ions were not the most toxic to *P. glomerata*.

8.5.2 Gold and platinum uptake

Gold and platinum uptake were both characterised by the mobilisation of precipitated ions. At 16 and 30 hours the gold and platinum ions detected in solution had increased to the concentrations originally added to the media (Figures 8.4, 8.5, Appendix E, Figures E1, E2, E9 and E10). Mobilisation of the ions resulted in their rapid removal from solution, similar to the uptake observed in the copper solutions (Chapter 7). While the effect of fungal metal mobilisation has been predominantly studied on solid media, such as the bioleaching of ores, similar mobilisation techniques apply to metals present in wastewaters (Gadd, 2007). As outlined previously (Chapter 2), these techniques include chelation, methylation and leaching and can mobilise metals from oxides, sulphides and phosphates amongst others (Gadd, 2004).

Mobilisation of gold at pH 2 and 4 was not observed, however, more precipitate was generated at these two pH values than at pH 6, and mobilisation may have occurred at a later stage between two time intervals chosen for sampling and analysis. Alternatively, as it was not observed, there may have been no mobilisation at these pH values, further supporting the idea that the chemical interplay between proteins and metals is a complicated process which is not easily elucidated. Interestingly, the mobilisation of platinum occurred at all initial pH values; however it occurred slightly later in the growth cycle when the *P. glomerata* biomass was well into log phase of growth. The mobilisation of platinum at all pH values may be related to the similarity in mass of *P. glomerata* growing in the platinum solutions at all three pH values (Table 8.3) and the induction effects on metabolism observed during the toxicity analysis (Figure 8.2).

There is little material in the public domain with regard to the removal of precious metals from solution, and none dealing specifically with the active fungal bioaccumulation of gold and platinum, however, a few biosorption studies have been published.

Nakajima (2003) investigated the biosorption of gold by a number of different bacterial and fungal species. Unfortunately, since specific uptake of gold could not be calculated in this study and specific uptake was the only value reported by the author, direct comparison between fungal biomass could not be made, however the author used micromolar concentrations of gold, while complete removal of gold with a maximum concentration of 0.23 mM was achieved by *P. glomerata*, suggesting that *P. glomerata* may be a superior accumulator of gold ions. The author indicated that the fungal biomass underwent a colour change to purple after the sorption of gold as the Au^{3+} ions were reduced to Au^{0} within the cells, corroborating observations made in this study (Figure 8.3).

Similarly to gold, the published data with regard to platinum removal from solution deals specifically with biosorption, and specific uptake values are provided in all the studies (Guibal *et al.*, 1999; Ma *et al.*, 2006) however one study involved the bioaccumulation of platinum by the plant *Lolium multiflorum* (Lesniewska *et al.*, 2004). The authors found that the plant roots contained the highest concentration of platinum, however it was most likely bound to the root surface in a biosorptive mechanism as only 1.13 % of the total concentration was detected in the leaves of the plant.

Although no direct comparison can be made between the gold and platinum removal from solution by *P. glomerata* and other biomass, when comparing the removal of the two ions from solution to that of other metals (Dönmez and Aksu, 2000; Magyarosy *et al.*, 2002; Congeevaram *et al.*, 2007), the fact that less than 5 $mg.l^{-1}$ of metal ions remained in solution at the end of the analysis period

indicates that *P. glomerata* can effectively bioaccumulate precious metals and most cases accumulate higher concentrations of metal ions.

8.7 Conclusions

Gold ions appeared to have the most toxic effect on *P. glomerata* when compared to any of the other metal ions. However, this toxicity was contradicted by the growth of the fungal isolate at higher pH values. This implied that gold toxicity was linked to pH and therefore speciation and it was likely that if the pH of the toxicity test was higher, the effect would have been reduced.

Platinum ions induced respiration at all concentrations, except 150 $mg.l^{-1}$. This phenomenon may be explained by the normal response observed when an organism is exposed to a toxic compound which is present at sublethal concentrations. In addition, platinum speciation did not appear to affect the *P. glomerata* as similar growth was observed at pH 2, 4 and 6.

The interaction of precious metals with the surrounding environment is a complex process which is not well understood and creates some confusion as to the state in which the metal ions are found.

The *P. glomerata* biomass was able to mobilise gold and platinum ions from precipitates and subsequently remove almost all the ions from solution.

Although there are no data available with regard to the fungal bioaccumulation of gold or platinum, the uptake of these metals by *P. glomerata* is favourable when compared to uptake of base metals by other fungal species.

The ability of the fungal isolate to mobilise metal ions from a solid substrate implied enormous promise for the treatment of wastewaters, as complex waste streams almost invariably result in the precipitation of various metal ions. Provided the pH is above 4, *P. glomerata* can successfully remove high concentrations of gold and platinum ions from solution.

CHAPTER 9

METAL DEPOSITION AND LOCALISATION WITHIN THE CELLS OF *PHOMA GLOMERATA*

9.1 Introduction

Micro particle induced X-ray emission (micro-PIXE) and transmission electron microscopy (TEM) have established themselves as sensitive, qualitative, visual metal analysis techniques (Weiersbye *et al.*, 1999; Gericke and Pinches, 2006). Most conventional metal analysis techniques are useful for the determination of bulk metal concentrations. These include atomic absorption spectroscopy, atomic flame spectroscopy (AFS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) to name a few (Garman and Grime, 2005). However, more specific information with regard to metal localisation and concentrations is required for the analysis of interactions of metals with living cells (Przybyłowicz *et al.*, 2004).

Metals not only interact with living cells, they have the ability to form complexes with various biological metabolites (Gadd, 2000). Microorganisms are known to secrete a number of low molecular weight extracellular polymeric substances (EPS) into the environment; these include amino acids, proteins, vitamins and carbohydrates. The EPS have a number of crucial roles, such as enzyme and substrate entrapment (Hu *et al.*, 2003) and protection against various toxic compounds such as metals (Comte *et al.*, 2006). The interactions between EPS carbohydrates and metal ions are important as carbohydrates are involved in metal transport, storage and detoxification (Yang *et al.*, 2005). Investigations into the relationship between carbohydrates and metal ions have increased rapidly in the past two decades as increasing numbers of carbohydrate complexes are finding applications in the agricultural, chemical and pharmaceutical sectors (Gyurcsik and Nagy, 2000).

9.2 Hypothesis

Removal of metal ions from solution by *P. glomerata* is due to both biosorption on the cell surface and active deposition of metal within the cell, the location of which is determined by the specific metal.

9.3 Aims and objectives

The chief aim of this study was to confirm that metal ions being removed from solution were being bioaccumulated by *P. glomerata* and secondly to determine if there was a link between metal

removal and production of carbohydrate by the fungal isolate. The following objectives were set to achieve these two aims:

- Mico-PIXE analysis was carried out on the fungal isolate to confirm the binding of mixed metals in solution to the biomass.
- The localisation of each of the metals in single metal solutions was assessed through TEM.
- The production of carbohydrate by *P. glomerata* when exposed to different concentrations of each metal at various pH values was monitored through the use of the dinitrosalicylic acid (DNS) assay.

9.4 Materials and methodology

Unless otherwise stated, all growth media and reagents were reagent grade Biolab and Saarchem brand respectively, supplied by Merck Chemicals (Pty) Ltd (South Africa) and used as received. All glassware was acid washed in 5 % HNO_3 for a minimum of 5 hours, rinsed in deionised water overnight and dried before use.

9.4.1 Micro-PIXE analysis

Malt extract broth (Section 5.4.1) in Erlenmeyer flasks was inoculated with *P. glomerata* and allowed to grow at 28 ℃ with shaking at 150 rpm on a bench top Orbital shaker (Labcon) for four days, after which copper, nickel, gold and platinum solutions (Section 4.4.2) were added to generate a final concentration of 100 $mg.l^{-1}$ of each metal. After an additional two days biomass samples were removed for micro-PIXE analysis.

The biomass specimen was prepared by homogenising one mycelial ball in distilled water, mounting on a double 1 % formvar (Sigma-Aldrich, South Africa) frame and cryofixing (Leica EM CPC, Austria) in instrument grade liquid propane (Afrox, South Africa). The specimen was then freezedried at -90 ℃ (Leica EM CFD, Austria) for 2 4 hours.

Microanalysis of the specimen was performed using a nuclear microprobe (Materials Research Group, iThemba Labs, South Africa) (Prozesky *et al.*, 1995). A proton beam with a current of 300 pA and 3.0 MeV was focused on a 5 × 5 µm area of the specimen and raster scanned. Backscattering (BS) and PIXE were used concurrently with an external 155 µm Kapton absorber placed between the PIXE Si(Li) detector and the specimen. Data were collected using a XSYS data acquisition system and further processed with GeoPIXE-II software (Ryan *et al.*, 2002). Quantitative elemental maps were produced using the 'dynamic analysis' method (Ryan *et al.*, 1995). Average elemental concentrations for the specimen were calculated on the basis of the PIXE spectra obtained from the selected Area and analysis of the corresponding BS spectra using

a RUMP simulation package and non-Rutherford cross-sections for isotopically natural carbon and oxygen at a laboratory angle of 170 ℃ provided the data on matrix composition and areal density.

9.4.2 Transmission electron microscopy

Transmission electron microscopy was carried out as described by Cross *et al.* (2001). Fungal samples used for TEM were prepared in single metal solutions as laid out above (Section 9.4.1) and fixed and dehydrated as described in Section 5.4.4 for SEM analysis. Between fixing and dehydration, specimens were further fixed with osmium tetroxide for 60 min before undergoing two ten minute wash steps with 2.5 % gluteraldehyde in 0.1 M sodium phosphate buffer (pH 7). Once the samples were dehydrated the ethanol was discarded and replaced with propylene oxide (Sigma-Aldrich, South Africa) and left for fifteen minutes, after which it was replaced with fresh propylene oxide for a further fifteen minutes. The propylene oxide was discarded and the specimens exposed to a series of propylene oxide:resin (v/v) mixtures with increasing concentrations of resin (75:25, 50:50, 25:75) each of which was allowed to infiltrate for 90 minutes. The specimens were each transferred to pure resin and allowed to infiltrate overnight.

Samples were placed at 60 ℃ for 36 hours to allow the resin to polymerise after which they were sectioned and viewed with a transmission electron microscope at 90 kV (JEOL 1210, Japan).

9.4.3 Carbohydrate production

Carbohydrate analysis was carried out on the supernatant of the samples generated during the metal uptake studies in Chapters 7 and 8 (Sections 7.4.2 and 8.4.2) Carbohydrate composition was determined as a function of reducing sugars secreted into solution with the use of the dinitrosalicylic acid (DNS) assay (Miller, 1959). A reaction mix was prepared containing two grams of NaOH and 2 g of 3, 5 dinitrosalicylic acid which were each dissolved separately in 100 ml deionised water and mixed. To this 40 g Rochelle salts tetrahydrate, 0.4 g phenol and 0.1 g sodium bisulphite were added. The assay contained 600 µl reaction mix and 300 µl of sample. A standard curve was set up using glucose as the substrate in the concentration range between 0 and 1 $g.l^{-1}$ and the final concentration of reducing sugars in the media was expressed as glucose molar equivalent of the carbonyl moiety of the terminal aldehyde and ketone groups of the reducing sugars (Appendix F, Figure F2).

9.5 Results

9.5.1 Micro-PIXE

After preparation of the specimen, it was discovered that the single hyphae were too thin and too narrow to determine localisation patterns of the copper, nickel, gold and platinum ions within the cell, and an area with a high hyphal density was selected for evaluation of bulk metal concentrations.

The specimen yielded two distinct areas of elemental concentration, designated as a and b, as seen in Figure 9.1. Areal densities were determined to be 0.65 $mg.cm^{-2}$ and 0.73 $mg.cm^{-2}$, respectively. All further determinations of elemental concentrations were calculated based on these values.

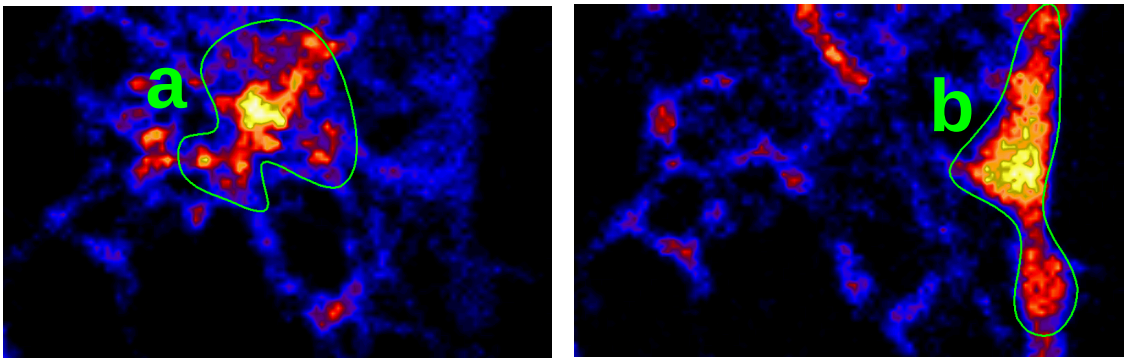

Figure 9.1 Areas selected on the hyphal specimen of *P. glomerata* for bulk metal analysis of copper, nickel, gold and platinum with micro-PIXE.

Quantitative maps for physiologically essential elements, in particular potassium, sulphur, calcium, phosphorus and chloride, were created to confirm the sample area to be analysed comprised biological material. Figure 9.2 shows the distribution of each of these elements with relative concentrations; the darker the colour, the lower the concentration.

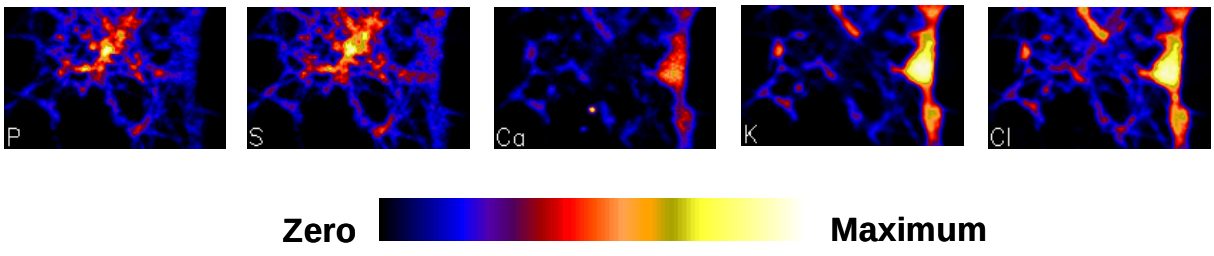

Figure 9.2 Quantitative maps showing the occurrence and relative concentrations of phosphorus, sulphur, calcium, potassium and chloride within the *P. glomerata* hyphal material.

Phosphorus and sulphur were predominantly concentrated in Area a, while calcium and potassium were localised in Area b and chloride was distributed throughout the biomass, but concentrated in Area b. The highest concentrations detected for each the elements are listed in Table 9.1.

Table 9.1 Concentrations of phosphorus, sulphur, calcium, potassium and chloride within the *P. glomerata* hyphal material based on PIXE and BS spectra. Proton energy 3.0 MeV, Si(Li) detector shielded with 155 µm thick Kapton filter. Values followed by ± indicate 1 σ.

Areas extracted from maps		**Area a**	**Area b**
Matrix composition		$C_{57}O_{11}H_{23}N_9$	$C_{55}O_{14}H_{22}N_9$
Areal density (mg.cm^{-2})		0.65	0.73
Elemental concentrations (mg.kg^{-1})	**Phosphorus**	15476 ± 641	6196 ± 229
	Sulphur	2560 ± 102	1425 ± 1425
	Calcium	4625 ± 38	10201 ± 52
	Potassium	3384 ± 18	14902 ± 68
	Chloride	332 ± 9	1275 ± 31

In Figure 9.3 quantitative maps of the *P. glomerata* specimen for copper (A), nickel (B), gold (C) and platinum (D) can be seen. The base metals appeared to be largely situated in Area b with copper and nickel concentrations as high as 1211 and 1136 mg.kg^{-1} respectively; while the precious metals were concentrated in Area a with the maximum detected concentrations for gold and platinum being 5617 and 6339 mg.kg^{-1} respectively.

