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DEFINING A SPECTRUM OF METALS BIOSORBED BY *PAENIBACILLUS CASTANEAE* WITH RESPECT TO HEAVY METAL CONTAMINATION IN GAUTENG

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A research project submitted to the Faculty of Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences (Coursework and Research Report)

Johannesburg, 2016

DECLARATION OF INDEPENDENT WORK

I, **NOKUTHULA CHINHOGA** (**1021787**), am a student registered for the degree of Master of Science in Environmental Sciences (Coursework and Research Report, Full Time) in the academic year 2015.

I hereby declare the following:

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- The information used in the Research Report **HAS NOT** been obtained by me while employed by, or working under the aegis of, any person or organisation other than the University.
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Signature: ___ Nkhinhoga _____ Date: 04/07/2016

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ABSTRACT

Paenibacillus castaneae isolated from acid mine decant (Gauteng, South Africa) was previously shown to tolerate high concentrations of lead (Pb). The ability of the bacterium to tolerate/resist other heavy metals is probable and suggests a role for *P. castaneae* as a biosorbent for their removal from contaminated wastewaters. The current study aimed at determining whether the bacterium is also resistant to other common metal contaminants specifically, zinc (Zn) and nickel (Ni), found in South African wastewaters for biosorption by *P. castaneae*. Additionally, the influence of the external factors pH and competing cations on the uptake of these metals by the bacterium was evaluated. Specific rates of metal uptake (*Q*) were calculated indirectly from quantifying (by spectroscopy) the residual ion concentrations post exposure to 3 mM metal after various treatments. *P. castaneae* was found to tolerate Zn but showed vulnerability towards Ni. In a binary metal system, the bacterium showed a preferential metal uptake in the order Zn>>Co> Mn with a highest *Q* of 26 mg Zn/g biosorbent biomass recorded in the presence of Mn at pH 7. On the contrary, in a multimetal complex solution, the order of preference shifted to Co>>Zn with no absorption of Mn at the same pH. The results indicate that both pH and the presence of cations have an effect on the uptake of Zn by *P. castaneae* that could favour or inhibit its biosorption. The present study confirms the ability of *P. castaneae* to remove additional metals such as Zn, Mn and Co. These findings further suggest the potential of *P. castaneae* as a biosorbent for greener clean-up strategies of contaminated water facilities around Gauteng in the way of bioremediation.

Keywords: *P. castaneae*, biosorption, specific metal uptake, zinc, lead, nickel

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NOMENCLATURE

Å	− angstroms
AMD	− acid mine drainage
BLLs	− blood lead levels
CSIR	− Council for Scientific and Industrial Research
DWAF	− Department of Water Affairs and Forestry
EPS	− extra polymeric substances/exopolysaccharide
EU	− European Union
g	− relative centrifugal force
h	− hour
ICPOES	− Inductively Coupled Plasma Optical Emission Spectrometry
INECAR	− Institute of Environmental Conservation and Research
IQ	− intelligence quotient
LB	− Luria Bertani
MICs	− minimum inhibitory concentrations
mins	− minutes
OD	− optical density
OD_{600}	− optical density reading at 600 nm wavelength
PCD	− programmed cell death
ppm	− parts per million
Q	− specific metal uptake (mg metal/g biosorbent biomass)
rpm	− revolutions per minute
USA	− United States of America
USEPA	− United States Environmental Protection Agency
UV/Vis	− ultraviolet visible
WHO	− World Health Organisation

CHAPTER 1: INTRODUCTION

Heavy metal contamination is a serious threat to the environment and human population. It is of great concern in South Africa and especially in Gauteng. This is as a result of mining activities and other industrial practices such as metallurgy, electronics and plating that release heavy metals into the environment (Zabochnicka-Świątek & Krzywonos, 2014). The challenge of heavy metal contamination is the efficient removal of these heavy metals from the environment so that it becomes safe for the consumption and therefore survival of humans and animals as well as crop production (Plaizier and Sprott, 2012).

Mining is the main cause of heavy metal contamination in Gauteng whereby untreated or poorly treated acid mine drainage (AMD) reaches the natural water sources and contaminates them with heavy metals (CSIR, 2010; McCarthy, 2011). This has been noted particularly in the water bodies that are close to the Witwatersrand (Durand, 2012). The water from the natural sources is used for irrigation of crops resulting in the accumulation of these toxic heavy metals in plants that are consumed by humans. In some cases, the contaminated water is used for drinking and cooking by the people from the nearby communities further harming their health (Oelofse, 2008).

Chemical and physical methods are routinely used to remove heavy metals from the environment especially water. However, these methods are ineffective and costly, often generating secondary toxic sludge. Biological methods such as bioaccumulation and biosorption offer attractive alternatives to traditional metal removal strategies (Zabochnicka-Świątek & Krzywonos, 2014) because they are cheap and eco-friendly. These biological methods use plants or microorganisms such as fungi and bacteria to remove heavy metals.

Biosorption is a passive process that uses microbial biomass to bind heavy metals on their surface and is used in the removal of non-biodegradable heavy metals from contaminated wastewaters (Vijayaraghavan & Yun, 2008). There are several reports on the biosorption of heavy metals like Pb, Zn, Ni, manganese (Mn) and cobalt (Co) by microorganisms. The most common biosorbents are bacteria and several species have been used to remove heavy metals. Such species include *Bacillus subtilis* (Joo et al., 2010), *Pseudomonas aeruginosa* (Clausen, 2000), *Staphylococcus saprophyticus* (Ilhan et al., 2004) and *Paenibacillus jamilae* (Pérez et al., 2008).

Bacterial biosorption has been used successfully and has shown promise for integration into existing water treatment facilities. However, the commercialisation of the process has been rather slow. This is largely due to lack of knowledge on what real production costs would be if biosorbents were to be converted to suitable applicable granules (Volesky & Naja, 2007). Nonetheless, biosorption is a competitive strategy to traditional ion-exchange technology and promises to be both efficient and low-cost (Zouboulis et al., 2004). Therefore the search for new biosorbent-based processes is ongoing.

1.1 Problem statement

Paenibacillus castaneae was previously isolated from AMD and showed promise as a biosorbent for Pb by the Environmental Biotechnology Research Group at the University of the Witwatersrand. An important trait of bacterial biosorbents is their range of metal affinities that enables the removal of more than one toxic metal from complex multimetal solutions such as industrial wastewaters (Puranik & Paknikar, 1999). Currently there is no such information for *P. castaneae* which is critical for developing a bioremediative strategy. The study sought to expand the metal spectrum of *P. castaneae* by focusing on the common metal pollutants of wastewaters in Gauteng. Knowledge generated from this study would contribute to the development of a water treatment strategy that includes *P. castaneae* as a biosorbent for environmental metal contaminants.

The following questions became evident to conduct this research project:

- Can *P. castaneae* biosorb Zn and Ni in addition to Pb as these metals are also found in elevated concentrations in acid mine decant in Gauteng?
- How would pH affect metal uptake by *P. castaneae* as different metals are soluble within different pH ranges?
- Would other metal cations that are not necessarily toxic but found in wastewaters compete with Zn and Ni during biosorption?

To answer these research questions the following aims and objectives were set out.

1.2 Aims and objectives of the study

1.2.1 Aim

The aim of this study was to establish a spectrum that represents the preferential biosorptive capacity of *P. castaneae* for common heavy metal contaminants of water in Gauteng as a result of mining activities.

1.2.2 Objectives

To fulfil the aim of the study, the following objectives were stated, namely:

i. To observe the growth pattern of *P. castaneae* in a rich culture medium.

ii. To calculate the specific metal uptake of Pb, Ni and Zn by *P. castaneae.*

iii. To evaluate the effect of pH on the specific metal uptake of Ni and Zn and the cations Co and Mn on the specific metal uptake of Zn.

1.3 The present study

P. castaneae was first isolated from the phyllosphere of *Castaneae sativa* Miller (the sweet chestnut trees) cultivated in the Sierra de Francia region of Salamanca province, Spain. It is an aerobic, Gram variable rod that grows optimally at 30 ^{o}C and pH 7 although it has been reported to grow at an acidic pH of 5.7 (Valverde et al., 2008). The isolate of *P. castaneae* used in this study was cultivated from acid mine decant (pH 5.4) that originated in the West Rand Basin of Gauteng and flowed into the Tweelopiespruit. An elemental analysis of the decant revealed elevated concentrations of Co (6 mg/L), Fe (500 mg/L), Mn (160 mg/L), Ni (30 mg/L) and Pb (2 mg/L) (Kondiah, 2015) inferring the ability of this organism to survive metal toxicity.