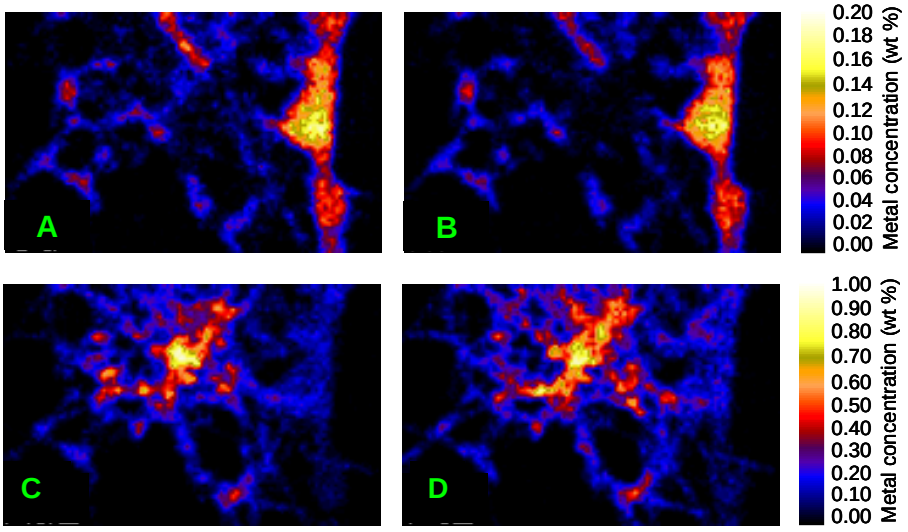

Figure 9.3 Quantitative maps showing the occurrence and concentrations of copper (A), nickel (B), gold (C) and platinum (D) (mg.kg^{-1}) within the *P. glomerata* hyphal material.

Table 9.2 shows the highest concentrations of copper, nickel, gold and platinum in each area on the specimen.

Table 9.2 Concentrations of copper, nickel, gold and platinum within the *P. glomerata* hyphal material based on PIXE and BS spectra. Proton energy 3.0 MeV, Si(Li) detector shielded with 155 µm thick Kapton filter. Values followed by ± indicate 1 σ.

Areas extracted from maps		**Area a**	**Area b**
Matrix composition		$C_{57}O_{11}H_{23}N_9$	$C_{55}O_{14}H_{22}N_9$
Areal density (mg.cm^{-2})		0.65	0.73
Elemental concentrations (mg.kg^{-1})	**Copper**	388 ± 10	1211 ± 23
	Nickel	241 ± 6	1136 ± 21
	Gold	6339 ± 80	1890 ± 30
	Platinum	5617 ± 59	1971 ± 28

While the base and precious metals were localised in specific areas on the specimen, the highest concentrations of both gold and platinum in Area b were higher than those of copper and nickel (1890, 1971, 1211 and 1136 mg.kg^{-1} respectively). The detection of higher concentrations of gold and platinum than copper and nickel on the biomass corroborated the initial findings with regard to metal uptake during the screening process of the three different fungal isolates (Chapter 5) and indicated that the biomass had a higher affinity for precious metals than for base metals.

9.5.2 Transmission electron microscopy

Transmission electron microscopy was carried out on *P. glomerata* growing in single metal solutions of copper, nickel, gold and platinum to ascertain if the metals were being transported into the cell or binding to the cell surface alone. The presence of metals was detected by TEM as electron dense areas (dark areas) on the biomass specimens (Yamato *et al.*, 1997). Figure 9.4 shows *P. glomerata* biomass grown in the presence of copper (A), nickel (B), gold (C) and platinum (D) and the subsequent metal deposition on the outside of the cell wall (indicated by the arrows) in contrast to the control with no electron dense areas around the cell wall (Appendix F, Figure F1). Metal deposits on the cell wall appeared to be of a specific size and shape, rather than to have an erratic binding pattern.

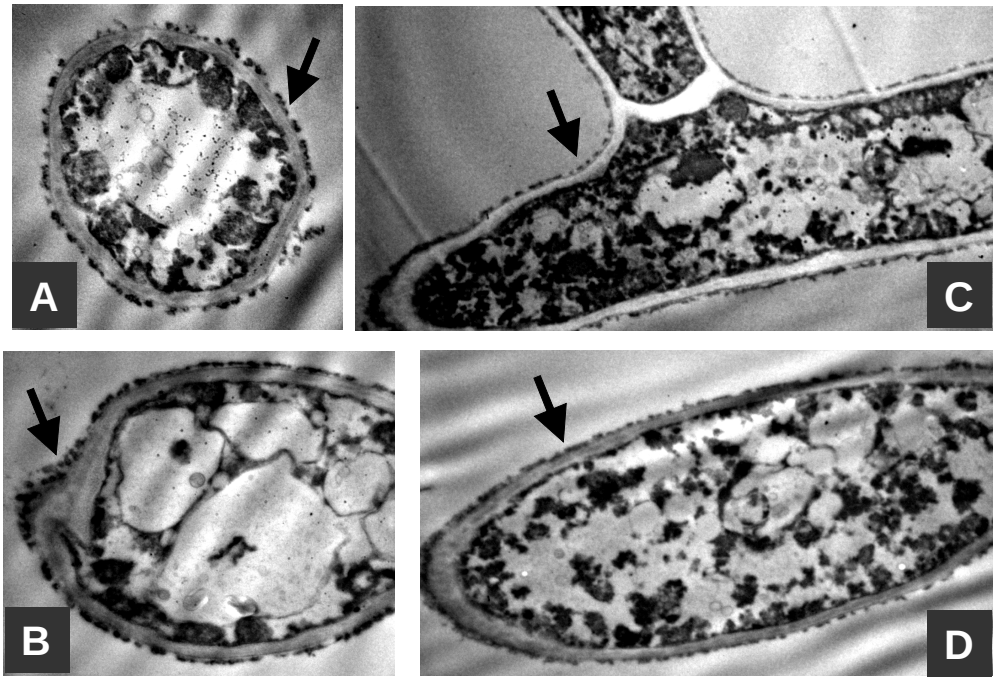

Figure 9.4 Copper (A), nickel (B), gold (C) and platinum (D) deposited on the surface (indicated by the arrows) and within the cells of *P. glomerata* viewed by TEM with a magnification of 8000 times.

Metals ions were deposited within the cytoplasm of the fungal cells as discrete particles. They appeared to be evenly distributed throughout the cytoplasm, rather than being associated with a particular cellular structure. However, staining with osmium tetroxide allows for the visualisation of cellular organelles by binding to the structures and creating electron dense areas, which may have a masking effect on target metal ions bound to these organelles.

9.5.3 Carbohydrate production

Hydroxyaldehyde and hydroxyketone groups of carbohydrates are known to interact with and bind metal cations (Allscher *et al*., 2007). The production of reducing sugars containing these functional groups by *P. glomerata* during growth in single metal solutions (copper, nickel, gold and platinum) at varying initial metal ion concentrations and pH values was monitored to determine if the presence of reducing sugars affected metal ion removal from solution. Figure 9.5 represents the concentration of carbonyl groups (mM) equivalent to glucose produced by *P. glomerata* while growing in a copper solution of 55 $mg.l^{-1}$. At 16 hours a significant spike in reducing sugar production could be observed at all pH values, and within 24 hours no further carbohydrate production could be detected. In contrast the concentration of reducing sugars increased exponentially between 16 and 24 hours, after which carbohydrate production stopped, resulting in a plateau in the detection of reducing sugars.

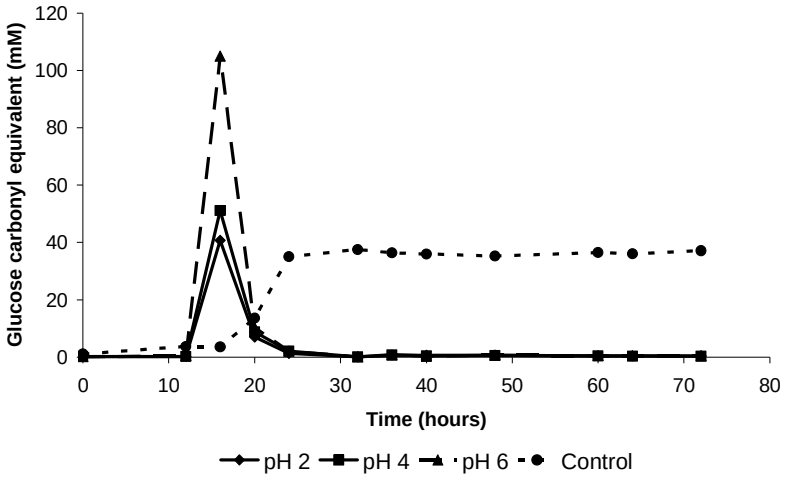

Figure 9.5 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a copper solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was less than 5 % of the mean.

The production of reducing sugars appeared to be pH dependent as the higher the pH, the greater the concentration (Table 9.3). This was found to be true at initial metal concentrations of 25 and 40 mg.l^{-1} (Appendix F, Figures F3 and F4). When the production of reducing sugars at the same pH and different initial copper concentrations was compared (Figure 9.6) it was found that the same concentration of carbohydrate was secreted into solution irrespective of the copper concentration.

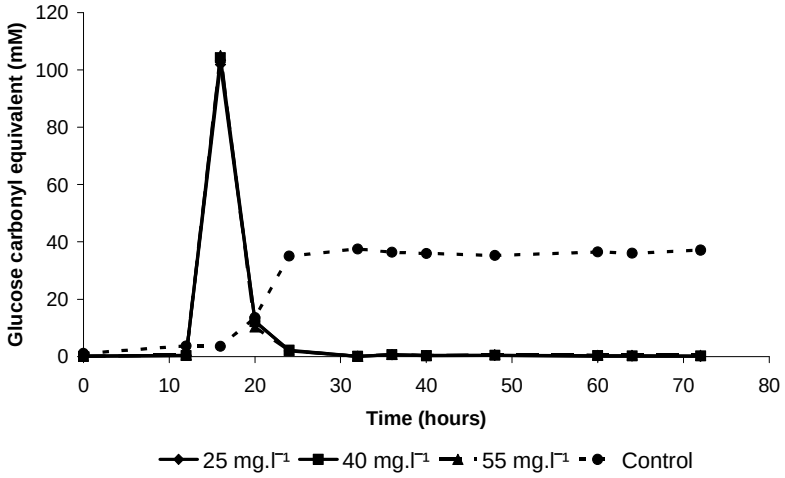

Figure 9.6 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a copper solution with an initial pH of 6 and copper concentrations of 25, 40 and 55 mg.l^{-1}. At each time interval the concentrations of glucose carbonyl equivalents at each initial copper concentration were almost identical. Standard deviation was less than 5 % of the mean.

Production of identical concentrations of reducing sugars at the different initial copper ion concentrations confirmed that in copper solutions, carbohydrate production was pH dependent and not a response to initial metal concentrations.

The production of reducing sugars by *P. glomerata* in the nickel ion solutions also generated one concentration peak; however, it occurred at 56 hours rather than 16 hours. As with the copper solutions, the carbohydrate production in the nickel solutions was pH dependent (Figure 9.7, Appendix F, Figures F5 and F6).

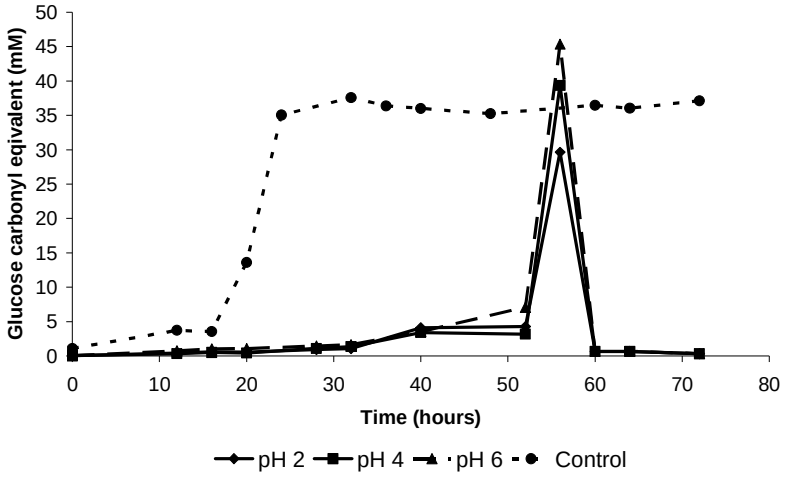

Figure 9.7 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a nickel solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

In contrast to the effect of initial copper concentrations on reducing sugar production, increased nickel concentrations had a clear retardation effect on the production of the carbohydrate (Figure 9.8). Unlike the two base metals, gold and platinum did not generate a distinct reducing sugars concentration peak, but instead a concentration curve similar to that of the control was observed (Figures 9.9 and 9.12).

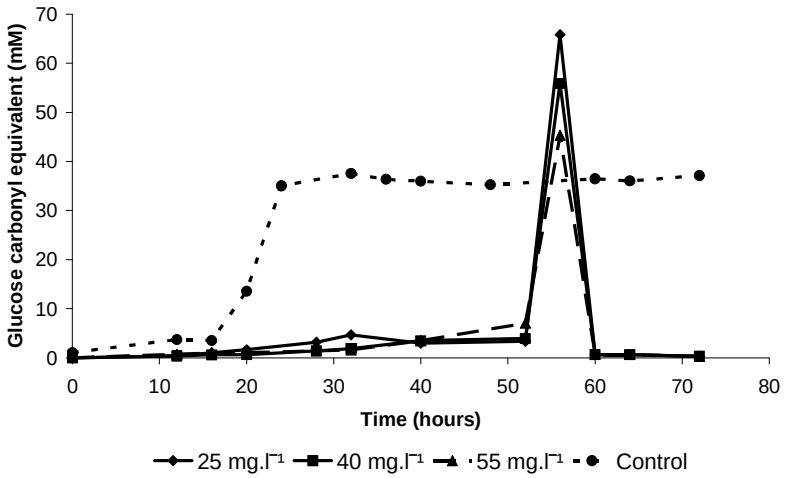

Figure 9.8 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a nickel solution with an initial pH of 6 and nickel concentrations of 25, 40 and 55 mg.l^{-1}. Standard deviation was 5 % of the mean.

In Figure 9.9 it can be seen that the production of reducing sugars in the 55 mg.l^{-1} gold solution was slightly slower than that of the control, however, a higher concentration of carbohydrate was detected at all initial pH values. At pH 2, lower gold concentrations were correlated with lower carbohydrate production, while production of reducing sugars at pH 4 and 6 occurred concurrently with the control (Appendix F, Figures F7 and F8).

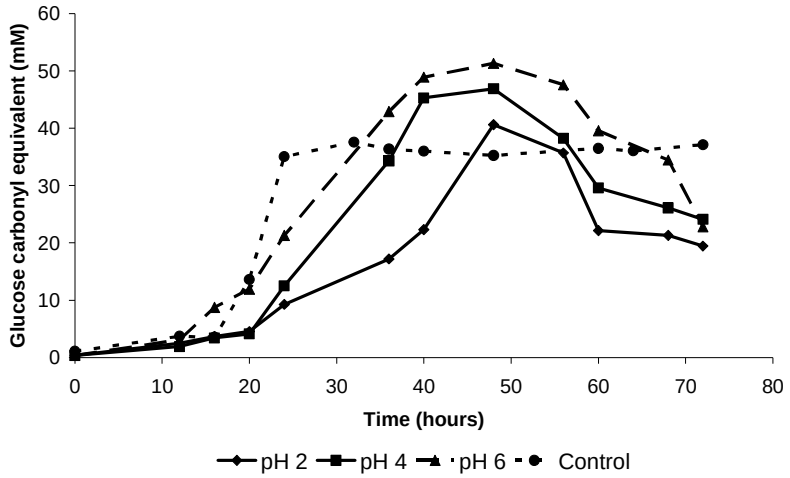

Figure 9.9 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a gold solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

Figure 9.10 illustrates the effect of initial gold concentration on the production of reducing sugars at pH 6.