Microorganisms from *Paenibacillus* genus have been used in the bioremediation of heavy metals (Çolak et al., 2013). Pérez et al. (2008) reported on the use of EPS produced by *P. jamilae* for the biosorption of harmful heavy metals (Pb, Ni, Zn, Cu, Co and Cd). The EPS was able to bind all the metals but showed a higher affinity to Pb by biosorbing it ten times more than the other metals that were under investigation.

P. polymyxa isolated from metal-contaminated soil showed tolerance to different concentrations of heavy metals where minimum inhibitory concentrations (MICs) ranged

from 25 µg/mL to 1600 µg/mL for Ni and Cu. It was more effective in Ni and Cu removal and has potential to be used as a biosorbent in the removal of heavy toxic metals from contaminated wastewater (Çolak et al., 2013). In a separate study, *P. validus* Strain MP5 showed adsorption of Zn, Ni, Co, Cd, Cr, and Pb (Rawat & Rai, 2012). The strain was isolated from waste sites of brass, electroplating and textiles industries and has potential to be used in the cleanup of heavy metal polluted sites. Similarly, *Paenibacillus* sp. strain TeW was able to grow in the presence of Pb, Cu, Zn and Ni and showed the same growth pattern in the presence and absence of tellurite (Chien & Han, 2009).

Species belonging to the genus *Paenibacillus* show a great potential for their application in bioremediation processes due to their tolerance for heavy metals. Although similar studies have not been reported in literature for *P. castaneae*, preliminary data has shown the capacity for this isolate to tolerate up to 5500 mg/L Pb and reduce the concentration of metal (5 mg/L Pb) in growth media by 64% within 48 h (Vallabh, 2014). Using these results, it can be inferred that *P. castaneae* could be a potential biosorbent for heavy metals and find application in bioremediative strategies for heavy metal contamination in Gauteng.

1.4 Chapter outline

Chapter 1 is a short introduction to the environmental concerns that this study attempts to address. It includes a problem statement and outlines the research questions, aim and objectives.

The topic is expanded on fully in Chapter 2 by discussing heavy metal contamination in Gauteng and the effects of some toxic heavy metals on life. The chapter continues by explaining the different methods applied for treatment of heavy metal contamination with a focus on bacterial treatment. A section on current literature describing the application of *Paenibacillus* to remove heavy metals concludes the literature review.

Materials and methods that were used during this research are listed and explained in Chapter 3.

The data that was obtained from this research is outlined and analysed in Chapter 4. Furthermore, an in depth discussion on the rate of specific metal uptake and the effect of pH and cations on biosorption of the heavy metals is provided.

Chapter 5 summarises the study while drawing up appropriate conclusions. The chapter also includes recommendations for future work building on the knowledge produced during this study.

CHAPTER 2: LITERATURE REVIEW

2.1 Heavy metal contamination threat in Gauteng, South Africa

Progress in industrial technology and development as well as population growth continues to increase and this is resulting in an increase in the release of toxic heavy metals into the environment (Diagomanolin et al., 2004; Majid & Argue, 2001). Commercial enterprises, for example, mining, metallurgy, electronics and electroplating utilise heavy metals and their technological procedures discharge them as waste into the environment (Zabochnicka-Świątek & Krzywonos, 2014). Other sources of heavy metal contamination include vehicle emissions, automobile battery production, production of fertilisers and pesticides as well as fly ash from incineration processes (Majid & Argue, 2001). The release of these metals consequently pollutes the environment (Zabochnicka-Świątek & Krzywonos, 2014).

Mining has been the backbone of South Africa's economy for a long time and continues to be so today (Sutton et al., 2006). Gold mining in Johannesburg led to the conception of the name 'city of gold' and this is what made Gauteng a prosperity province (McCarthy, 2010). However, the mining activities are resulting in AMD which has become a great concern in South Africa because the polluted water from the mining sites reaches the natural sources without being treated adequately. AMD results from the reaction between iron disulphide (pyrite) and oxygenated water to produce orange-red ferric hydroxide, ferrous sulphate and sulphuric acid (McCarthy, 2011). During normal weathering conditions, the mineral deposits can produce the acid at a very low rate such that it can be neutralised by natural processes (Blowes et al., 2003). This case is not the same during mining activities because the rock is greatly damaged and this increases the surface area and the rate at which acid is produced (McCarthy, 2011). The acidic water then solubilises the other metals in the rock and salts which in turn prompt heavy metal contamination. The contaminated acidic mine water then flows to the ground surface leading to AMD (Adler & Rascher, 2007). The water from mining deposits reaches the natural water bodies with its salts, acid, toxic heavy metals such as Co and cadmium (Cd) and radioactive elements. This process is evident in the Witwatersrand region where groundwater in the area is highly contaminated with heavy metals as a result of gold mining (CSIR, 2010).

All living organisms require some mineral elements in trace amounts for growth and survival such as Zn, iron (Fe), manganese (Mn), sulphate (SO_4) and potassium (K) but there are other metals which do not have any benefits to living organisms (Soetan et al., 2010). The essential

mineral elements are required in very small quantities and become toxic when in excess. Excessive accumulation of both essential and non-essential metals causes serious diseases and even death to humans, plants and animals (Hogan, 2010). Metallic elements with a high density above 5 g/cm^3 (Nies, 1999) and are toxic or poisonous even at very low concentrations are referred to as heavy metals (Lenntech, 2004). The most toxic heavy metals are mercury (Hg), Pb, chromium (Cr), arsenic (As), copper (Cu), Ni, beryllium (Be), tin (Sn), Cd, Co, Zn and Mn (Velásquez & Dussan, 2009).

Water is the most valuable natural resource existing on our planet and without it life on Earth would be non-existent. This fact is widely recognized but pollution of water bodies by human activities such as mining continues to increase drastically (Vijayaraghavan & Yun, 2008). AMD in South Africa continues to pollute water facilities such as the Vaal and Limpopo rivers which result in unpleasant environmental and social impacts. The presence of radioactive elements and toxic heavy metals in water pose a serious threat to human health, aquatic life, plants and animals (Andreazza et al., 2010).

Communities built around these polluted natural water facilities consume water with a pH as low as 2 for their domestic activities (Oelofse, 2008) such as irrigation and water for animals. During irrigation, heavy metals are deposited into agricultural soils and are further absorbed by the plant roots into the tissues of the plant where they build-up (Trüby, 2003). Animals feeding on the contaminated plants and drinking water from contaminated water facilities accumulate heavy metals in their tissues and milk if lactating (Peplow, 1999). When humans consume these plants or animals (and their products) they become exposed to toxic heavy metals and this is a severe threat to their health. Similarly during shortages of clean drinking water, people are forced to consume the water directly from polluted rivers and streams thereby exposing themselves to these toxic heavy metals (Duruibe et al., 2007). Heavy metals have the ability to cumulatively build up in the environment for a very long time. Consequently they result in long term to lifelong adverse effects after the sources of contamination have been destroyed hence the need to dispose safe water into the surrounding natural water sources (Babin-Fenske & Anand, 2011).

A study by Gzik and co-workers (2003) showed levels of Cr, Ni and Pb that were above the acceptable levels in plant parts (roots, fruit and shoots) collected in Rustenburg. During this study, middle aged female chickens and free living adults of the red toads were also tested and found to contain Pb levels between 0.8 and 2.8 mg/kg dry mass with one sample carrying

5.3 mg/kg dry mass. The assumed causes of these high levels of Pb are the trucks and traffic cars from the highway emphasising the need to test agricultural products from this region before human consumption to ensure safety (Gzik et al., 2003).

Concentrations of metal ranging from 1.6-9.3, 2.1-2.5, 2-3 and 10.5-20.1 µg/L for Cd, Zn, Cu and Pb respectively, have been reported in the Dzindi, Madanzhe and Mvudi rivers found in Thohoyandou of the Limpopo province (Okonkwo & Mothiba, 2005). The higher levels of Pb were attributed to the use of leaded petrol vehicles in the area. Additionally, high levels of Pb in Madanzhe and Mvudi rivers might have been influenced by the nearby sewage treatment plant and a waste dumping site respectively. Levels of Cd in this study were thought to have been caused by agricultural activities since fertilisers may contain metal impurities and some pesticides which contain metals. Zn and Cu had the lowest concentrations and Zn contamination was assumed to have been from the cheap Zn roof tops of the houses in this area (Okonkwo & Mothiba, 2005).

An investigation by Brandl (2011) on the levels of heavy metals around Ebenezer Dam in Limpopo showed that Pb, Zn, Cu, Cr and As in the water were present in concentrations below the maximum acceptable limiting values showing the water was safe for agriculture and domestic use. However, the soil, rock and sediment samples (surrounding the dam) had elevated concentrations of these heavy metals. Overall concentrations of Zn, Pb, As, Cu and Cr in the entire study area were 157, 57, 73, 313 and 888 mg/g respectively (Brandl, 2011).

2.2 Toxic effects of the most common heavy metal pollutants

Common metals that are known to pollute water include As, Cd, Cu, Cr, Hg, Ni, Pb and Zn (Wang & Chen, 2006). Elemental analysis of mine polluted water on the West Rand Basin of Gauteng, South Africa showed Co, Mn, Ni, Pb and Zn to be present at concentrations exceeding acceptable limits that are reflected in other mining areas within Gauteng (K. Kondiah, personal communication). These metals are the focus of the present study and their hazardous effects on human health are outlined below.

2.2.1 Lead

Pb is considered to be the most harmful toxin among other heavy metals (Ferner, 2001). Common sources of Pb are fuel additives, batteries, fishing pigments and roofing (Moeletsi et al., 2004). The effects of Pb poisoning in adults include kidney failure, poor foetus development, damages to the cardiovascular system and the central nervous system (Duruibe

et al., 2007). It also results in poor development of the grey matter in the brain and this leads to a poor intelligent quotient (IQ) in young children (Udedi, 2003). A survey that followed-up on Blood Lead Levels (BLLs) in children in primary schools around Johannesburg, Cape Town and Kimberly in South Africa from 1995-2008 raises concerns over Pb contamination and its subsequent health risks in these areas. The 2002 survey showed a decrease in BLLs among 7-11 year-olds from an average of 11.9 μg/dL in 1995 to 9.1 μg/dL in 2002 (Mathee et al., 2003). However, BBLs as low as 3 μg/dL are markedly high to cause adverse effects in children (Canfield et al., 2003; Kootbodien et al., 2012) so it is critical to find ways to reduce Pb levels in such environments. The 2007-2008 survey showed an increase in number of children with BLLs <5 μg/dL for all the three cities when compared to the 2002 survey despite the discontinuation of leaded-fuel. The assumed major contribution to these results was poverty – parents working in jobs that use high levels of Pb, poor levels of nutrition and limited resources to prevent Pb exposure (Naicker et al., 2013).

A Rustenburg study on heavy metal contamination was conducted by Gzik and co-workers showed that the concentration of Pb in plant samples had an average of 11 mg/kg dry weight which is higher than the acceptable amounts of 0.2 mg/kg of Pb (EU Commission, 2001). The animal samples did not show any traces of Pb in them (Gzik et al., 2003). An assessment of heavy metals contamination in Dzindi River, Limpopo also showed elevated concentration of Pb with an average of 0.03 mg/L which is toxic to aquatic life, animals, plants and humans (Edokpayi et al., 2014).

Heavy metal contamination in vegetables (cabbage, carrots, spinach, onion and tomato) and soil samples in Alice town in the Eastern Cape Province have been reported (Bvenura & Afolayan, 2012). The samples from this site were collected randomly around the town and analysed for the presence of heavy metals. Pb was not found in the vegetable samples but in the soil samples. The concentration was between 5.15 mg/kg and 14.01 mg/kg of Pb dry mass and according to the World Health Organisation (WHO) standards this does not pose a threat to human health (Bvenura & Afolayan, 2012).

Ayeni and co-workers (2010) analysed soil samples from the adjacent soil and the river bank of the lower Diep River in Cape Town for the presence of heavy metals. They showed that of the 4 sites where soil samples had been collected from, one had a concentration of 71.7 mg/kg. This is a cause of concern for Pb contamination in this area (Ayeni et al., 2010).

2.2.2 Zinc

Zn is a heavy metal that is considered non-toxic when taken orally but excess amounts result in impairment of growth and reproduction (INECAR, 2000; Casper & Malter, 2015). Sources of the metal include pharmaceutical industries, galvanising, textiles, domestic wastes and cosmetics (Moeletsi et al., 2004). Acute toxicity of Zn results in abdominal pain, nausea, vomiting and diarrhoea (Schwartz et al., 2005) and chronic toxicity results in reduced iron function, reduced immune function and weakens pancreatic enzymes (Nriagu, 2007).The signs of illness associated with Zn poisoning are similar to those of Pb and can be mistakenly diagnosed as Pb poisoning (McCluggage, 1991).

The study by Edokpayi et al. (2014) on Dzindi River, Limpopo also showed Zn to be present at a concentration (0.1 mg/L) higher than the approved guidelines for aquatic life but lower than that for irrigation (DWAF, 1996). While Bvenura & Afolayan (2012) reported the absence of Pb in vegetable samples around Alice Town (Eastern Cape), they found elevated level of Zn in the same samples. Their study reported between 4.27 mg Zn/kg and 89.88 mg Zn/kg with tomatoes, onions and spinach having unacceptable levels that are a cause for concern. The average concentration of Zn from the adjacent soil and river bank samples of Diep River were 27.8 mg/kg and 97.6 mg/kg respectively making the soil unsuitable for agriculture (Ayeni et al., 2010).

2.2.3 Nickel

Alloys, Ni-Cd batteries, electroplating and launderettes are the sources of Ni contamination in the environment (Cempel & Nikel, 2006). Ni is classified as a carcinogenic metal and it is an environmental and occupational pollutant. Short-term exposure severely damages the reproductive system (Forgacs et al., 2012) and can result in infertility, miscarriage and birth defects (Apostoli & Catalani, 2010). Long-term exposure results in increased chances of lung cancer, neurological deficiency, poor development in children and high blood pressure (Chervona et al., 2012). Additionally, Ni exposure also damages the kidneys and the liver (Das et al., 2008). In Egypt 25 workers who worked in a nickel-plating industry had their kidney functioning tested and the results showed that all of them were suffering from a compromised liver function (El-Shafei, 2011).

In a separate assessment of heavy metal contamination in Kroondal (close to Rustenburg) by Gzik's group (2003), soil and plant samples carried increased concentrations of Ni considered

too toxic for human health. The soil contained Ni between 146-593 mg/kg dry mass while plant samples had an average of 79 mg/kg. Agricultural activities carried out on such soils enable the passage of heavy metals to humans and animals via the food chain.

2.2.4 Manganese

Industries like iron and steel production and the burning of fossils fuels are the main sources of Mn contamination (Williams et al., 2012). Although the human body contains approximately 10 mg of Mn which is used as an enzyme cofactor, excessive concentrations result in anaemia and increased chance in hypertension for people who are over 40 years of age. Patients suffering from severe hepatitis and heart attacks have been associated with high levels of Mn suggesting a role in detrimental effects on human health (Blaurock-Busch, 1997).

Blood samples of grade-one school children from Johannesburg and Cape Town were analysed by Röllin and co-workers for the presence of Mn with the consent of parents. While majority of the Mn concentrations were below the maximum allowed value of 14 µg/L, 12.5% and 4.2% of the children from Johannesburg and Cape Town, respectively, registered higher blood Mn concentrations (Röllin et al., 2005).

Once again, Mn concentrations higher than the recommended standards of 0.05 mg/L set by DWAF for domestic water use (DWAF, 1996) were found. A research that was conducted in Dzindi River in Limpopo Province showed that the concentrations of Mn had an average value of 0.15 mg/L (Edokpayi et al., 2014). Additionally, agricultural soils along Diep River contained 16.2 mg/kg and 26.4 mg/kg of Mn respectively Ayeni et al. (2010) further confirming the threat to humans and animals by food chain transfer.

2.2.5 Cobalt

Co forms an integral part of vitamin B12 that is necessary for the formation of healthy red blood cells. It is required in the human body in very small amounts but at increased levels, it has been associated with neurological (Tower, 2010), cardiac and endocrine symptoms (Brock & Stopford, 2003).

The main source of Co contamination is mining (Barałkiewicz & Siepak, 1999). Mitileni et al. (2011) conducted a study on the dissemination of heavy metals in deposits around New Union Gold Mine Tailings in Limpopo and Co was found to be present at an average

concentration of 18.8 mg/kg. Although this is lower than the South African recommendations (Steyn et al., 1996), according to the USEPA Sediments quality recommendations (USEPA, 2000) such concentrations are hazardous to human health. These findings indicate the need for continuous monitoring and consistency within legislation surrounding environmental safety.

It has been estimated that the amount of people in the world that are exposed to very high concentrations of toxic heavy metals is approximately one billion and some millions from this number are estimated to be suffering from subclinical metal poisoning (Nriagu, 1988). There is evidence that consumption of food or water contaminated by toxic heavy metals exposes humans to several diseases and some of them are chronic ones. Carin Smit, a clinical metal toxicologist mentioned that "when metals enter the body, they attach themselves to the sulphur and hydrogen in proteins which are found in the enzymes and hormones of the body thereby disabling these catalytic elements randomly or purposely leaving neuro-immuno-endocrinological devastation" (Smit, 2009).