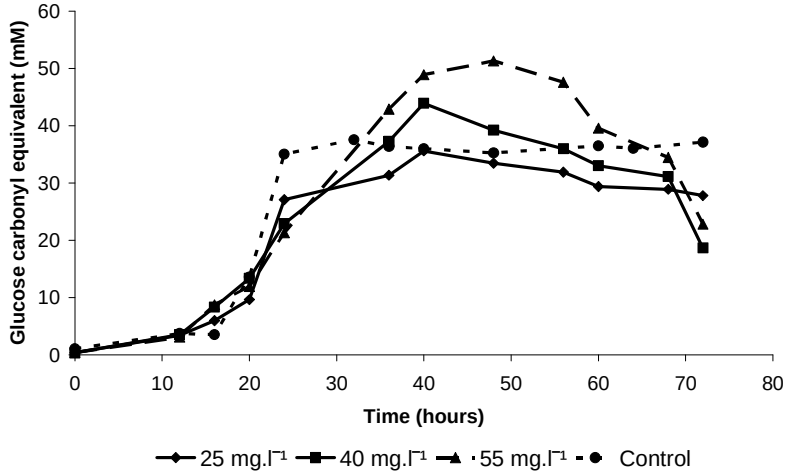

Figure 9.10 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a gold solution with an initial pH of 6 and gold concentrations of 25, 40 and 55 mg.l^{-1}. Standard deviation was 5 % of the mean.

At the lowest concentration (25 mg.l^{-1}) there was a slight suppression of carbohydrate production, while at pH 4 and 6 the concentration of reducing sugars was elevated when compared to the

control. The concentration of reducing sugars produced at each initial metal concentration at pH 2 and 6 increased with higher concentrations of gold, while the carbohydrate concentration at pH 4 remained the same (Table 9.3).

In a solution of 25 mg.l^{-1} of platinum, production of reducing sugars at pH 4 and 6 occurred at the same rate and carbohydrate concentrations at all initial pH values exceeded that of the control (Appendix F, Figure F9). At 40 mg.l^{-1} of platinum, the concentration of reducing sugars at pH 2 and 4 were lower than those at 25 mg.l^{-1}. However, the carbohydrate concentration at pH 6 had increased (Appendix F, Figure F10 and Table 9.3). At an initial platinum concentration of 55 mg.l^{-1} (Figure 9.11), the production of reducing sugars at pH 2 and 4 was even further retarded; the final concentration was the same as that of the control, however carbohydrate production was slower. In contrast, the production of reducing sugars at pH 6 exceeded those of the lower initial platinum concentrations (Table 9.3).

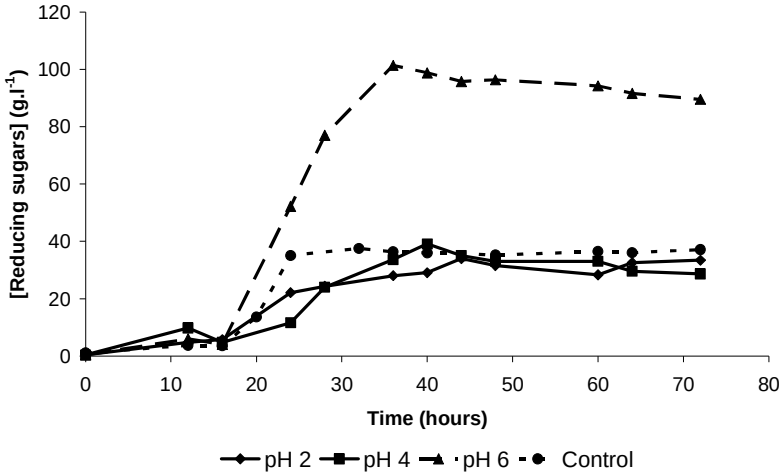

Figure 9.11 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a platinum solution with an initial concentration of 55 mg.l-1 at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

When the reducing sugar concentration at pH 6 at all initial platinum concentrations was compared, it was observed that initial metal concentration had very little effect on carbohydrate production (Figure 9.12). There was a stronger link between initial pH and metal concentrations at pH 2 and 4 (Table 9.3).

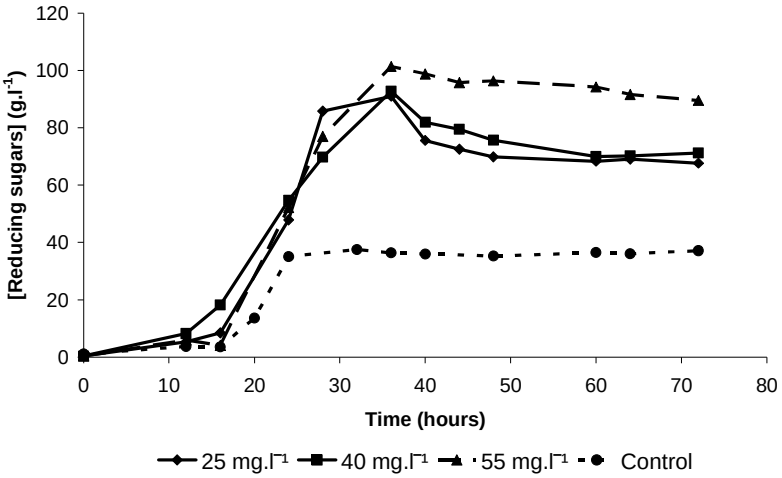

Figure 9.12 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a platinum solution with an initial pH of 6 and platinum concentrations of 25, 40 and 55 mg.l^{-1}. Standard deviation was 5 % of the mean.

Table 9.3 summarises the various concentrations of reducing sugars at each initial pH and metal concentration as a function of glucose equivalents of the carbonyl group.

Table 9.3 Mean glucose equivalents of the carbonyl groups on the reducing sugars produced by *P. glomerata* during a 72 hour period at 28 ℃ in copper, nickel , gold and platinum solutions of 25, 40 and 55 mg.l^{-1} and initial pH values of 2, 4 and 6 (in triplicate). Standard deviations were less than 5 % of the mean.

Metal ion	Initial metal concentration (mg.l^{-1})	Initial pH	Time (hours)	Glucose carbonyl equivalent (mM)
control	**0**	**5**	30	37.56
copper	**25**	**2**	16	36.96
		4	16	42.21
		6	16	101.85
	40	**2**	16	37.55
		4	16	45.45
		6	16	104.27
	55	**2**	16	40.73
		4	16	51.12
		6	16	104.98
nickel	**25**	**2**	56	32.44
		4	56	37.54
		6	56	65.82
	40	**2**	56	25.98
		4	56	31.93
		6	56	55.89
	55	**2**	56	29.63
		4	56	39.37
		6	56	45.34

Table 9.3 Continued. Mean glucose equivalents of the carbonyl groups on the reducing sugars produced by *P. glomerata* during a 72 hour period at 28 ℃ in gold and platinum solutions of 25, 40 and 55 $mg.l^{-1}$ and initial pH values of 2, 4 and 6 (in triplicate). Standard deviations were less than 5 % of the mean.

gold	25	2	48	16.82
		4	48	45.15
		6	40	35.66
	40	2	56	27.55
		4	40	42.32
		6	40	43.91
	55	2	48	40.61
		4	48	46.87
		6	48	51.31
platinum	25	2	36	58.99
		4	36	89.76
		6	36	90.98
	40	2	36	29.54
		4	36	68.42
		6	36	92.76
	55	2	44	33.98
		4	40	39.13
		6	36	101.39

The highest concentration of reducing sugars produced by *P. glomerata* occurred in the platinum and copper solutions, while the nickel and gold solutions had similar concentrations to the control and production of carbohydrate in each solution was found to be related to the initial pH.

9.6 Discussion

9.6.1 Micro-PIXE

In the last eight years all work published with regard to fungal-metal interaction analysis with micro-PIXE has been undertaken with arbuscular mycorrhizal fungi and their respective plant associations (Weiersbye *et al.*, 1999; Jurkiewicz, *et al.*, 2001; Scheloske *et al.*, 2001; Turnau *et al.*, 2001).

The fundamental difference between the assessment of elemental distribution of metals within mycorrhizal fungi and the results reported here is the respective size of the fungal structures. Weiersbye *et al.* (1999) examined a specimen with a 1000 × 1000 μm^2 area, while Turnau *et al.* (2001) used a scan size of 10 × 10 μm^2. The size of the individual *P. glomerata* hyphae were determined to be approximately 1 µm wide, smaller than the individual beam spot of the instrument (3 × 3 μm^2). For this reason elemental maps of single hyphae could not be produced to determine the localisation of copper, nickel, gold and platinum within individual cells. The focus of the analysis

therefore shifted to bulk metal determination of a cluster of hyphae. Other metal analysis techniques such as ICP-AES, AAS and AFS would have provided quantitative data with regard to copper, nickel, gold and platinum concentrations of digested samples (Przybyłowicz *et al.*, 2004). However, these techniques would have provided data similar to those in Chapters 7 and 8 and a qualitative, visual confirmation of metal uptake was required.

Weiersbye *et al.* (1999) assessed the ability of micro-PIXE to measure and map elemental concentrations of arbuscular mycorrhizas associated with the roots of the grass *Cynodon dactylon*, isolated from gold and uranium mine tailings. The authors reported the highest concentration of copper and nickel localised within the hyphae to be approximately 750 and 200 $mg.kg^{-1}$ respectively. Unfortunately, they did not report on the localisation of gold ions within the hyphae; however copper and nickel concentrations detected in the *P. glomerata* biomass were substantially higher (1211 and 1136 $mg.kg^{-1}$). Similarly, copper concentrations accumulated by *P. glomerata* were three times higher than that detected in *Suillus luteus* by Turnau *et al.* (2001). Nickel concentrations reported by the authors were less than 46 $mg.kg^{-1}$.

To date there are no reports in the public domain regarding the detection of precious metals in fungal biomass with micro-PIXE, so a comparison of metal uptake and detection capabilities was not possible. However, considering the elemental maps in Figure 9.3, higher concentrations of gold and platinum (1890 and 1971 $mg.kg^{-1}$ respectively) than copper and nickel were detected in Area b (Figure 9.1), suggesting *P. glomerata* biomass could accumulate more precious metals than the mycorrhizas listed above.

The localisation of precious and base metals in different areas of the *P. glomerata* biomass implied that isolate age may have a role in metal binding. This may be as a result of metabolites produced in the later stages of growth; however, there is no literature with regard to copper, nickel, gold or platinum bioaccumulation by any *Phoma* species. In addition, attempts to determine the selective bioaccumulation of the metals from a mixed metal solution proved unsuccessful. No biomass growth or metal uptake occurred in metal concentrations as low as 10 $mg.l^{-1}$.

Upon examination of elemental maps in Figure 9.2 it was observed that phosphorus and sulphur were present in substantially higher concentrations in Area a than Area b (Figure 9.1) and the opposite was true for potassium and calcium, which may also be a function of biomass age. Chloride was distributed throughout both areas, but was more concentrated in Area b.

In the fungal cell, phosphorus occurs as organic or inorganic phosphates and is required for the synthesis of phospholipids and nucleic acids, and sulphur is present in a number of vitamins such

as biotin, lipoic acid and thiamine and plays a structural role in the amino acids cysteine and methionine. Microorganisms have the ability to chemically transform sulphur molecules with ease as most sulphur in the cell originates from chemical sources. Potassium is an essential element in all organisms as cofactors in enzymes and for maintenance of cell homeostasis and optimal functioning (Madigan and Martinko, 2006). Cytosolic free calcium plays a crucial role in signal transduction in the cell; however, in fungi it is present in nanomolar concentrations (Miller *et al.*, 1990). The high concentration of calcium detected in the *P. glomerata* biomass may, in part, be due to the presence of calcium in the growth medium. The presence of these elements therefore confirmed that the specimen being analysed was biomass rather than metal precipitate or ions in solution.

In addition to the physiological occurrence of the elements mentioned above, the distribution patterns of phosphorus, sulphur, potassium and chloride may be explained by ligand-metal interactions. Copper and nickel both form chloride complexes ($NiCl_2$ and $CuCl_2^-$) and may explain the concentration of chloride detected in Area b (Table 9.1). Copper and nickel also interact with potassium and form stable copper sulphate and nickel sulphide complexes, e.g. $KCuCl_2$, $K_4Ni(CN)_4$, $CuSO_4$ and N_3S_2. In addition nickel has the ability to interact with phosphorus as phosphine complexes, $Ni(PF_3)_4$ (Lee, 1991).

Gold readily binds chloride ions forming complexes such as $AuCl_2$ and $AuCl_4^-$ (Lee, 1991) and has been reported to exist as part of a phosphine molecule (Bruce *et al.*, 2006). Platinum can form similar chloride and phosphine complexes ($PtCl_6^-$ and $Pt(PPh_3)_4$) in addition to potassium complexes such as K_2PtC_{l4} and K_2PtC_{16} and sulphur compounds ($Pt(SO_4)2•2H_2O$) (Lee, 1991).

It is unlikely that many of these complexes exist as biomolecules within the cell. However; the ability of the metals to interact with phosphorus, sulphur, potassium and chloride chemically, provides confirmation of the ability of the metals to form complexes with biomolecules containing the ligands in the cellular environment.

The higher concentrations of precious metals detected in the hyphae corroborates the preliminary findings in Chapter 5, and data in Chapter 7 and 8. In addition, the enhanced precious metal uptake makes wastewater treatment with *P. glomerata* a favourable alternative to other fungal biomass as recovery of economically valuable metals can be achieved (www.basemetals.com; www.thebulliondesk.com).

9.6.2 Transmission electron microscopy

Elemental metal present in biological material may be visualised through TEM. Metal deposits are identified as electron dense areas in the tissue and appear as dark regions (Yamato *et al.*, 1997). In Figure 9.4 the metal deposits around the cell wall are visible. The deposits appeared to have an ordered arrangement in terms of size and shape and were evenly distributed over the surface area. In addition, small discrete metal particles could be seen within the cytoplasm of the cell (particularly clear in Figure 9.4C).

These particles have been documented by Gericke and Pinches (2006) as being metal nanoparticles produced by microbes in the presence of metal ions; the size and shape of which are dependent on the organism and culture conditions. There did not appear to be any specific localisation of the copper, nickel, gold and platinum in the cell. Particles were distributed evenly through the cytoplasm. There may have been binding to cellular structures, however osmium tetroxide staining was necessary in order to visualise the biomass, and this may have masked possible metal localisation as the tissue became darker (Cross *et al.*, 2001). Transmission electron microscopy provides an indication of the presence of elemental metal. It is possible that some of the metal ions taken up by the *P. glomerata* biomass were not in the elemental form, but formed complexes with various biomolecules instead and as a result could not be visualised. Nonetheless, metal deposition was clearly visible on the cell surfaces and possibly within the cytoplasm. This is supported by Gericke and Pinches (2006) who screened a range of fungi, yeast and bacteria for their ability to metabolically produce gold nanoparticles. The authors found that the fungus *Verticillium lutteoalbum* and the yeast *Pichia jadinii* produced the highest concentration of nanoparticles and that the biomass turned dark purple after a few hours of exposure to $HAuCl_4$ as the gold ions were transported into the cell. This finding corroborated the results obtained in Chapter 8 with regard to the *P. glomerata* biomass' ability to remove gold precipitate from solution (Chapter 8, Figure 8.5).

The deposition of nanoparticles in the cytoplasm has been described above; however, none of the organisms presented by Gericke and Pinches (2006) deposited metal ions on the outside of the cell wall. Conversely, Macaskie *et al.* (2005) investigated the production of palladium crystals by the bacterium *Desulfovibrio desulfuricans* and reported that the nanoparticle crystals were only formed on the outside of the cell and Tunali *et al.*, (2006) confirmed the biosorption of copper and lead to the surface of inert biomass (*Bacillus* sp.) through SEM and EDAX. Chen *et al.*, (2003) reported that *Phoma* sp.3.2883 was able to generate silver nanoparticles after exposure to silver nitrate. They did not utilise living mycelia, but freeze dried hyphal material. Analysis of the biomass through TEM after application of silver nitrate revealed the presence of scattered particles.

However, it is unclear as to how the nanoparticles were formed as the analysis was carried out in a biosorptive fashion and nanoparticle production was not discussed.

Based on Figure 9.4, it is not yet possible to determine if the metal binding is a function of biosorption or nanoparticle production, however, the production of nanoparticles was not a focus in this study and the TEM imaging has successfully confirmed copper, nickel, gold and platinum uptake and localisation by *P. glomerata*.

9.6.3 Carbohydrate production

Production of carbohydrate has been linked to metal cation removal from solution, in particular the metal ion binding by aldehyde and ketone functional groups (Koukal *et al.*, 2007). As mentioned in Chapter 2, in the last twenty years there has been an increased interest in metal-carbohydrate interactions (Gyurcsik and Nagy, 2000). Biosorption of metals by EPS has become a popular alternative to the use of living organisms for metal removal (Guibaud *et al.*, 2001). While a number of authors have described the mechanisms of cation binding to polysaccharides (Gyurcsik and Nagy, 2000; Comte *et al.*, 2006; Lu and Guo, 2007; Allscher *et al.*, 2007; Koukal *et al.*, 2007) none discuss the metal uptake ability in conjunction with the mechanism of removal in fungal systems.

The DNS assay was selected as a representative indicator of the production of carbohydrate and metal-carbohydrate interactions as it detects the presence of the carbonyl in aldehyde and ketone groups (Miller, 1959). During interaction between the reducing sugar and metal ion, the double bond is oxidised to a hydroxyl group (Koukal *et al.*, 2007) and the assay will no longer detect that particular carbohydrate molecule, in this way a direct correlation between carbohydrate and metal cation concentration could be made.

The peaks in reducing sugar concentration in the copper solutions all occurred between 12 and 24 hours, and the concentration of carbohydrate appeared to be related to the initial pH of the solution. However, comparison of the reducing sugar production and the *P. glomerata* biomass growth (Chapter 7) revealed a correlation between the two. As described in Chapter 7 and 8, the initial pH of the solution had a profound effect on biomass growth in all metal solutions at all initial concentrations. The lack of effect of initial copper concentration on reducing sugar production at each pH (Table 9.3) was because at this stage (early log phase) all the biomass concentrations at each initial copper concentration for a given pH were similar (Appendix D, Figures D1 to D3).

Between 20 and 24 hours there was a marked decrease in the concentrations of copper ions in solution (Appendix D, Figures D4 to D6), particularly at pH 4 and 6. This occurred at the same point at which no further reducing sugars could be detected in solution (Figure 9.5). The swift

decrease in the concentrations of both the copper and the reducing sugars was due to the stability of Cu^{2+} in aqueous solution as a free cation (Lee, 1991). As a result the metal ion will interact readily with the aldehyde or ketone groups on the reducing sugars and be removed from solution. An 8 hour lag period between the rapid production of reducing sugars (12 hours) and the removal of copper ions from solution was observed (20 hours). This delay in metal uptake was attributed to the development of a high diffusion coefficient as the polysaccharides were secreted into solution (Volesky, 2003). The polysaccharide mimics a porous material that is highly permeable to ions, but as such, increases the intraparticle diffusion coefficient.

The production of reducing sugars by *P. glomerata* in nickel ion solutions further confirmed the relationship between biomass concentration and carbohydrate production. In this case the production of reducing sugars began at 30 hours, but the concentrations remained below 5.0 mM glucose carbonyl equivalents until the concentration peaked at 56 hours (Figure 9.8); the beginning of log phase of growth. As illustrated in Chapters 7 and 8, the toxicity of nickel to *P. glomerata* was much greater than that of copper and platinum and this resulted in retardation in biomass growth in the nickel solutions. It follows that the higher the concentration of nickel and the lower the pH, the greater the toxic effect, and this can be observed by the growth of the biomass at nickel concentrations of 25, 40 and 55 $mg.l^{-1}$ (Appendix D, Figures D16 to D18). Therefore, it was observed that at 56 hours, the lower the initial metal concentration; and the higher the initial pH, the higher the biomass concentration. This correlates with the findings related to the production of reducing sugars as the higher the initial pH, the more concentrated the carbohydrate in solution (Figure 9.7). In addition, the toxicity of nickel ions was further corroborated by the decreased reducing sugars concentration at increased initial nickel concentrations and the marginal increase in carbohydrate concentration when compared to the control.

As with copper, nickel exists as a free ion in solution (Lee, 1991) and will readily bind the carbonyl functional group. However, the main difference between the metal uptake of copper and nickel was that the spike in polysaccharide concentration did not correspond to a significant decrease in the nickel ions in solution. This was probably due to there being almost twice as much carbohydrate in the copper solution than in the nickel. This, in conjunction with the toxicity of the nickel, indicated that the majority of nickel removal from solution occurred via a biosorption mechanism (Comte *et al.*, 2006). This implied that copper uptake was a result of polysaccharide binding, biosorption onto the cell wall and active copper uptake into the cell itself.

In Chapters 4 and 8 it was outlined that gold and platinum ions exist as anionic complexes in solution (Godlewska-Żyłkiewicz, 2003; Nakajima, 2003) and cannot interact with the carbonyl complex on aldehyde and ketone functional groups (Volesky, 2003). For this reason interactions

between gold or platinum and the carbohydrate would not disrupt the detection of reducing sugars with the DNS assay. The anions may interact with other functional groups such as amine, imine and imidazol groups (Volesky, 2003) on amino acids and proteins which are secreted as part of the EPS (Hu *et al.*, 2003); however the presence of these functional groups during growth was not assessed. As stated in Chapter 2, the amino groups in the chitin and chitosan of the fungal cell wall would sorb a large concentration of the anions and the majority of the gold and platinum ions would probably be localised on the biomass surface. This idea is supported by the surface deposition seen in Figure 9.4C and D.

As expected, reducing sugar concentrations in gold and platinum solutions increased with the onset of log phase (Appendix E, Figures E5 to E10), and plateaued, much like the control, further confirming the interaction between the carbonyl group and the copper and nickel ions.

The growth of *P. glomerata* in gold solutions at pH 4 and 6 was very similar (Appendix E, Figures E5 to E7) and production of reducing sugars seemed more dependent on the initial metal concentration and pH of the solution than on the biomass growth. At the initial gold concentrations of 25 and 40 $mg.l^{-1}$, carbohydrate concentrations at pH 4 remained more or less the same (i.e. at a similar concentration to the control), while at pH 2 and pH 6 the higher the gold concentration the more reducing sugars were produced (Table 9.3). The highest concentration of reducing sugars at each pH was detected in solutions of 55 $mg.l^{-1}$ of gold. One of the functions of EPS is to overcome toxicity (Koukal *et al.*, 2007) and gold was found to be the most toxic to *P. glomerata* of the metal ions investigated (Chapters 7 and 8). The increased carbohydrate production at the higher initial concentrations may have been in response to the toxic effects of the gold ions, even though the concentrations were not as elevated as those of copper when compared to the control. This can be further corroborated by the concentration of reducing sugars produced in the platinum solutions by *P. glomerata*.

In Chapter 8, platinum was found to have a stimulatory effect on *P. glomerata* respiration (Figure 8.2). A similar effect was found with the production of reducing sugars at pH 6; three times as much carbohydrate was detected in the platinum solution than in the control (Table 9.3). The production of reducing sugars at pH 2 and 4 was reduced as the platinum concentration was increased, but the opposite was observed for pH 6 (Figure 9.11 and Appendix F, Figure F9 and F10). Although there was no significant toxic effect of platinum at any initial concentration and pH, there was a slight decrease in the production of biomass at pH 2 and 4 as the initial metal ion concentration increased (Appendix E, Figures E8 to E9). Increasing the platinum concentration at pH 6 did not have the same effect; in addition, there was less growth at pH 2 than pH 4 at all initial platinum concentrations. Increasing the platinum ions in solution at the lower pH values may result

in inhibition of the production of EPS, however the presence of platinum ions did not appear to inhibit other aspects of *P. glomerata* growth.

Although the presence of platinum ions in solution induced a greater production of reducing sugars (pH 2 and 4 at 55 $mg.l^{-1}$ being the only exception) than gold ions, carbohydrate production in each metal solution followed a similar trend, i.e. at pH 2 and 4 the greater concentrations were correlated with the lower carbohydrate concentrations, and at pH 6, the higher metal concentrations were linked to higher carbohydrate production.

The increased production of reducing sugars with increasing metal ion concentrations at pH 6 is most likely due to the gold and platinum speciation. As outlined in Chapter 8, at pH 6 the chloride ions bound to each of the metal ions are displaced and the complexes are less toxic; gold exists as $Au(OH)_4^-$ (Nakajima, 2003) and platinum as $PtOH•H_2O$ (Lustig *et al.*, 1998). The reduced toxicity of gold and platinum at pH 6, the solubilisation and complete removal of both metal ions from solution at this pH (Chapter 8) and the pH of the wastewaters in Dam 2 and 4 (Chapter 6) indicate that *P. glomerata* would be an ideal living biomass for the mobilisation and accumulation of gold and platinum from solution and from the metal precipitates suspended therein. In addition, metal ions may be entrapped within the EPS, resulting in an uncomplicated separation of biomass and supernatant for metal recovery.

9.7 Conclusions

Confirmation of copper, nickel, gold and platinum uptake into the *P. glomerata* biomass was obtained through micro-PIXE and TEM.

Enhanced copper and nickel removal by *P. glomerata* in comparison to mycorrhizal fungi, when analysing with micro-PIXE, was reported. Transmission electron microscopy confirmed the presence of both biosorptive and bioaccumulative mechanisms of *P. glomerata* onto the cell wall and in the cytoplasm respectively.

The reducing sugars produced naturally by *P. glomerata* had a function in copper and nickel ion binding. The EPS may have bound gold and platinum ions in addition to the base metals, and cationic functional groups would have to be analysed for the sorption of precious metals. Additional mechanisms of copper, nickel, gold and platinum uptake were also involved as elemental metals were observed within the *P. glomerata* cells.

Production of reducing sugars in gold and platinum solutions was pH dependent: the higher the pH, the less toxic the gold and platinum species, the more carbohydrate was produced.

The production of EPS which is capable of binding metal ions implies that *P. glomerata* may be an attractive bioaccumulator of metals from wastewaters. Provided the pH is maintained at approximately 6, high concentrations of EPS will be produced which can then be separated from the biomass to recover the metal ions. In conjunction with the metal mobilisation abilities of the fungus at this pH, the use of *P. glomerata* may be a desirable method of recovering metal ions from precious metal refinery wastewaters.

CHAPTER 10

CONCLUSIONS

10.1 Assessment of the optimum conditions for the biosorption of copper, nickel, gold and platinum ions

Initially it was planned that the biosorption of metal ions by plant material of the nickel hyperaccumulator *Berkheya coddii* would be undertaken (Chapter 4). The rationale behind this particular direction in the metal removal investigation was that *B. coddii* is currently being used by Anglo Platinum to recover nickel from process dumps. The biomass is harvested and added to the smelter to recover the metal ions. A biosorption step using the harvested *B. coddii* as the biosorbent was proposed in order to load the biomass with as high a metal concentration as possible before recovery took place. The metal ions of interest would be removed from the refinery wastewaters, where they currently remain unrecovered in evaporation dams.

One of the first aspects of the biosorption process to be investigated was the nature and concentration of functional groups on the biomass surface; this was achieved in part through an acid-base titration (Section 4.5.1). These functional groups are responsible for interacting with the metal ions and hence, metal recovery. Under normal circumstances the pH of the biosorbent would change more gradually than that of the buffer and a correction for the presence of the buffer must be made. In this case the pH of the biosorbent changed more rapidly than that of the buffer (Figure 4.2), making it impossible to correct for its effect.

When the optimal pH for biosorption of copper, nickel, gold and platinum by plant biomass was investigated (Section 4.5.2), it was observed that *B. coddii* had poor specific uptake values when compared to those reported in literature. In addition, the concentration of nickel in the sorbate increased after the equilibration period. It was concluded that since *B. coddii* harvested in the wild was used as a biosorbent, nickel deposits in the leaves were being solubilised. If the nickel was bound in an ion exchange mechanism, its release from the leaves probably resulted in ion exchange with hydroxide ions in solution, which accounted for the rapid increase in pH of the biosorbent during the titration when compared to the buffer alone. The increased nickel concentrations interfered with the sorption of the other three metal ions and resulted in reduced uptake capacity of the biomass. In addition, the leaching nickel ions into solution would result in the production of a secondary waste that would require further treatment. Biosorption of metal ions from precious metal refinery wastewaters with wild *B. coddii* was found to be an unsuitable metal recovery technology due to the low specific uptake of copper, gold and platinum and the release of nickel ions into solution.

10.2 Fungal-metal interactions and bioaccumulation of copper, nickel, platinum and gold ions

Some flasks used in the *B. coddii* biosorption study were left for 48 hours with the metal solutions still in them, after the 2 day period it was observed that several fungal isolates had colonised the metal solutions at a variety of concentrations and pH values. These fungi were subcultured until three pure strains were obtained (Chapter 5). Each strain was screened to assess its ability to remove copper, nickel, gold and platinum from solution through bioaccumulation, as well as its ability to grow in two precious metal refinery wastewaters (Section 5.5.2). It was observed that one particular fungal isolate had increased growth in each wastewater, when compared to the others. In addition, it was able to accumulate high concentrations of gold and platinum, with nickel being the metal ion with the lowest accumulation. These observations lead to further investigations being carried out with the particular fungal isolate and its subsequent identification.

A BLAST search of the ITS region of the fungal rRNA resulted in two possible species, namely *Phoma herbarum* and *Phoma glomerata*. Morphological comparison of the isolate was undertaken to determine which species was cultured from the metal solution. The shape of the pycnidia and colour of pigments produced by the isolate indicated that it was probably *P. glomerata*, however final confirmation was obtained when the presence of chlamydospores was observed. *Phoma herbarum* does not produce chlamydospores, while *P. glomerata* does.

The toxicity and metal uptake of copper, nickel, gold and platinum by *P. glomerata* were assessed (Chapters 7 and 8) to determine the maximum metal concentration that would not inhibit biomass growth and therefore metal uptake. Toxicity was reported as a reduction in respiration due to the presence of the metal ions over 15 hours. The toxicity information refers specifically to acute toxicity and does not take into account the acclimatisation abilities of the isolate, or the chronic toxicity of low concentrations of metal ions over a long period of time. It was found that the presence of gold ions resulted in the greatest reduction in respiration (Section 8.5.1), followed by nickel. Low concentrations of copper ions resulted in slight induction of fungal respiration, probably as it is an essential metal required in the functioning of a variety of enzymes (Section 7.5.1). In contrast, platinum induced respiration to such an extent that in some cases the relative activity was doubled. Only the addition of 150 $mg.l^{-1}$ of platinum resulted in inhibition of respiration. The toxicity of each metal is linked to the pH and metal solution chemistry. The toxicity tests were carried out at low pH values, and gold toxicity was shown to decrease as the pH increased. Had the toxicity tests been carried out at higher pH values, it may have been found that nickel was the most toxic of the four metal ions investigated, however, the pH used was selected as it represented the pH conditions of the refinery wastewater.

Nickel toxicity was observed when the metal ion uptake was investigated (Section 7.5.2). Nickel uptake by *P. glomerata* was minimal and biomass growth at all initial pH values was substantially inhibited by the presence of nickel ions when compared to the control and other metals. In contrast, the removal of copper ions increased and toxicity decreased with increasing pH. Increasing the initial copper concentration resulted in a slight decrease in uptake efficiency, however, as concentrations of free copper were increased, more copper was bioaccumulated.

The preparation of both gold and platinum solutions resulted in precipitation of the metal ions. However, in both cases, *P. glomerata* was able to mobilise the metal from the precipitate, after which rapid removal occurred. The accumulation could be observed visually in the case of gold as the precipitate was dark in colour. Within 24 hours, it was possible to see the gold precipitate localised within the biomass mycelia and removed from the bulk liquid.