It is very important to find a method that will be applied to treat heavy metals before they are released into the environment so as to reduce the threat to human life. Heavy metal contamination does not necessarily have to be a problem because there are chemical methods to treat it however, these methods have their drawbacks such as high cost and production of toxic substances post-treatment hence the need to find other effective ways of treating it (Plaizier & Sprott, 2012).

2.3 Biological treatment of heavy metals from contaminated water

Several chemical and physical methods are being used to treat heavy metal contamination (Khosravi et al., 2005) namely filtration, chemical precipitation, evaporation, oxidation/reduction, ion-exchange, membrane technology and reverse osmosis (Zabochnicka-Świątek & Krzywonos, 2014). Currently, South Africa has one reverse osmosis plant in Witbank which operates at a commercial scale to treat heavy metal contamination. However, this method has a limitation of high cost just like the other chemical and physical methods (McCarthy, 2011). Alternatively, promising effective methods such as biosorption and bioaccumulation which use biological materials (microorganisms or plants) can be applied to treat heavy metal contamination. Biological methods for the removal of contaminating metals offer several advantages over chemical and physical methods as outlined in Table 1.

Table 1: The advantages and disadvantages of physicochemical and biological methods for the removal of heavy metal contaminants from the environment.

Method	Advantages	Disadvantages	References
Physicochemical:			
Reverse osmosis	• Releases pure effluent	• Expensive • Uses high pressure • Membrane fouling	Zouboulis et al., 2004
Chemical precipitation	• Easy to operate • Affordable • Safe to operate	• Difficult to separate • Produces toxic sludge as end product • Ineffective	Zouboulis et al., 2004 Barakat, 2011
Ion-exchange	• Effective, therefore metal removal is very possible • Metal recovery	• Sensitive to pH of the solution • Non-selective • Resins are expensive	Zouboulis et al., 2004 Barakat, 2011
Electrochemical treatment	• Metal recuperation	• Can only be used at very high metal concentrations • Sensitive to some compounds	Zouboulis et al., 2004
Biological:			
Biosorption	• Low cost • Efficient and rapid • Operational in broad conditions (pH and temperature) • It does not produce chemical toxins • Highly selective • Uses both living and non living microorganisms	• When non living cells are used the process cannot be improved as they will not be metabolising • Early saturation during the binding of the metal might be a problem • Poor selectivity	Zabochnicka-Świątek & Krzywonos, 2014 Karakagh et al., 2012 Pardo et al., 2003 Ahluwalia & Goyal, 2007
Bioaccumulation	• Highly selective	• Uses living microorganisms only • More expensive than biosorption	Zabochnicka-Świątek & Krzywonos, 2014

When microorganisms are to be used for biosorption or bioaccumulation, bacteria is mostly preferred as it has outstanding advantages over others and these include high surface area to volume ratio, high concentration of potentially active chemosorption sites in their cell walls and they are also found abundantly in the environment (Mullen et al., 1989). The biological material can bind to heavy metals thereby removing them from the contaminated water. However, the way the biological material binds in these two processes are different. During the process of biosorption, the biological material binds the heavy metals on its surface whereas during bioaccumulation the biological material allows the heavy metals to be transported inside it (Zabochnicka-Świątek & Krzywonos, 2014).

Non living microorganisms are more efficient to use in water treatment because they:

- do not require a continuous supply of nutrients which makes the process more affordable than using living organisms.
- are not affected by toxic wastes.
- can be used more than once (Zabochnicka-Świątek & Krzywonos, 2014).
- have the ability to take up metals in high concentrations (Karakagh et al., 2012) as they lack protons that are present during metabolism (Pardo et al., 2003).

The structure of the cell wall of biosorbent used in biosorption plays a very crucial role as it is involved in the binding of metal to the biosorbent. Specifically, the functional groups found within the cell wall of bacteria such as phosphate, amine, hydroxyl, carboxyl, and sulfhydryl groups are able to bind metal cations (Stumm & Morgan, 1996). Gram negative and Gram positive bacteria have different biosorption capacities because the components of their cell structure are different. It is interesting to note that Gram negative bacteria have shown an increased metal biosorption capacity than Gram positive bacteria. This is because Gram positive bacteria have a complex cell structure while Gram negative bacteria have a simple structure which exposes all the proteins and functional groups responsible for metal binding on its cell surface (Joo et al., 2010).

Factors such as pH (for both biomass and metal solution), temperature, incubation time, concentration and type of biomass used and the presence of other cations in the solution influence biosorption rates (Özdemir et al., 2009). Therefore, it is very crucial for these factors to be monitored during biosorption research studies. The pH in which the cells and metal solutions are conditioned govern the surface charge of the microorganism and chemical

state of the metal ion, respectively (Randall et al., 1979). As a result, the attraction of the metal to the microorganism during biosorption is dependent on the pH of both solutions. Different metals have different optimum pH for high metal uptake (Dönmez et al., 1999; Saeed & Iqbal, 2003). For effective and efficient rates of metal removal using microorganisms it is therefore important to consider the pH at which the process of bioremediation will occur. While working on the biosorption of Cu and Cd using *Sphaerotilus natans*, Esposito et al. (2001) concluded that the amount of metal absorbed increases as pH also increased. They further explained that the reasons for this include "competition for the hydrogen ions which will be in low concentration and the weakly acidic nature of the active sites on the adsorbent whose deprotonation with increasing pH favours the metal uptake".

The presence of other heavy metals and soluble cations during biosorption compete with the metal of interest for the binding sites on the surface of the cells. This in turn will have an effect on the amount of metal that will be removed (Naja et al., 2010). Heavy metals cannot be found individually in the contaminated soils or water (Modak et al., 1996), subsequently it is better for any biosorbent to be able to remove a range of metals. Such metal preferences can easily be defined by calculating the maximal rates of adsorption by a specific biosorbent for different metals. According to Engl & Kunz (1995) when *Saccharomyces cerevisiae* was used for biosorption of Zn, Cu, Cd and Pb the affinity of this fungus was greatest for Cu followed by Cd, Pb then Zn. However when equimolar bimetal solutions were used by adding Pb to each metal, the amount of metal removed decreased significantly for Cu, Cd and Zn but *S. cerevisiae* showed a greater affinity for Pb. The study of biosorption of heavy metals in multimetal solutions help in understanding the interaction between the metals in the solutions and also investigating their competition for the biosorbent binding sites (Engl & Kunz, 1995).

Biosorbents of heavy metals can be classified as bacterial, yeast, fungal or algal (Chen et al., 2009; Mythili & Karthikeyan, 2011) but for the purpose of this study, the focus was on bacterial biosorbents.

2.3.1 Bacterial treatment of heavy metals from contaminated environments

There are several studies that have been carried out to analyse the ability of bacterial species to take up heavy metals in the search for commercialisable biosorbents. Among these, *Bacillus*, *Pseudomonas* and *Streptomyces* are reported to be potent biosorbents (Das et al., 2008; Vijayaraghavan & Yun, 2008). Table 2 summarises some of the common bacterial

biosorbents and their biosorption capacities. It is noteworthy to mention that the table is not an exhaustive list due to the extensive number of studies that have been reported on metal uptake by bacteria. Furthermore, direct comparisons are impossible as each study was conducted under different conditions.

Table 2: A summary of some important bacterial species that have been reported in literature as good biosorbents for heavy metals.