Phoma glomerata secretes EPS into the environment during normal growth. The concentration of reducing sugars in the EPS was monitored during the metal ion bioaccumulation study to determine if the presence of metals had an effect on EPS production. The assay used determined carbohydrate concentration as a function of the terminal aldehyde or ketone groups with the carbonyl double bond still intact (Chapter 9). The copper and nickel ions had the ability to interact and bind to the carbonyl group of the aldehyde and ketone moieties. As the carbonyl double bonds were broken, a decrease in carbohydrate concentration could be observed which generated a distinct peak in carbohydrate concentration. The production of carbohydrate was linked to the log phase of fungal growth and the occurrence of the peak depended on the toxicity of the metal ion. As anticipated, the decrease in carbohydrate production was not observed in gold and platinum solutions. This was due to each metal's chemistry in solution. Both exist as chloride anions and cannot interact with the negatively charged carbonyl group. They may have bound to other components of the EPS such as the amine groups on peptides and proteins. The presence of metal ions induced the production of carbohydrate in excess to that of the control, indicating that production of carbohydrate may be a defence mechanism of the organism against metal toxicity.

The increased uptake of precious metals when compared to base metals, reported in Chapter 8, corroborated the preliminary findings in Chapter 5. Increasing the pH decreased the metal toxicity and increased the metal uptake irrespective of the metal ions being investigated. The mobilisation of the gold and platinum ions indicated that *P. glomerata* was able to alter the bioavailability of the metal. This has a significant impact on the bioremediation of wastewaters in which metals have precipitated, because if the fungal isolate can mobilise metals, they may be recovered from otherwise inaccessible forms. The production of the EPS and its ability to bind metal ions

independently from the biomass suggests a promising method of metal recovery as harvesting the EPS could allow for metals to be reclaimed from wastewaters.

10.3 Confirmation of metal uptake by *P. glomerata*

The micro-PIXE analysis reported in Chapter 9 provided bulk metal analysis and confirmed that *P. glomerata* was able to remove metal ions from a mixed metal solution. Localisation analysis was carried out using TEM with single metal solutions. The metal binding appeared to be predominantly around the outside of the cell wall and within the cytoplasm of the cells as discrete entities. Binding to the outside of the cell may suggest a biosorptive mechanism of metal uptake, however, metals visualised through TEM are in the form of elemental ions, which indicated that the copper, nickel, gold and platinum were biologically reduced to their elemental form during deposition. This indicated that the process was more complicated than normal biosorption. Once again preferential precious metal uptake was observed when confirmation of metal uptake was achieved with the use of micro-PIXE.

10.4 Precious metal refinery wastewater pre-treatment by *P. glomerata*

Nutrient investigations during the growth of *P. glomerata* in precious metal refinery wastewater obtained from two dams (reservoirs) at Anglo Platinum precious metal refinery revealed that if the fungal isolate required nutrient supplementation, addition of sucrose and yeast extract would be optimal for growth (Chapter 6). The advantage of these two substrates is their availability. Sucrose can be obtained cheaply from any commercial sugar producer and yeast extract is produced as a waste by-product during fermentation of beer. In addition to elucidation of the optimal nutrient supplementation, it was also found that *P. glomerata* could grow in 100 % (v/v) of both wastewaters. This suggests that the fungal isolate may be an attractive option for metal removal from the wastewaters as they do not need to be diluted. It was also shown that established biomass was able to grow in both wastewaters without nutrient supplementation.

Packed bed reactors were inoculated with *P. glomerata* and then exposed to the wastewaters to assess survival of the fungus and effluent quality. The reactor effluent was found to require an additional treatment step to decrease the dissolved salt concentrations below the guideline limits required for water reuse in DWAF category 4 applications. It was also observed that the *P. glomerata* survived and thrived in the packed beds. The ability of *P. glomerata* to remove metal ions from solution and survive and grow in 100 % (v/v) wastewater makes it an attractive preliminary treatment step to reduce metal toxicity for other processes and mobilise and recover economically valuable metal ions from wastewaters generated during the refining process.

This project has shown that harvested *B. coddii* biomass would not be a suitable biosorbent for the recovery of mixed metals from the wastewaters stored in evaporation dams at the precious metal refinery. It has discovered, isolated and identified a fungus which had previously been considered as a contaminant and demonstrated its potential to preferentially recover precious metals from mixed metal solutions. The fungus, *P. glomerata*, has been shown to actively accumulate metal, even mobilising metals in precipitates, and to withstand the hostile environment of undiluted refinery wastewater, thus demonstrating its potential as the basis for a unit operation in the process train. In order to fulfil this potential, further work is required as described in the following section.

CHAPTER 11

FUTURE RECOMMENDATIONS

11.1 Reactor scale up

Optimisation of the packed bed reactor and investigations into other reactor systems needs to be carried out to determine if *P. glomerata* is capable of decreasing the high salt and organic compound content of the two wastewaters. In addition, the effects of nutrient supplementation should be investigated as the fungal isolate was able to remove over 90 % of ammonium ions under batch conditions, supplemented with sucrose and yeast extract. Screening of additional technologies for salt and organic compound removal should be undertaken in order to achieve holistic wastewater treatment for reuse in DWAF category 4 processes.

11.2 Metal uptake

The metal removal from solution undertaken in this study was carried out with *P. glomerata* biomass which had not been acclimatised to any of the metal ions investigated. The metal uptake capability and tolerance may have been increased through exposure to low copper, nickel, gold and platinum ion concentrations. It has been reported that the nutrient availability affects the metal uptake by living organisms, with this in mind, the effects of different nutrients on metal uptake should be determined. Low concentrations of mixed metal solutions completely inhibited non-adapted *P. glomerata* growth. The toxicity and uptake capacity of copper, nickel, gold and platinum by acclimatised biomass should be investigated to determine the effect of a mixed metal solution on *P. glomerata*. Lastly, metal uptake of other precious and base metals should be carried out to determine the extent of metal bioaccumulation of *P. glomerata*.

APPENDIX A

Table A1 Average change in pH and *q* values for 100 µM and 100 mg.l^{-1} copper solutions after biosorption by *B. coddii* for 30 min and 1 hour respectively. Final biomass concentration of 1 g.l^{-1}.

	Initial pH	Average Δ pH	Standard deviation of Δ pH	Average *q* (µM.g^{-1})	Standard deviation *q* (µM.g^{-1})
100 µm	2.0	0.03	0.05	98.14	0.74
	3.0	0.20	0.03	99.05	0.83
	4.0	0.40	0.09	99.54	0.79
	5.0	0.18	0.48	100.00	0.00
	5.5	-0.37	0.51	100.00	0.00
100 mg.l^{-1}	2.0	0.14	0.05	405.25	58.97
	3.0	0.26	0.03	457.38	32.23
	4.0	0.15	0.07	486.32	37.84
	5.0	-0.60	0.29	514.77	18.85
	5.5	-0.40	0.04	556.16	53.90

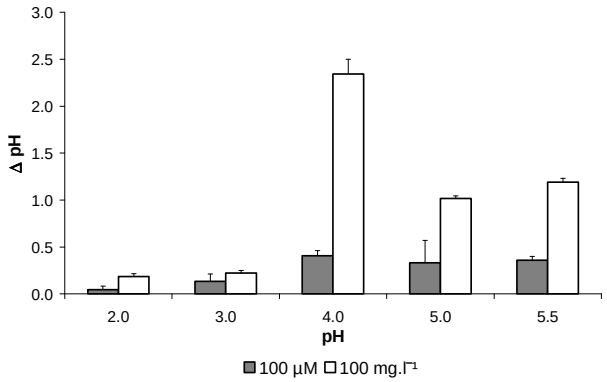

Figure A1 Average difference between initial and final pH for 100 µM (5.86 mg.l^{-1}) and 100 mg.l^{-1} (1703.86 µm) nickel solutions after biosorption by *B. coddii* for 30 min and 1 hour respectively. Final biomass concentration of 1 g.l^{-1}. Error bars represent standard deviation.

Table A2 Average change in pH and *q* values for 100 µM and 100 mg.l^{-1} gold solutions after biosorption by *B. coddii* for 30 min and 1 hour respectively. Final biomass concentration of 1 g.l^{-1}.

	Initial pH	Average Δ pH	Standard deviation of Δ pH	Average *q* (µM.g^{-1})	Standard deviation *q* (µM.g^{-1})
100 µM	2.0	-0.02	0.03	78.95	17.69
	2.5	0.09	0.03	88.04	7.15
	3.0	0.21	0.03	91.81	3.99
	3.5	0.34	0.04	89.91	2.47
	4.0	0.40	0.01	89.83	2.58
100 mg.l^{-1}	2.0	0.08	0.03	124.27	9.19
	2.5	0.06	0.03	120.26	2.87
	3.0	0.21	0.04	119.53	2.81
	3.5	0.25	0.02	115.55	9.22
	4.0	0.06	0.08	126.37	25.78

Table A3 Average change in pH and *q* values for 100 µM and 100 mg.l^{-1} platinum solutions after biosorption by *B. coddii* for 30 min and 1 hour respectively. Final biomass concentration of 1 g.l^{-1}.

	Initial pH	Average Δ pH	Standard deviation of Δ pH	Average *q* (µM.g^{-1})	Standard deviation *q* (µM.g^{-1})
100 µM	0.5	0.03	0.06	59.16	2.70
	1.0	-0.08	0.00	57.85	0.89
	1.5	-0.17	0.03	58.05	0.54
	2.0	-0.08	0.06	57.23	1.09
	2.5	-0.02	0.32	57.30	1.19
100 mg.l^{-1}	0.5	0.08	0.01	293.90	10.00
	1.0	-0.12	0.06	292.71	8.91
	1.5	-0.11	0.03	290.68	12.42
	2.0	-0.01	0.02	282.46	12.95
	2.5	0.17	0.01	274.60	1.52

APPENDIX B

Table B1 Buffer recipes for the extraction of the fungal genome.

Buffer	pH	Reagent	Concentration (µM)
Buffer A	8	Tris HCl	2 000
		EDTA	60
		Sodium chloride	2 000
		Sodium citrate	2
		Calcium chloride	10
PBS	1.2	Sodium chloride	136
		Potassium chloride	2
		Disodium hydrogen phosphate	10
		Potassium dihydrogen phosphate	1.4

Table B2 Buffer recipes for PCR amplification of the fungal ITS rRNA region.

Master mix 1		**Master mix 2**	
Volume (µl)	**Reagent**	**Volume (µl)**	**Reagent**
2	ITS1-F primer (5 mM)	2	Mg-free buffer
2	ITS4 primer (5mM)	1.5	$MgCl_2$
1	DNTPs (10 mM)	0.2	Taq polymerase
1	BSA (50 $mg.l^{-1}$)	8.3	Water
2	DMSO (25 %)		
2.5	Water		

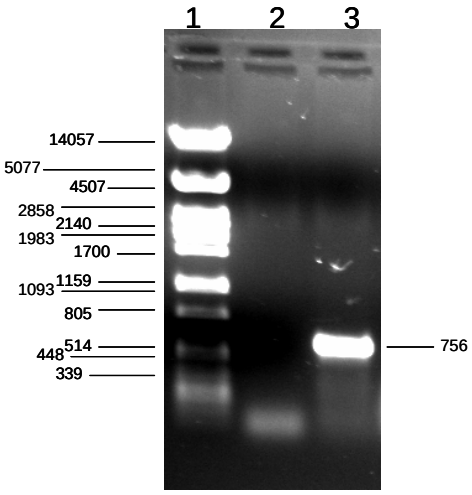

Figure B1 PCR of the ITS region of the 18s rRNA of the grey isolate amplified with the ITS1-F and ITS4 primers. Lane 1, Lambda *pst* molecular weight marker, Lane 2 negative control and Lane 3 PCR product.

Table B3 Reaction mix for the ligation of the fungal ITS region into the pGEM-T Easy vector (Promega, USA).

Volume (µl)	Reagent
1	Buffer
0.2	pGEM-T Easy vector
0.2	DNA Ligase
0.6	PCR product

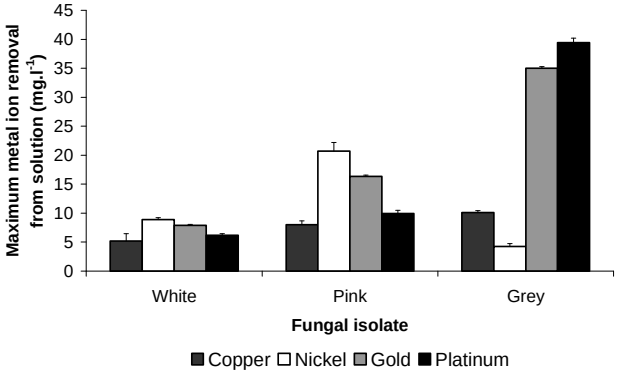

Figure B2 Comparison of the copper, nickel, gold and platinum ion removal by the three fungal isolates grown in malt extract over a period of 72 hours. Error bars indicate standard deviation.

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  1 CGAGCATGCT CCGGCCGCCA TGGCGGCCGC GGGAATTCGA TTTCCTCCGC
 51 TTATTGATAT GCTTAAGTTC AGCGGGTATC CCTACCTGAT CCGAGGTCAA
101 GAGTGTAAAA ATGTACTTTT GGATGTCGTC GTTATGAGTG CAAAGCGCGA
151 GATGTACTGC GCTCCGAAAT CAATACGCCG GCTGCCAATT GTTTTAAGGC
201 GAGTCTACAG GAGACAAACA CCCAACACCA AGCAGAGCTT GAAGGTACAA
251 ATGACGCTCG AACAGGCATG CCCCATGGAA TACCAAGGGG CGCAATGTGC
301 GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA CTACTTATCG
351 CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
401 AAGTTGTAAC TATTAAKTTT TTTCAGACGC TGATTTCAAC TGCAAAGGGT
451 TTAAGTTTGT CCAATCGGTG GGCGAACCCA CCGAGGAAAC GTAAGGTACT
501 CAAAAGACAT GGGTAAGAGA TAGCAGGCAA AGCCTACAAC TCTAGGTAAT
551 GATCCTTCCG CAGGTTCACC TACGGAAACC TTGTTACGAC TTTTACTTCC
601 TCTAAATGAC CAAGAATCAC TAGTGAATTC GCGGCCGCCT GCAGGTCGAC
651 CATATGGGAG AGCTCCCAAC GCGTTGGATG CATAGCTTGA GTATTCTATA
701 GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT
751 GAATGK
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Figure B3 Genetic sequence of the ITS region of the 18s rRNA PCR product from the genomic extraction of the grey isolate.

APPENDIX C

For full BLAST search please see accompanying CD.