Microorganism	Metal removed	% or amount removed	References
Geobacillus thermodenitrificans	• Fe • Cr • Co • Zn • Pb	• 79.9 mg/g • >70.7 mg/g • >69.76 mg/g • >48.26 mg/g • >32.26 mg/g	Chatterjee et al., 2010
P. aeruginosa ASU 6a	• Zn	• 83.3 mg/g	Joo et al., 2010
Rhodococcus opacus	• Pb • Co • Mn	• 95.2 mg/g • 13.4 mg/g • 6.91 mg/g	Cayllahua & Torem, 2010; Abdel-Ghani & El-Chaghaby, 2014
Staphylococcus saprophyticus	• Pb • Cr • Cu	• 100% • 24.2% • 14.5%	Ilhan et al., 2004
Bacillus sp.	• Pb	• 50%	Varghese et al., 2012
B. firmus	• Pb • Zn • Cu	• 98.3% • 61.8% • 74.8%	Salehizadeh & Shojaosadati, 2003
B. cereus AUMC B52	• Zn	• 66.6 mg/g	Joo et al., 2010
	• Mn • Pb	• 43.5 mg/g • 21.1 mg/g	Joo et al., 2010 Çolak et al., 2011
B. pumilus	• Pb	• 28.06 mg/g	Wang & Sun, 2013
B. licheniformis	• Cu • Fe • Cr	• 14-95% • 95% • 52%	Samarth et al., 2012
B. licheniformis	• Cu	• 93%	Clausen, 2000

CCO1	• Cr	• 52%	
P. putida	• Zn	• 6.9 mg/g	Pardo et al., 2003
	• Pb	• 270.4 mg/g	Uslu & Tanyol, 2006
	• Cu	• 96.9 mg/g	Uslu & Tanyol, 2006
S. rimosus	• Zn	• 80 mg/g	Mameri et al., 1999
	• Ni	• 32.6 mg/g	Selatnia et al., 2004
B. subtilis IAM 1026	• U	• 52.4 mg/g	Nakajima & Tsuruta, 2002

Much of the information on bacterial biosorption is derived from toxicity studies where the effect of metal uptake on cellular metabolism was evaluated. In one such study, soil bacterial species namely *P. fluorescens* and *B. mycoides* were able to tolerate 700 mg/mL Cr and 500 mg/mL Ni because they could grow unaffected in these concentrations (Jankiewicz & Kuzawinska, 2009). The same study also reported that *B. mycoides* was tolerant to 40 mg/mL Cd. *Citrobacter* Strain MCM B-181 showed a favourable order of metal sorption at pH 4.5 and pH 6 when exposed to equimolar concentrations of multimetal cations (Pb, Cd, Zn, Ni, Co and Cu). The pH of the solution affected the specific metal uptake but the order of metal uptake did not change (Puranik & Paknikar, 1999).

According to Silambarasan & Abraham (2014) there are quite a number of biosorption studies that confirm the efficiency of bacterial strains in removing heavy metals and also their survival in a wide range of metals concentrations. Bacterial isolates (164) from soil, sediments and water samples were assessed for heavy metal resistance and about 45% of them showed a relatively high tolerance to Pb (>6000 µg/mL), an average tolerance to Ni and Cr (1000-1500 µg/mL) and low tolerance to Zn (<500µg/mL).

Microorganisms isolated from samples collected around contaminated soils and water bodies show more tolerance to increased levels of heavy metals than those isolated from uncontaminated areas (Moberly et al., 2010). The microorganisms are able to adapt, grow, survive and multiply in such conditions (Chatterjee et al., 2010; Chien & Han, 2009; Ilhan et al., 2004) as a result of defence systems that are activated in the presence of accumulated metal ions. These include active processes such as enzymatic detoxification, intra and extracellular sequestration and active reduction of metal ions as well as passive interaction with the cell wall and extracellular polymeric substances (EPS) (Akcil & Koldas, 2006; Hookoom & Puchooa, 2013; Permina et al., 2006). Subsequently, most microorganisms that

are used for bioremediation are sourced from wastewaters and soils that contain heavy metals. The bacteria of interest in this study, *P. castaneae*, was isolated from acid mine decant (containing heavy metals) from the West Rand Basin in Gauteng (K. Kondiah, personal communication).

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of individual metal stocks

All growth media, metal salts and nitric acid were purchased from Sigma-Aldrich, USA. Both metal salts and nitric acid were of ≥99.999% trace metals basis to prevent external metal contamination.

A 10 mM stock solution of each metal was prepared by dissolving metal salts of $Pb(NO_3)_2$, $NiCl_2 \cdot 6H_2O$, $ZnCl_2$, $MnCl_2.4H_2O$ and $CoCl_2.6H_2O$ individually in sterile de-ionised water. The stocks were filter sterilised through a 0.22 µm membrane and used at a working concentration of 3 mM.

3.2 Microbial strains and culture conditions

A growth profile of *P. castaneae* that was isolated from the West Rand basin was constructed (as described below) to determine a suitable time at which to harvest cells for the biosorption assay. Biosorption occurs maximally during stationary phase therefore it was important to establish the various growth phases of the isolate used in the study.

Freshly streaked Luria Bertani (LB) agar plates were used to inoculate a standard concentration of 1.5×10^8 cells/mL *P. castaneae* into LB broth. The standardised inoculum was prepared using the BBL™ Prompt™ Inoculation System (BD, USA) according to the supplied protocol. Briefly, five bacterial colonies (>1 mm in diameter) were picked using the inoculation wand supplied with the kit and resuspended in 1 mL sterile physiological saline by vortexing for 10 s. This resulted in an inoculum containing approximately 1.5×10^8 cells/mL. The inoculum was further diluted 1:100 in LB broth and incubated with shaking at 25 ºC for 30 h. All experimental work using *P. castaneae* was carried out at 25 ºC and incubated with shaking between 150 and 250 rpm unless otherwise stated. A 1 mL sample was removed regularly every 2 h and the optical density (OD_{600}) measured using a Libra S22 UV/Vis Spectrophotometer (Biochrom, UK). The assay was repeated in triplicate and the average readings used to construct a growth profile of OD_{600} against time.

3.3 Biosorption study

3.3.1 Preparation of biomass

P. castaneae was grown for 16 h at 25 ºC to stationary phase as determined from the results of the growth assay described in section **4.1**. Bacterial biomass was harvested by

centrifuging at 10 000 *g* for 10 mins. The cells were washed twice (2 500 *g* for 5 mins) in physiological saline and the supernatant discarded. The weight of the cell pellet was determined according to **Equation 3.1** below and the cells resuspended in sterile de-ionised water to a final concentration of 10 mg/mL.

Weight of (cell pellet + centrifuge tube) – Weight of centrifuge tube = Weight of cells only (**3.1**)

The dry weight of the cells was determined by drying 5 mL culture in a pre-weighed aluminium foil container in an oven at 60 ºC until a constant weight was reached. The weight of the dry biomass was calculated according to **Equation 3.2** below and used to correct for presence of moisture when calculating the rate of biosorption of metal.

Final constant weight (cells + aluminium foil container) – Initial weight (aluminium foil container) = Dry weight of cells (**3.2**)

3.3.2 Biosorption assay

Fifty milligrams of *P. castaneae* cells prepared in section **3.3.1** above was added to Pb solution to yield a final volume of 25 mL with a concentration of 3 mM Pb. The solution was incubated with shaking for 30 mins to enable the uptake of metal by the biomass. Following incubation, the biomass was separated by centrifuging at 17 000 *g* for 10 mins. This was done to separate the bound metal (to biomass) from the residual ions in solution. The pellet was discarded and the supernatant adjusted to a pH ≤2 using an equal mixture of nitric acid and sterile de-ionised water. Residual metal concentration was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES) at the School of Chemistry (University of the Witwatersrand).

The same procedure was followed using Zn and Ni individually. A control consisting of 3 mM metal without the addition of *P. castaneae* biomass was included for all three metals tested. Each biosorption assay was performed in triplicate to ensure statistical accuracy.

The specific metal uptake (*Q*) was calculated according to **Equation 3.3** below (Puranik & Paknikar, 1999):

$$Q = V (C_i - C_f)/1000M \quad (\mathbf{3.3})$$

where *Q* – specific metal uptake (mg metal/g biosorbent biomass)

V – volume of metal solution (mL)

C_i – initial concentration of metal in solution (mg metal/L)

C_f – final concentration of metal in solution (mg metal/L)

M – dry weight of biomass (g)

The percentage of metal biosorbed by *P. castaneae* was calculated by **Equation 3.4** below:

$$\% \text{ Biosorption} = \frac{(\text{Initial metal concentration} - \text{Final metal concentration})}{(\text{Initial metal concentration})} \times 100 \quad (\mathbf{3.4})$$

3.4 Effect of pH

pH of the solution influences metal solubility as well as availability of binding sites on bacterial cells. To observe the effect of pH on biosorption of Zn and Ni, both *P. castaneae* biomass and metal solutions were conditioned to pH 5, 6 or 7. Conditioned biomass (50 mg) was then added to the metal solution with the corresponding pH and the procedure for biosorption described in section **3.3.2** above was followed. Each pH was tested in triplicate and a respective control included.

The present study did not include experimental work on Pb as it formed part of a separate study within the research group. The results from this study were used to add to existing data accumulated within the research group with the long-term goal of optimising the metal biosorption process in *P. castaneae*.

3.5 Effect of cations

Cations are known to compete for the non-specific binding groups present on bacterial cells. The effect of cations on biosorption of Zn by *P. castaneae* was performed at pH 5, 6 and 7 using conditioned biomass and metal solutions. Studies were performed in binary and multimetal systems using Co and Mn as these metals are also present in high concentrations in polluted wastewater in Gauteng.