APPENDIX D

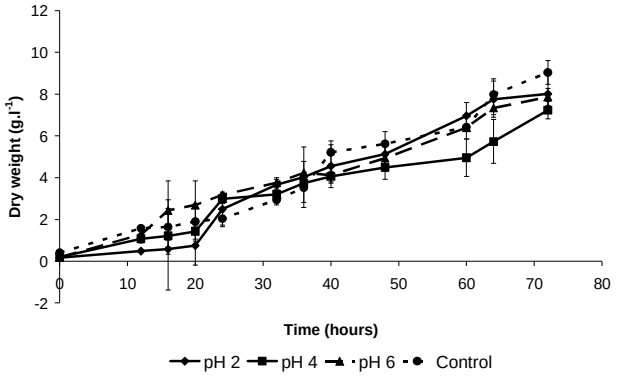

Figure D1 *P. glomerata* biomass growth at an initial copper concentration of 25 mg.l^{-1} of and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

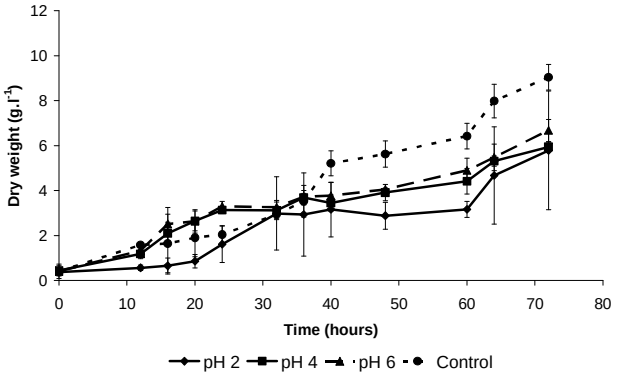

Figure D2 *P. glomerata* biomass growth at an initial copper concentration of 40 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

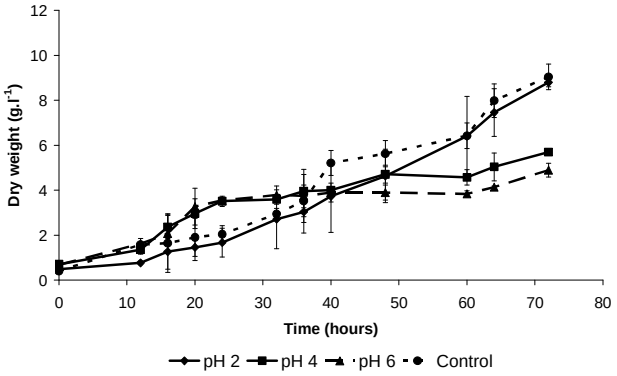

Figure D3 *P. glomerata* biomass growth at an initial copper concentration of 55 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

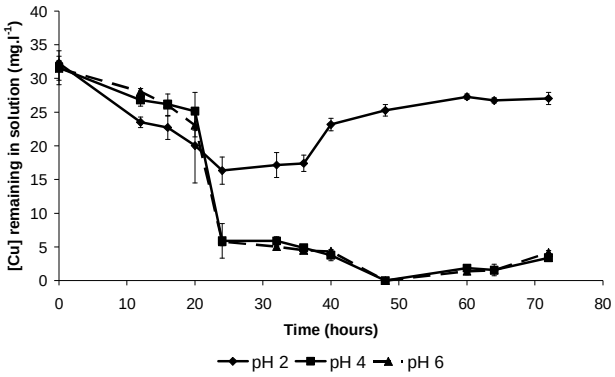

Figure D4 Concentration of copper ions remaining in solution during *P. glomerata* growth in copper solutions with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

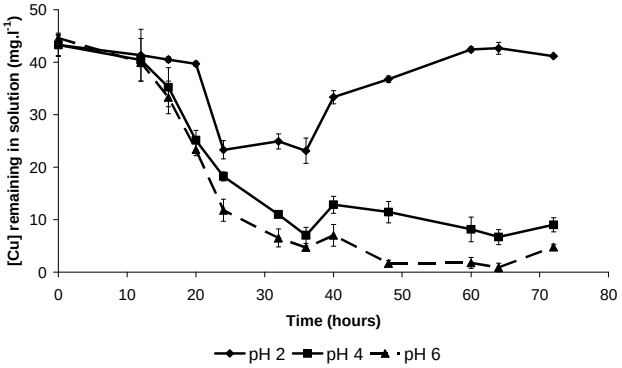

Figure D5 Concentration of copper ions remaining in solution during *P. glomerata* growth in copper solutions with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

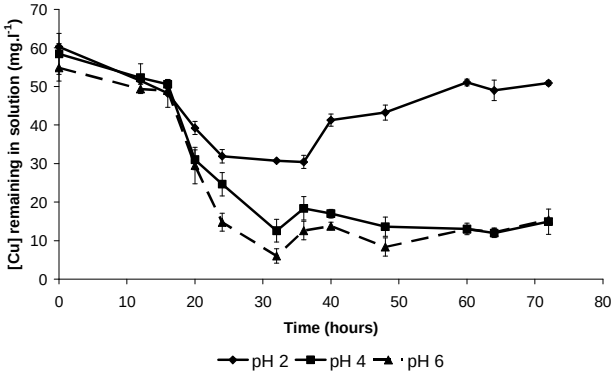

Figure D6 Concentration of copper ions in solution during *P. glomerata* growth in copper solutions with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

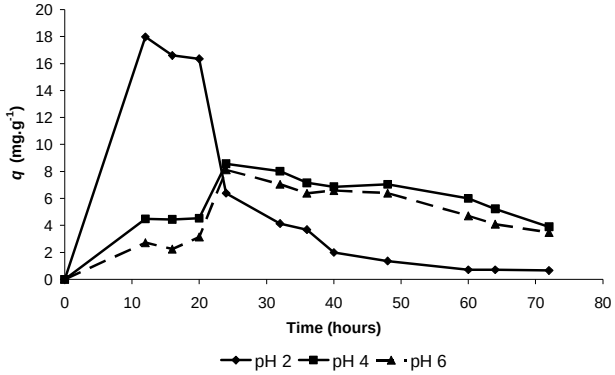

Figure D7 Specific uptake of copper by *P. glomerata* growing in a copper solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours.

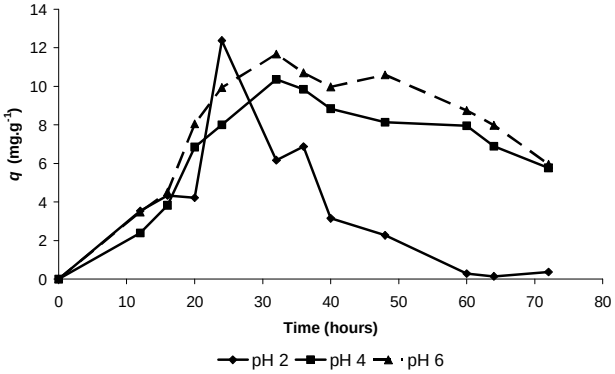

Figure D8 Specific uptake of copper by *P. glomerata* growing in a copper solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours.

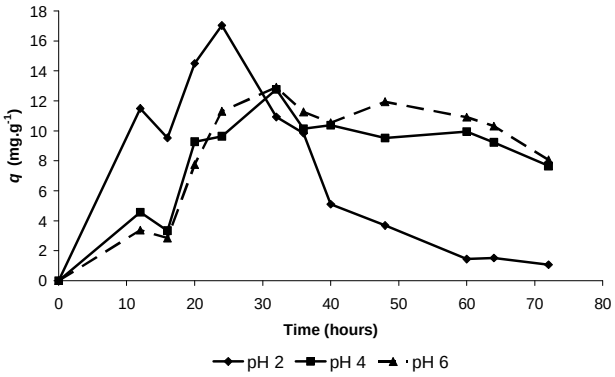

Figure D9 Specific uptake of copper by *P. glomerata* growing in a copper solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours.

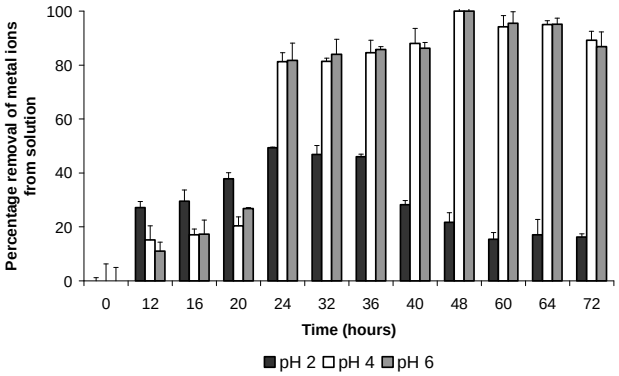

Figure D10 Uptake efficiency of copper by *P. glomerata* growing in a copper solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

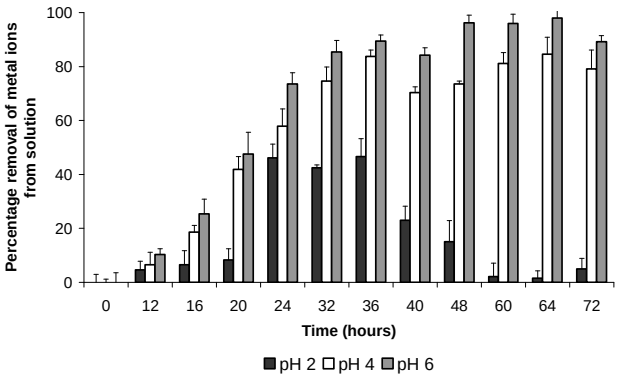

Figure D11 Uptake efficiency of copper by *P. glomerata* growing in a copper solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

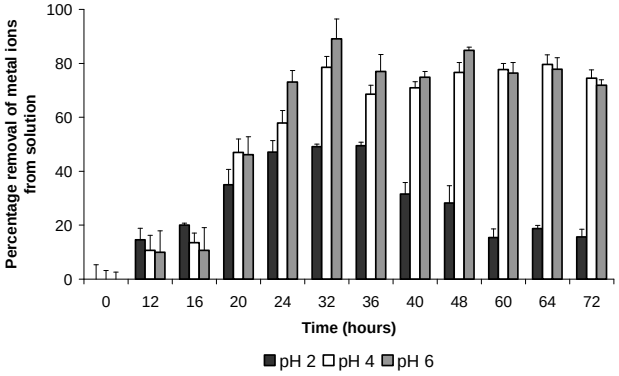

Figure D12 Uptake efficiency of copper by *P. glomerata* growing in a copper solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

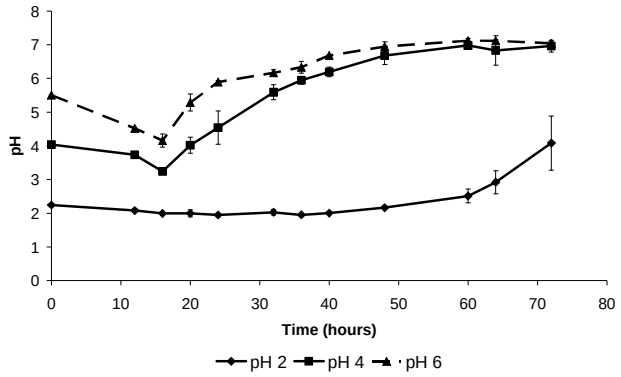

Figure D13 Change in pH during growth of *P. glomerata* growing in a copper solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

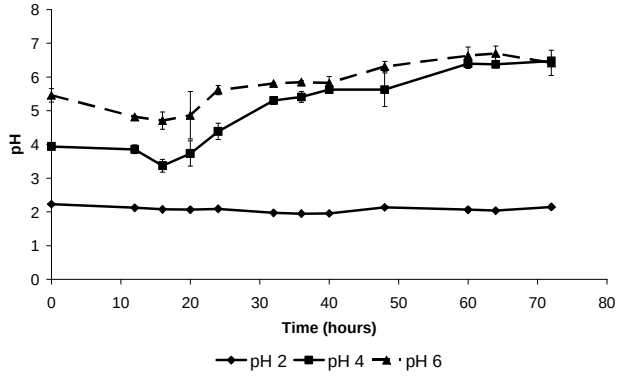

Figure D14 Change in pH during growth of *P. glomerata* growing in a copper solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

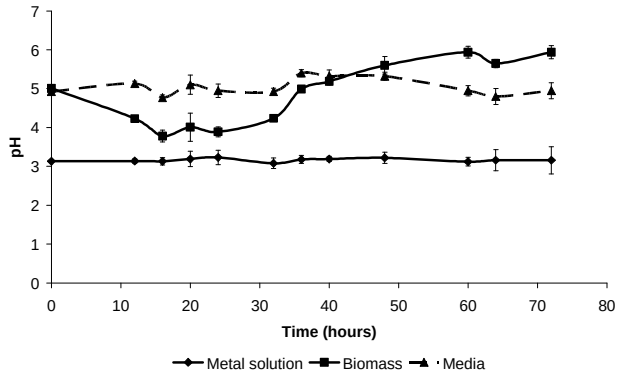

Figure D15 Change in pH during incubation of the media, media and metal and media and biomass controls at 28 ℃ for 72 hours. Error bars indicate standard deviation.

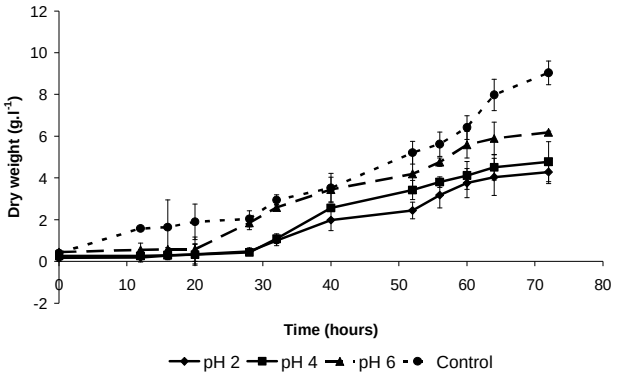

Figure D16 *P. glomerata* biomass growth at an initial nickel concentration of 25 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

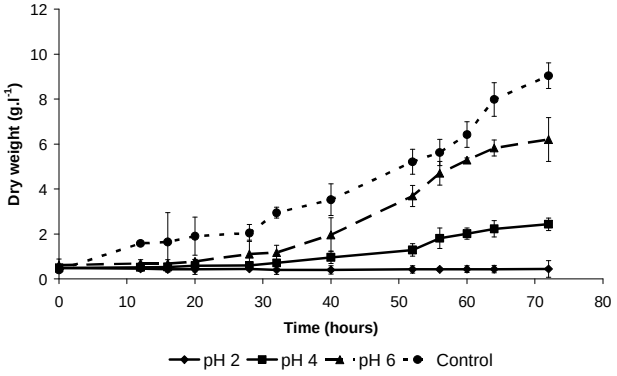

Figure D17 *P. glomerata* biomass growth at an initial nickel concentration of 40 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

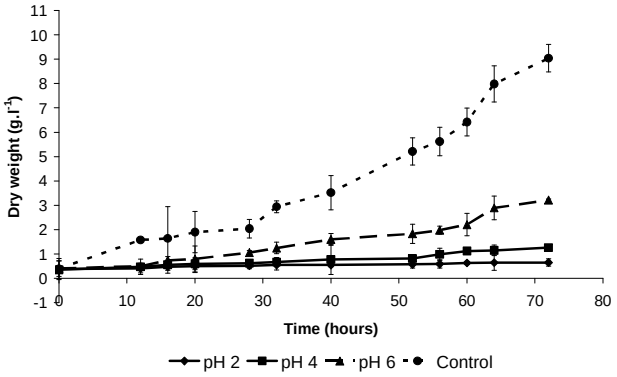

Figure D18 *P. glomerata* biomass growth at an initial nickel concentration of 55 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

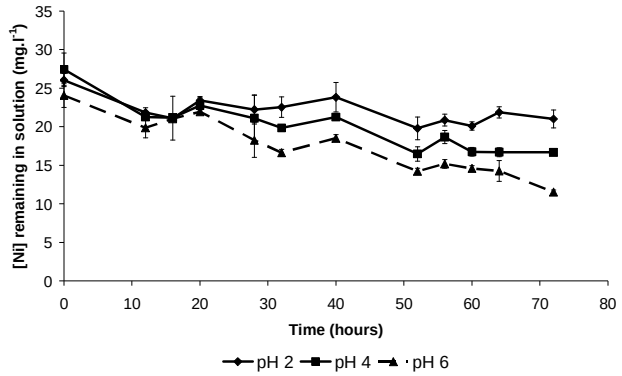

Figure D19 Concentration of nickel ions remaining in solution during *P. glomerata* growth in nickel solutions with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

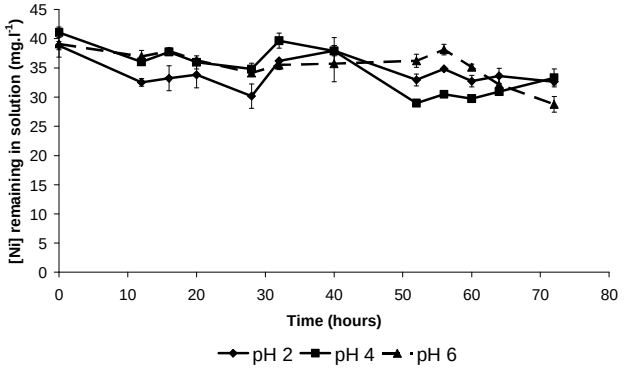

Figure D20 Concentration of nickel ions remaining in solution during *P. glomerata* growth in nickel solutions with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

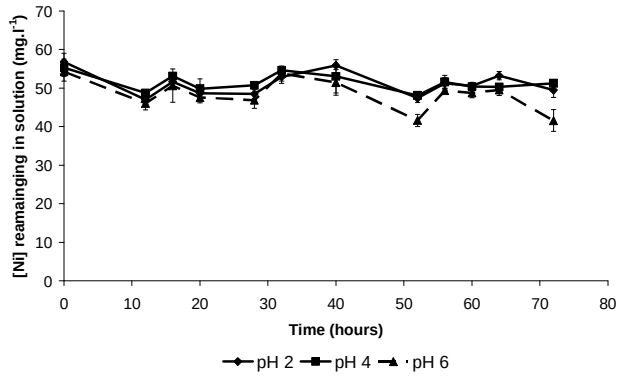

Figure D21 Concentration of nickel ions remaining in solution during *P. glomerata* growth in nickel solutions with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

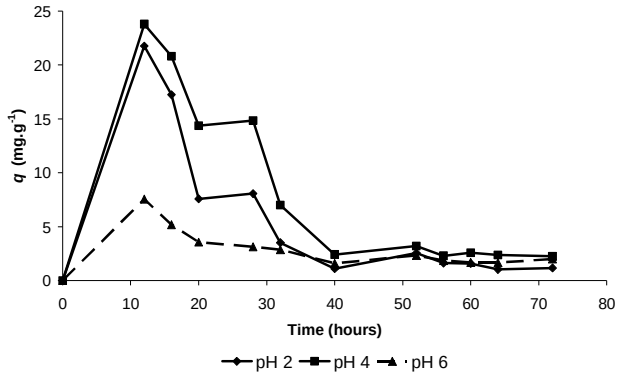

Figure D22 Specific uptake of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 25 $mg.l^{-1}$ at pH 2, 4 and 6 at 28 ℃ for 72 hours.

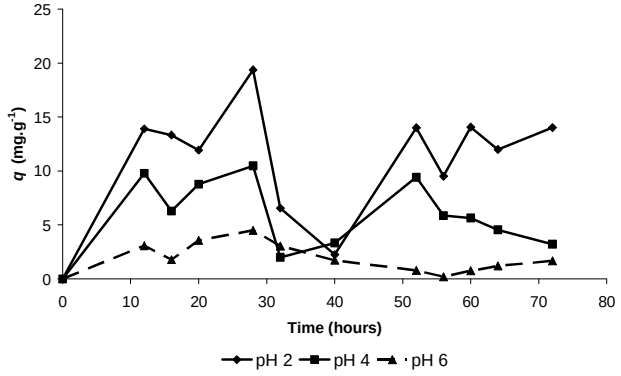

Figure D23 Specific uptake of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 40 $mg.l^{-1}$ at pH 2, 4 and 6 at 28 ℃ for 72 hours.

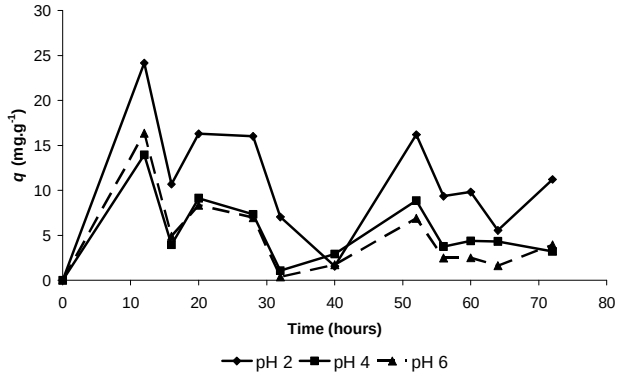

Figure D24 Specific uptake of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 55 $mg.l^{-1}$ at pH 2, 4 and 6 at 28 ℃ for 72 hours.

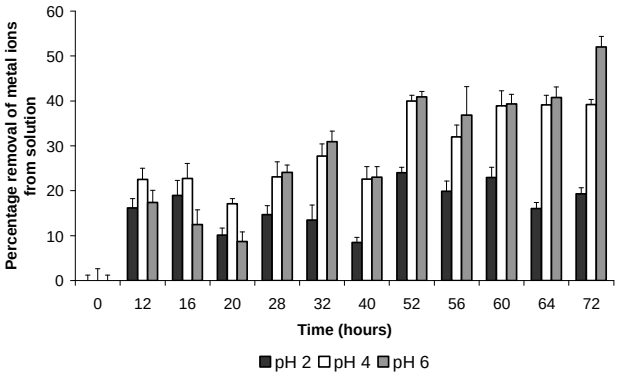

Figure D25 Uptake efficiency of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

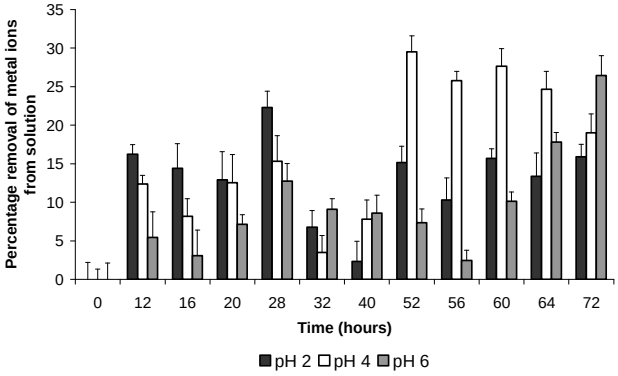

Figure D26 Uptake efficiency of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

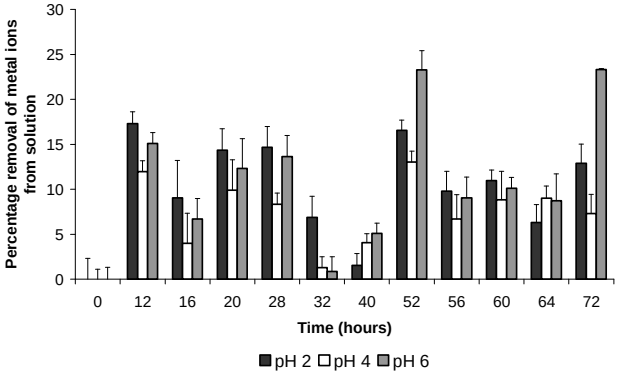

Figure D27 Uptake efficiency of nickel by *P. glomerata* growing in a nickel solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

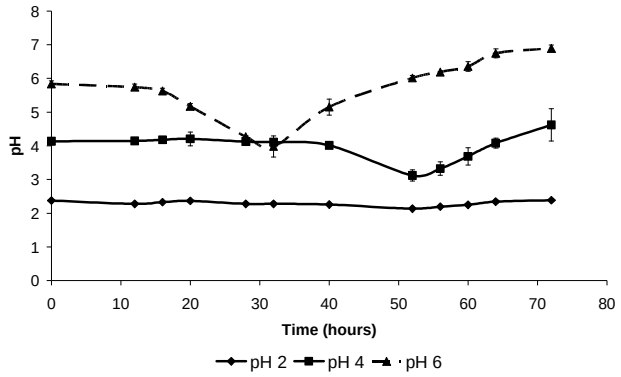

Figure D28 Change in pH during growth of *P. glomerata* growing in a nickel solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

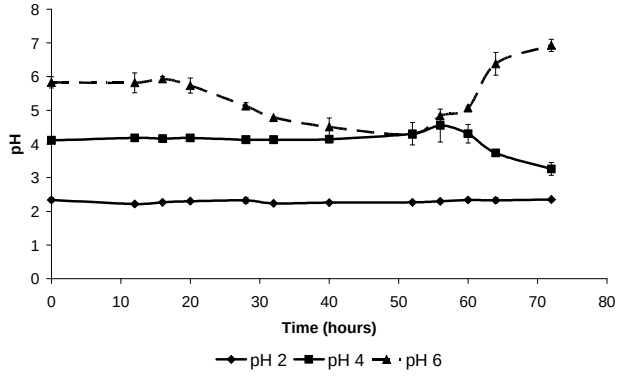

Figure D29 Change in pH during growth of *P. glomerata* growing in a nickel solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

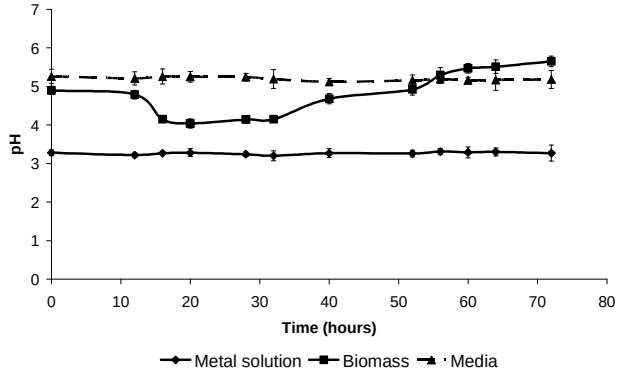

Figure D30 Change in pH during incubation of the media, media and metal and media and biomass controls at 28 ℃ for 72 hours. Error bars indicate standard deviation.

APPENDIX E

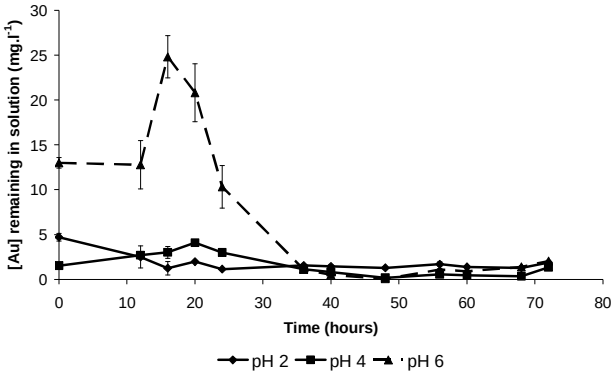

Figure E1 Concentration of gold ions remaining in solution during *P. glomerata* growth in gold solutions with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

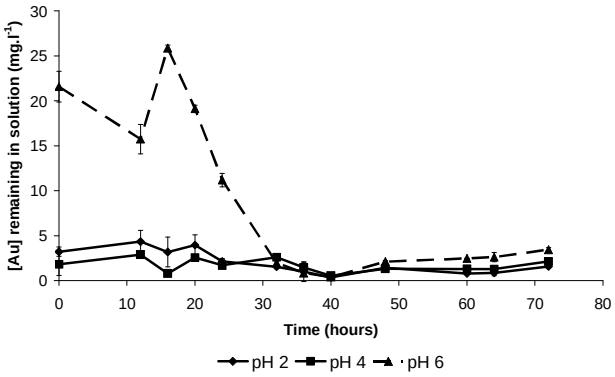

Figure E2 Concentration of gold ions remaining in solution during *P. glomerata* growth in gold solutions with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

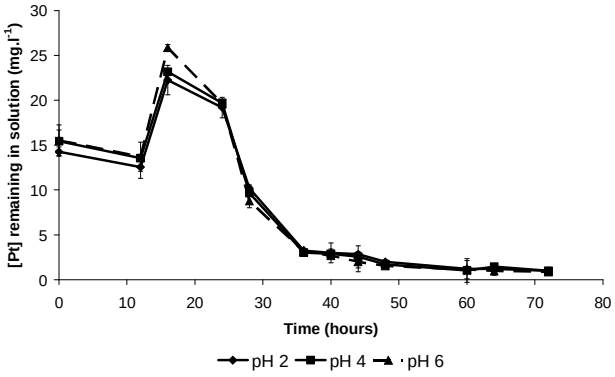

Figure E3 Concentration of platinum ions remaining in solution during *P. glomerata* growth in platinum solutions with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

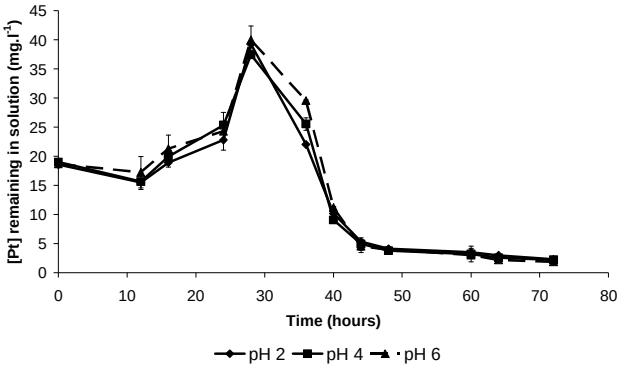

Figure E4 Concentration of platinum ions remaining in solution during *P. glomerata* growth in platinum solutions with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

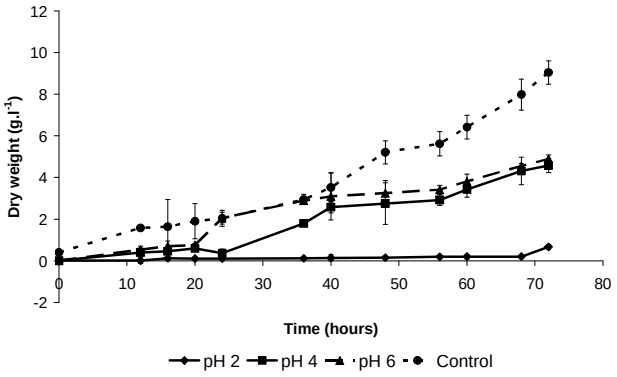

Figure E5 *P. glomerata* biomass growth at an initial gold concentration of 25 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

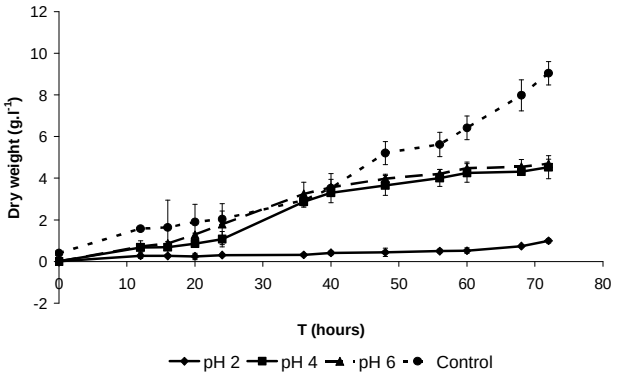

Figure E6 *P. glomerata* biomass growth at an initial gold concentration of 40 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

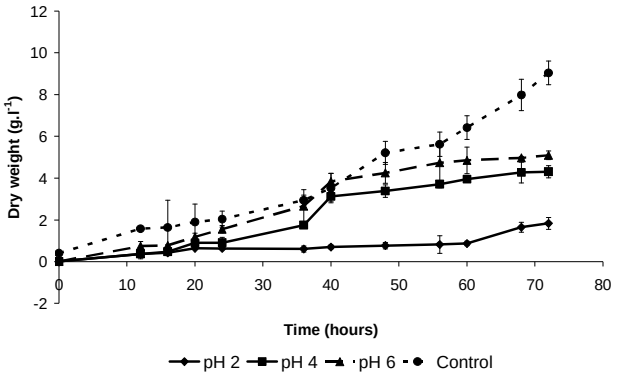

Figure E7 *P. glomerata* biomass growth at an initial nickel concentration of 55 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

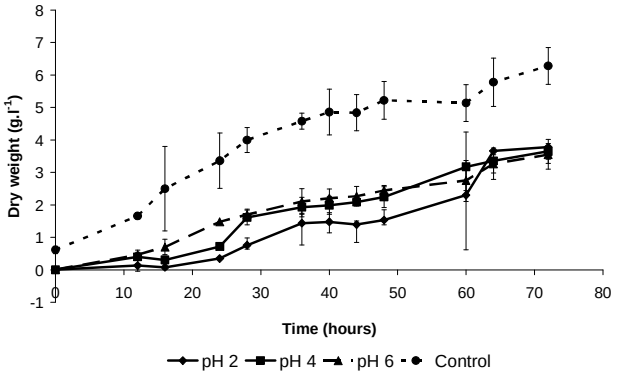

Figure E8 *P. glomerata* biomass growth at an initial platinum concentration of 25 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

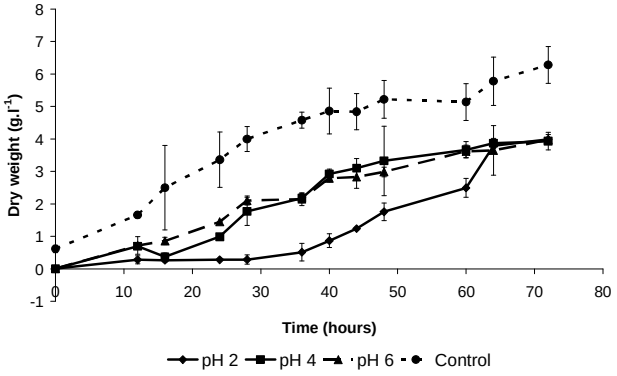

Figure E9 *P. glomerata* biomass growth at an initial platinum concentration of 40 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

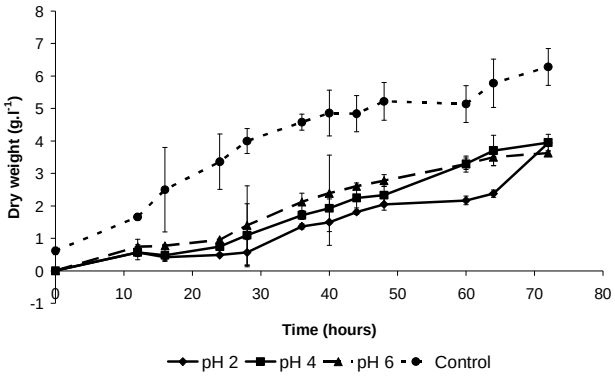

Figure E10 *P. glomerata* biomass growth at an initial platinum concentration of 55 mg.l^{-1} and pH of 2, 4 and 6 at 28 ℃ for 72 hours. Error b ars indicate standard deviation.