Conditioned biomass (50 mg) was exposed to binary metal solutions containing equimolar concentrations (3 mM) of Zn and Co or Zn and Mn, respectively, at each pH according to the method described in section **3.3.2**. Similarly, the biomass was exposed to a multimetal solution consisting of 3 mM concentrations of all three metals at the different pHs. Residual metal concentrations were determined by ICPOES after bound metal (biomass) was removed from the exposed solutions as described previously. Each binary/multimetal system was tested in triplicate at pH 5, 6 and 7 and included a metal solution control without added biomass.

The effect of pH and competing cations on the biosorption of Zn was assessed by calculating *Q* values for each treatment. These values were compared to that obtained for Zn under both neutral and non competitive conditions.

CHAPTER 4: RESULTS AND DISCUSSION

P. castaneae was previously shown to proliferate in nutrient medium containing high concentrations of Pb. This trait supports further characterisation of metal tolerance in *P. castaneae* with the long-term goal of finding suitable metal biosorbents for bioremediation. This research focused on Zn and Ni. The biosorption study showed that *P. castaneae* biosorbed the metals in the order of Pb>>Zn but was susceptible to Ni at a concentration of 3 mM. Subsequent to noting the vulnerability of the bacterium to Ni, the effect of competing cations was only done on Zn with Co and Mn. This chapter presents a thorough analysis and interpretation of the experimental results focusing on the rate of Zn uptake and the influence of external factors such as pH on its biosorption.

4.1 Growth assay

Biosorption occurs maximally when bacterial cells are in stationary phase (Fosso-Kankeu et al., 2009). This is because cells have grown to their full size and do not have to direct most of their energy to replication (Monod, 1949). Daughney et al. (2001) determined the exponential and stationary phases of *B. subtilis* by taking OD_{600} readings for 48 h so that this bacterium could be used for the removal of Cd and Fe. Similarly, to determine the time point between which the stationary phase in *P. castaneae* occurs at, a growth assay was performed as described in section **3.2**. The growth profile shown in Figure 1 was constructed from the average of three replicate OD_{600} readings taken every 2 h from culture grown at 25 ^{o}C for a period of 30 h.

It can be observed that *P. castaneae* experiences a typical bacterial growth process evidenced by the four distinct phases: lag, exponential (logarithmic), stationary and decline that microorganisms go through after being incubated into a nutrient medium culture (Widdel, 2007). At 25 ^{o}C, *P. castaneae* undergoes a lag period that lasts approximately 6 h (Fig 1) during which time cells are known to adjust to the new environment and start to develop structurally without increasing in number (Monod, 1949). This is immediately followed by the exponential phase when the cells start to reproduce by binary fusion resulting in a constant increase in the growth rate (Monod, 1949). In this study, *P. castaneae* was cultured in LB medium which is a rich medium carrying the optimum nutrients for its growth. As the exponential phase continued the *P. castaneae* cells were consuming the nutrients and producing waste (Monod, 1949). This was the beginning of stationary phase.

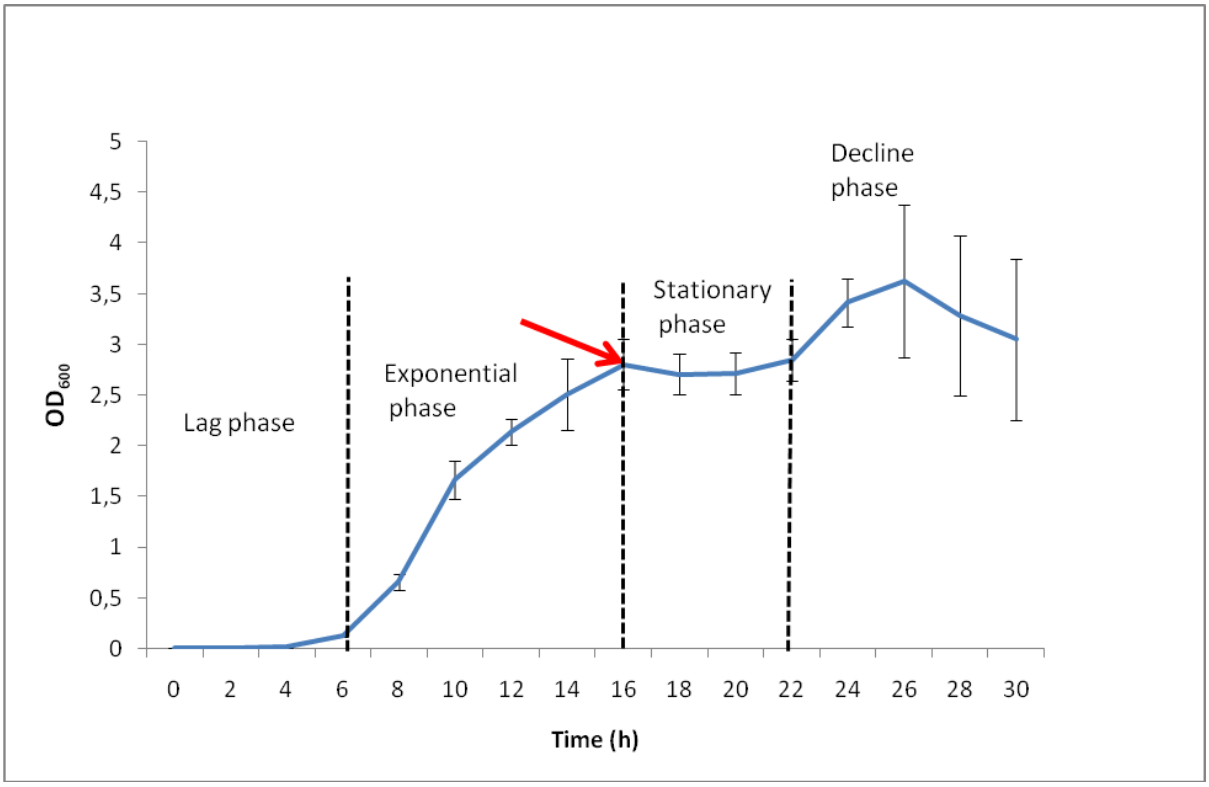


Figure 1: The growth profile of *P. castaneae* at 25 °C for 30 h. The profile shows the typical stages of microbial growth: lag, exponential, stationary and decline. This was used to establish the suitable time (indicated by the red arrow) to harvest the bacterial biomass to be used for biosorption studies.

The stationary phase was reached 16 h after incubation. This stage is characterised by a constant population of mature cells maintained through an equilibrium of dying and multiplying cells (Widdel, 2007). Determining the stationary phase was critical for all downstream experimental work because it is at this point that bacterial cells are best known to biosorb maximally (Fosso-Kankeu et al., 2009). Goyal et al. (2003) investigated the effect of biomass age on the removal of Cr by *S. cerevisiae*. The results showed that the cells that had reached early stationary phase removed more Cr from the solution than those that were still in the exponential phase. In a separate study, *B. subtilis* and *B. bacterium* were noted for maximum metal removal (Ag, Cr and Pb) at the early-stationary and late-stationary phases respectively (Fosso-Kankeu et al., 2009).

An interesting trend was observed after 22 h, where it appears that *P. castaneae* started actively replicating again but only for a short period of 4 h after which it rapidly declined in numbers signalling the death phase. This phenomenon could be as a result of programmed cell death (PCD) where suicidal cells undergo apoptosis releasing nutrients into the surrounding medium for subsequent use by the existing population (Ameisen, 2002; Lewis, 2000). Alternatively, Yetis et al. (2000) mentions that there are some microorganisms that

have two exponential phases and this is mainly caused by growth media with two carbon sources. When the first carbon source runs out during the growth, other microorganisms die which reduces the OD readings. However the microorganisms that remain adjust to the second carbon source and they continue to grow and increase again for a limited period thereby increasing OD readings (Kompala et al., 1986; Lee et al., 1974). The increase in OD after 22 h might mean that *P. castaneae* is one of the microorganisms with two exponential phases. From these results it was decided to use *P. castaneae* cells harvested at 16 h for the biosorption assays.

4.2 Biosorption study

Previous studies at the Environmental Biotechnology Research Group (University of the Witwatersrand) have focused on the tolerance and biosorptive ability of *P. castaneae* for Pb (Kondiah, 2015; Vallabh, 2014). However, the isolate used both in previous and the current study was isolated from acid mine decant containing significantly elevated concentrations of Zn and Ni in addition to Pb. The ability to grow in such an environment suggests that this isolate utilises various mechanisms to tolerate the concentrations in which these metals are present. Furthermore, one of these mechanisms could be biosorption and if so then *P. castaneae* could enhance the rate of metal uptake by existing bacterial consortia in the treatment of water.