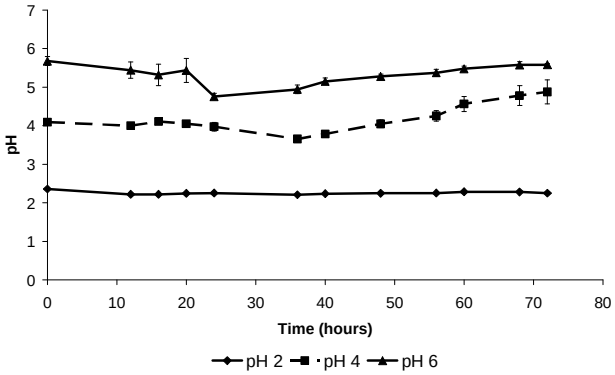

Figure E11 Change in pH during growth of *P. glomerata* growing in a gold solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

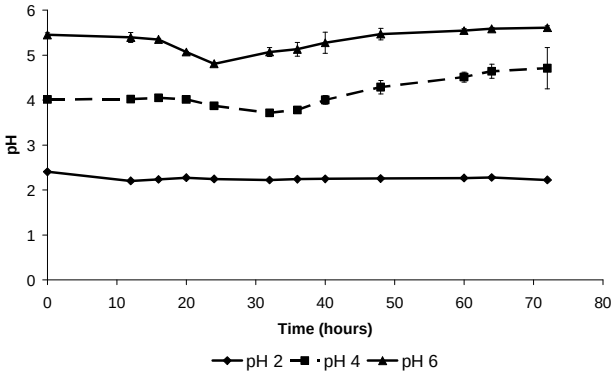

Figure E12 Change in pH during growth of *P. glomerata* growing in a gold solution with an initial concentration of 40 mg.l-1 at pH 2, 4 and 6 at 28 °C for 72 hours. Error bars indicate standard deviation.

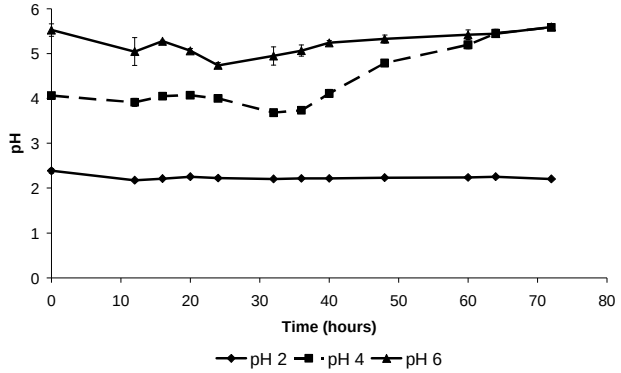

Figure E13 Change in pH during growth of *P. glomerata* growing in a gold solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

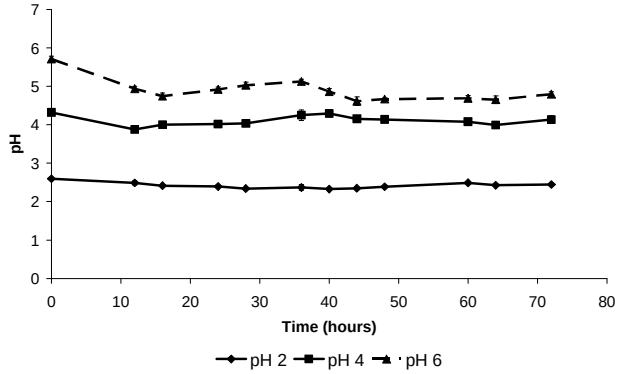

Figure E14 Change in pH during growth of *P. glomerata* growing in a platinum solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

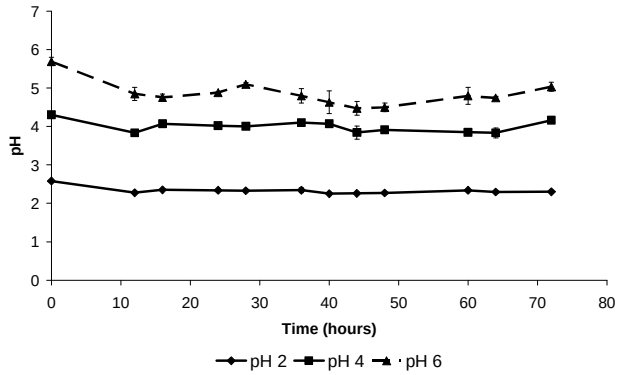

Figure E15 Change in pH during growth of *P. glomerata* growing in a platinum solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

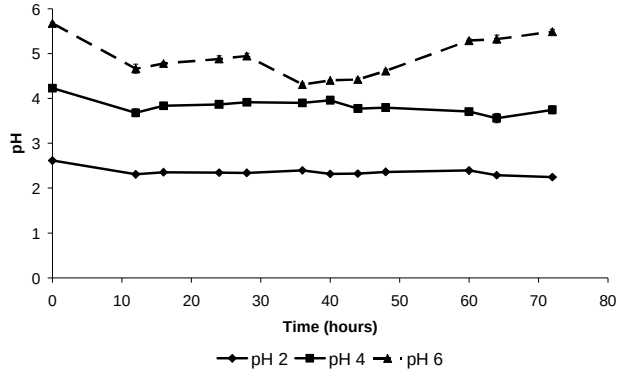

Figure E16 Change in pH during growth of *P. glomerata* growing in a platinum solution with an initial concentration of 55 mg.l^{-1} at pH 2, 4 and 6 at 28 ℃ for 72 hours. Error bars indicate standard deviation.

APPENDIX F

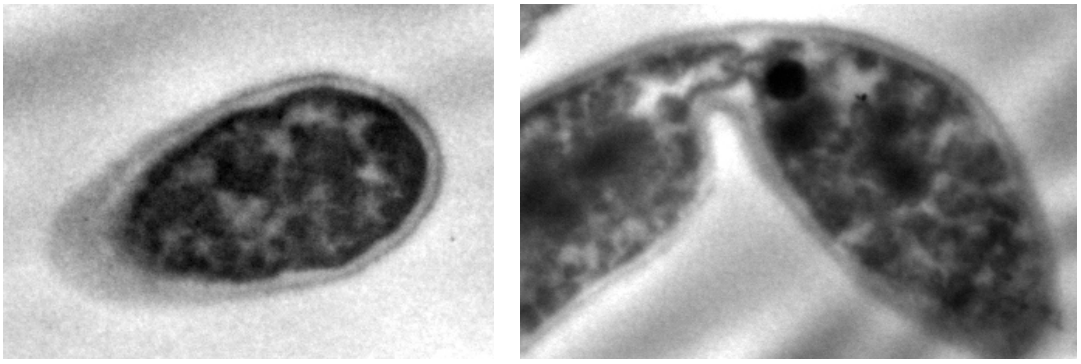

Figure F1 Control biomass of *P. glomerata* viewed by TEM with a magnification of 8000 times.

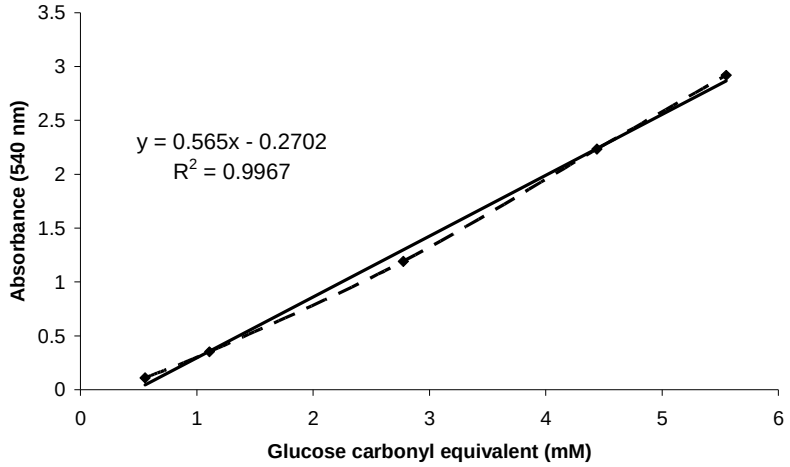

Figure F2 Standard curve of the glucose molar (mM) equivalents of the carbonyl moiety on terminal aldehyde and ketone functional groups on reducing sugars.

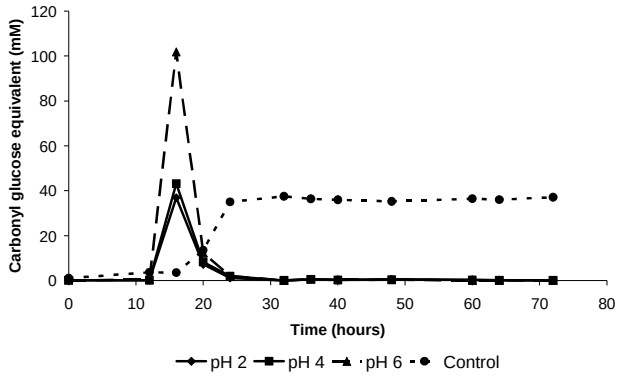

Figure F3 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a copper solution with an initial concentration of25 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

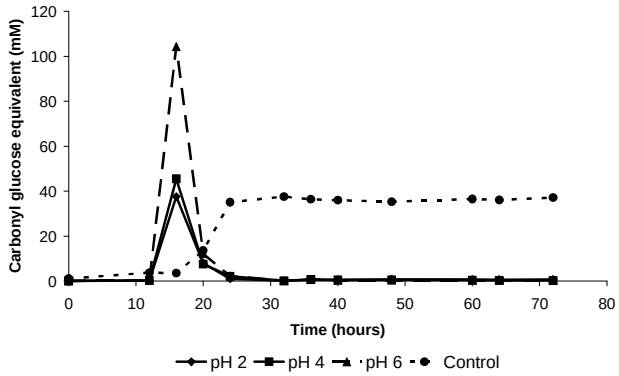

Figure F4 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a copper solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

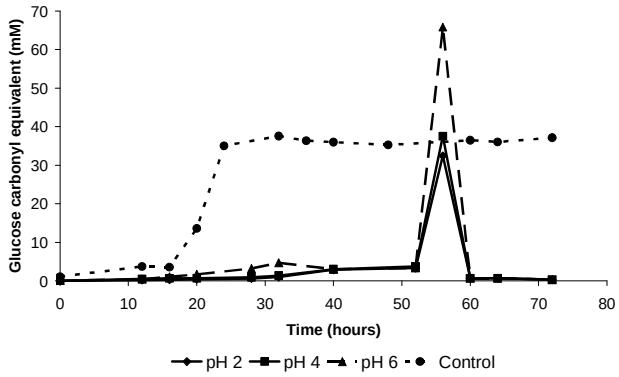

Figure F5 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a nickel solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

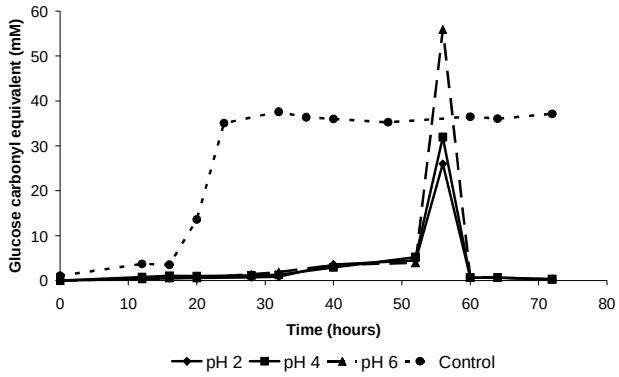

Figure F6 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a nickel solution with an initial concentration of 40 $mg.l^{-1}$ at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

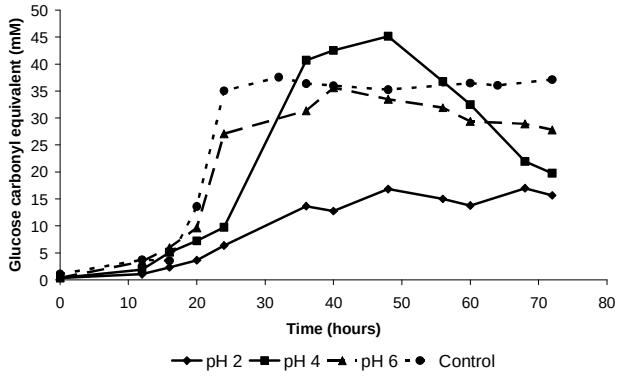

Figure F7 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a gold solution with an initial concentration of 25 $mg.l^{-1}$ at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

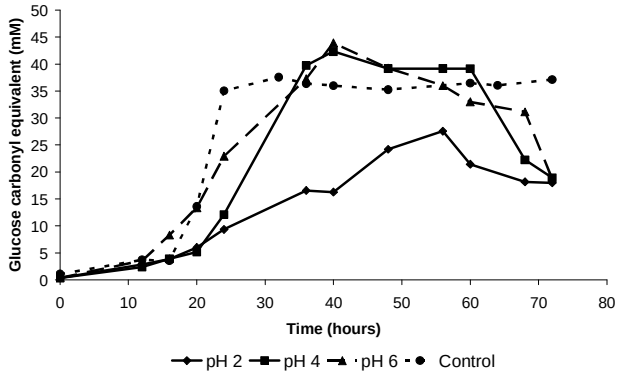

Figure F8 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a gold solution with an initial concentration of 40 $mg.l^{-1}$ at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

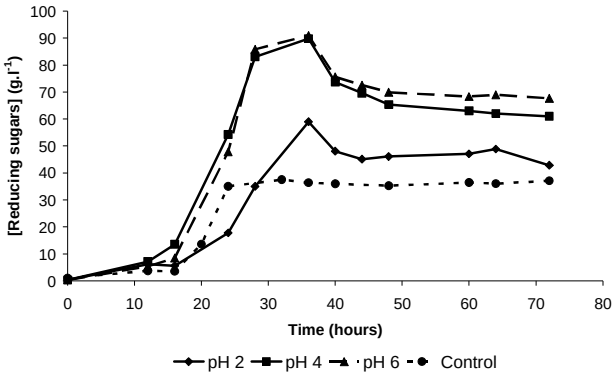

Figure F9 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a platinum solution with an initial concentration of 25 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

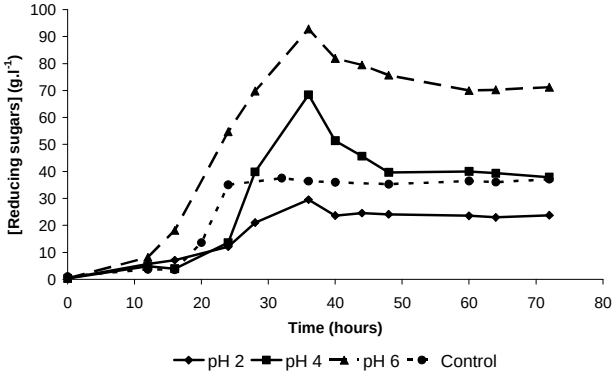

Figure F10 Production of reducing sugars during a 72 hour growth period at 28 ℃ by *P. glomerata* in a platinum solution with an initial concentration of 40 mg.l^{-1} at pH 2, 4 and 6. Standard deviation was 5 % of the mean.

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