To evaluate this aspect, *P. castaneae* was harvested at 16 h and the biomass exposed to 3 mM Zn or Ni for 30 mins at 25 ^{o}C followed by separation of the biosorbed metal from the residual metal ions. The rate of uptake was calculated by determining the residual ion concentration using ICPOES as this is the most sensitive chemical analytical technique to measure concentrations in the part per million (ppm) range (Hou Xiandeng, 2000).

ICPOES readings could accurately be used to calculate the specific rate of metal uptake *Q* by quantifying the metal ions remaining in the solution after exposure of the bacterial cells to the metal of interest. Therefore, the residual ion concentration indirectly relates to the amount of metal biosorbed. *P. castaneae* was able to biosorb both Pb and Zn in the order Pb>>Zn but could not biosorb Ni as no reduction in metal ion concentration was detected.

Figure 2 below is a graphical representation of the % metal biosorbed by *P. castaneae* and Table 3 reports the specific metal uptake *Q* for Pb, Zn and Ni under these conditions. *P. castaneae* biosorbed 57.3% of Pb within 30 mins reducing the metal concentration to 1.28

mM. The reduction occurred at a rate of 43.18 mg Pb/g biosorbent biomass (Table 3) which was significantly higher than that of Zn at 7.34 mg Zn/g biosorbent biomass. Only 7.3% of Zn was biosorbed within the same timeframe indicating a preference for Pb by *P. castaneae*.

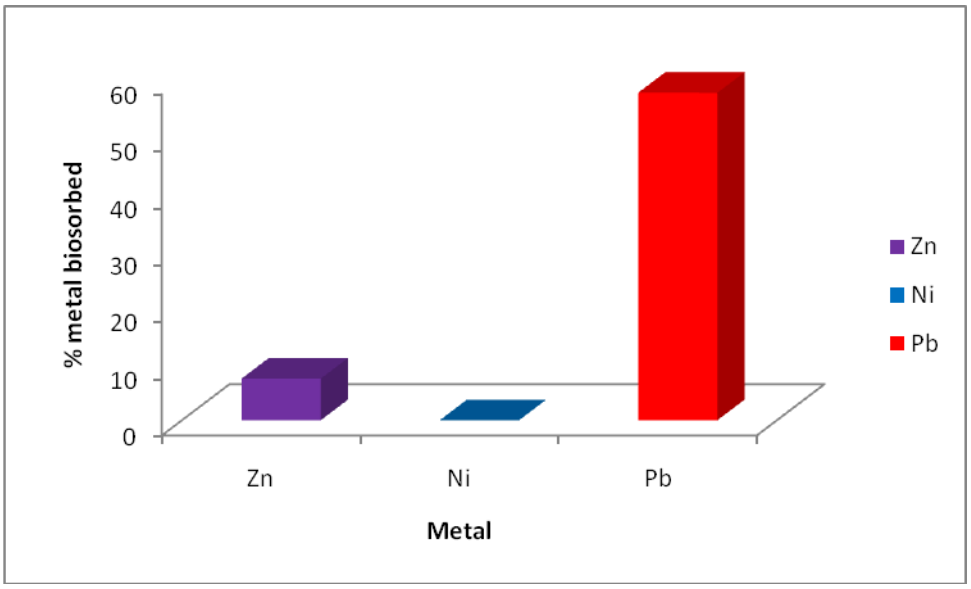


Figure 2: Percentage of metal that was biosorbed by *P. castaneae* (2 mg/mL) after exposure to 3 mM Pb, Zn or Ni at 25 °C for 30 mins. Pb was adsorbed the most, followed by Zn but there was no reduction in Ni concentration. Controls for individual metals at 3 mM were included and showed no decrease in concentration after the exposure period. The statistical data is represented in Appendix A.

Table 3: The specific rates of uptake for Pb, Zn and Ni by *P. castaneae* after 30 mins of exposure to 3 mM metal at 25 °C.

Metal	Initial concentration (mM)	Final concentration (mM)	Percentage of metal removal (%)	*Q* (mg metal/g biosorbent biomass)
Control[1]	3	3	0	0
Pb[2]	3	1.28	57.3	43.18
Zn	3	2.78	7.3	7.34
Ni	3	3	0	0

1 Controls for individual metals at a concentration of 3 mM were included and showed no decrease in concentration after the exposure period.

2 The experimental work for Pb was carried out as part of a BSc Honours research project by Ms. Daniella van Wyk.

These findings are similar to those reported previously for other species in the *Paenibacillus* family. One study showed the potential of *P. jamilae* in removing Pb, Ni and Zn, among other metals with a specific metal uptake recorded as 189.53, 5.5 and 15.73 mg metal/g biosorbent biomass respectively (Pérez et al., 2008). As noted from the results the highest *Q* was calculated for Pb and the lowest for Ni. The study was done at pH 3, 4.5 and 6 for 30 h and the highest *Q* values were observed at pH 6 (Pérez et al., 2008). An increase in contact

time has been reported to increase the amount of metal biosorbed by the biosorbent (Morillo et al., 2006).

Other authors have also reported preferential biosorption of Pb by different biosorbents (Özer & Özer, 2003; Puranik & Paknikar, 1999) because of the presence of bacterial exopolymers which are more favourable for Pb binding in comparison to other heavy metals (Cho et al., 2004). The results obtained were further validated by the inclusion of controls comprising individual metal solutions that lacked bacterial cells where no reduction in metal ion concentration was detected.

The biosorption of heavy metals has been reported to increase by increase in valence and atomic number of the metal to be removed (Holan & Volesky, 1994). Pb has an atomic number of 82 which is greater than that of Zn which is 30. It is possible to suggest that this may be another reason for the preferential uptake of Pb but would need to be further verified in a binary system. The current study attempted to do so, however there were significant discrepancies in the initial concentration of Pb between replicates hence the results not repeatable. Future work will include repeating the binary metal studies with Pb and Zn to confirm the order of metal preference in *P. castaneae*.

The favourable adsorption of both Pb and Zn in the present study suggests the potential for *P. castaneae* to be used as a biosorbent for these metals. However, the same does not hold true for Ni as results indicated that under the present experimental conditions, *P. castaneae* was unable to biosorb the metal (Figure 2 and Table 3). It may be likely that the concentration of Ni to which the biomass was exposed to was too high. There are several factors which influence biosorption including the initial metal concentration (Paknikar et al., 2003). High percentages of metal removal can be achieved using low initial concentrations of the metal of interest. Determining the MIC of Ni on *P. castaneae* may infer what concentrations of the metal may best be biosorbed. MIC is referred to as the lowest metal concentration that prevents the visible growth of the organism completely (Yilmaz, 2003). This is an important factor because if the concentration of Ni or any other metal in the contaminated water bodies is greater than that which *P. castaneae* can tolerate then the biosorbent cannot be used for the removal of the respective metal.

Contact time is another external factor that plays a vital role during biosorption. The initial passive binding of bulk metal ions to biomass occurs rapidly at the onset of mixing and

reaches equilibrium shortly after; about 5-15 mins in mesophilic organisms (Özdemir et al., 2009). During the experimental work *P. castaneae* was exposed to the metals for 30 mins only. Although *P. castaneae* is a mesophilic bacterium the contact time could have been a limiting factor for the cells to biosorb Ni. The cells may have been trying to adapt to the new metal-stressed environment therefore an increase in contact time may result in biosorption of Ni by *P. castaneae*. Similarly, increasing contact time may improve the biosorption capacity of Zn and other metals. A timeline experiment would be necessary to confirm this.

Some heavy metals may be completely toxic to bacteria. A study by Chien & Han (2009) showed that a strain of *Paenibacillus* was able to grow in the presence of Te, Pb, Zn and Cu however Cd and Cr were toxic to this bacterium. *Paenibacillus* sp. has also been shown to be vulnerable to Cu as growth inhibition of 50% was observed when it was propagated in as low as 0.011 mg/L Cu (Rathnayake et al., 2010). Pérez et al. (2008) on the biosorption of Pb, Cd, Co, Ni, Zn and Cu using *P. jamilae* showed that the biosorbent had a metal preferential order of Pb>>Cd>Zn>Cu>Co>Ni. The amount of Ni that was biosorbed by *P. jamilae* was 5.4 mg Ni/g biosorbent which was the least metal removed during the study. There is a possibility that *Paenibacillus* sp. are vulnerable to high concentrations of Ni. Since *P. castaneae* could not remove Ni as was observed from this research there exists a need for MIC determination.

In addition to contact time and initial metal concentration, several other factors are known to influence the binding of metal ions to the cell surface that include temperature, pH, competing cations and biomass concentration (Özdemir et al., 2009; Paknikar et al., 2003). Consequently, before a bioremediation strategy can be outlined, it is important to optimise the biosorption process. pH is considered to be one of the most important parameters during biosorption because it affects the solubility of the metal thereby making it available or unavailable for binding on the biosorbent (Saeed & Iqbal, 2003). The effect of cations during biosorption is also of paramount importance as wastewaters contain more than one heavy metal. The other cations have a tendency of competing with the metal of interest for the biosorbent binding sites though this will depend on the biosorbent affinity (Chojnacka, 2009). Due to the limited scope of this study, experimental work focused on the effect of pH and competing cations on the biosorption of Zn and Ni only by *P. castaneae*.

4.3 Effect of pH

Biosorption is a very pH-dependent mechanism and there is always a specific pH range for the different metals in which maximum metal uptake is achieved (Collins & Stotzky, 1992;

Collins & Stotzky, 1996). This is because metals become bioavailable when they are soluble and their solubility is pH dependent (Charles & Odoemelam, 2010). Additionally, pH is responsible for the charge on functional groups present in the cell walls (Friis & Myers-Keith, 1986) of the bacterial cells influencing the binding of soluble metal cations.

The general pattern that has been observed during previous biosorption studies is that as pH increases, the amount of metal biosorbed also increases (Esposito et al., 2001). This is because there are less hydrogen ions competing with the metal for the biosorbent binding sites. The other reason is the weakly acidic nature on the biosorbent binding sites assists metal uptake (Lopez et al., 2000; Pagnanelli et al., 2003). Increasing pH increases the negative charge on the cell surface of the biosorbent because of the ionisation of functional groups and this enhances metal uptake (Lu et al., 2006; Hughes & Poole, 1989).

However the increase in pH must be monitored because if the solution becomes too alkaline the metal precipitates forming complexes. This makes the metal unavailable for biosorption (Özer et al., 2000; Wang & Chen, 2006). An example was noted when *Trichoderma viride* and *Sarolegnia delica* were used to biosorb Zn. Increasing the pH above 8 resulted in reduced metal uptake of Zn by both biosorbents and as the solution became more alkaline, reduction in uptake continued as pH continued to be increased above 8 (Ali & Hashem, 2007).

Resolving the optimum pH range for the uptake of each metal is critical when designing a bioremediation strategy. This is necessary because the pH of wastewaters varies and treatment, especially of mine effluent, requires the addition of lime to neutralise the pH for effective metal removal by precipitation. Knowing the optimum pH range of a biosorbent for the uptake of various metals could allow for (I) using the biosorbent without pre-treatment if it can biosorb at low pH or (II) pre-treating the wastewater to bring it to the optimum pH within which the biosorbent can take up metals maximally and effectively.

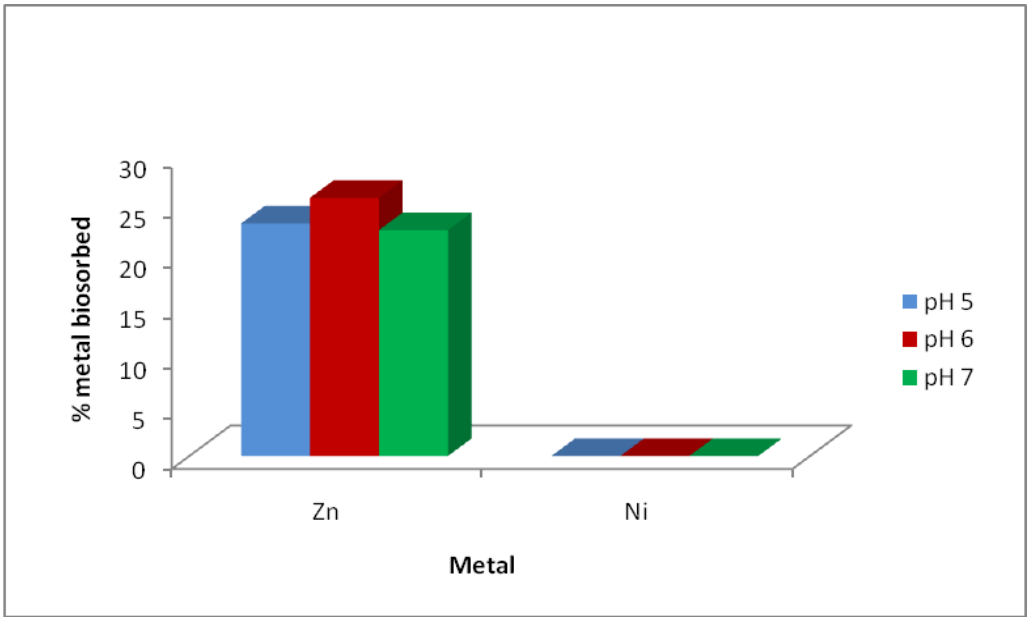


Figure 3: Percentage of the metal that was biosorbed after exposing Zn and Ni to *P. castaneae* for 30 mins at 25 °C. The biosorbent bound Zn at all three pHs with the most biosorption occurring at pH 6. Biosorption of Ni did not occur at any of the pHs. Control samples were included for both metals and there was no reduction in the concentrations. The statistical data is represented in Appendix A.

To evaluate the influence of pH on the biosorption of Zn and Ni, both the cells and metal solutions were conditioned to pH 5, 6 and 7.The results that were obtained after ICPOES analysis of the samples are shown in Figure 3 above and their specific rates of uptake indicated in Table 4 below.

Table 4: The specific rates of uptake for Zn and Ni at pH 5, 6 and 7 by *P. castaneae* after 30 mins of exposure to 3 mM metal at 25 °C.

Metal	pH 5		pH 6		pH 7	
	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)
Controls[1]	3	0	3	0	3	0
Zn	2.76	8	2.68	10.66	2.78	7.34
Ni	3	0	3	0	3	0

1 Controls for individual metals at a concentration of 3 mM were included and showed no decrease in concentration after the exposure period.

Biosorption of heavy metals occurs best between pH 5-8 (Özer & Özer, 2003; Puranik & Paknikar, 1997) hence the range of pHs tested in this study. Zn is most soluble around pH 6.5 while Ni becomes more soluble towards acidic pH of 5. It is therefore expected that at these

pHs, each of these metals will be bioavailable for sorption by the bacterial biomass and maximal uptake will occur (Fuerstenau & Palmer, 1976).

Figure 3 shows that under all three pH conditions *P. castaneae* was able to remove a marginal amount of Zn but could not do so with Ni. The concentration of Zn was reduced by 8%, 10.8% and 7.3% at pH 5, 6 and 7 respectively. The difference in the *Q* values (Table 4) was very marginal with the most Zn being biosorbed per gram biomass at pH 6. This is expected as Zn is most soluble at pH 6.5; further work could involve narrowing down the pH range to pinpoint the exact pH at which maximal biosorption occurs.

Several studies investigating the effect of pH on biosorption of Zn have also reported maximum biosorption at pH 6. *Streptoverticillium cinnamoneum* waste biomass was used for the removal of Zn and the highest *Q* value reported was 4.6 mg Zn/g biosorbent biomass after exposing the metal to the biomass for 1 h (Puranik & Paknikar, 1997). The amount of Zn removed by *S. cinnamoneum* at pH 6 was lower than that was removed by *P. castaneae* at all pH values from this study (8, 10.66 and 7.34 mg Zn/g biosorbent biomass) even though Zn was only exposed to *P. castaneae* biomass for 30 mins. This would infer that *P. castaneae* is a better biosorbent for Zn than *S. cinnamoneum*. Nonetheless, Jiang et al. (2006) observed in their study using tourmaline that the amount of Zn removed increased as pH increased and the highest *Q* of 67.25 mg Zn/g biosorbent biomass was observed at pH 7 which is much higher than what *P. castaneae* removed in this particular study. Similarly, the amount of Zn bound by *Citrobacter* strain MCM B-181 was found to increase rapidly between pH 5 and 6.5 and decrease as pH was increased above 6.5. At pH 6.5 the highest *Q* was 18 mg Zn/g biosorbent biomass (Puranik & Paknikar, 1999) which is also higher than the values obtained for *P. castaneae*.

Examples of other microorganisms like algae and fungi that have been used for the removal of Zn show that *Q* was highest in the pH range between 5 and 7. Özer et al. (2000) reported that the highest *Q* (45 mg Zn/g biosorbent biomass) of Zn by *Cladophora crispata* was recorded at pH 5. The most Zn was found bound to fungal biosorbent at a pH of 6.8 (Fourest & Roux, 1992) while *S. cerevisiae* biomass effectively removed the metal at pH 6 according to Zhao & Duncan (1997).

The experimental conditions used in the present study differ from what has been done previously restricting any comparisons made. Suffice to note that the optimum pH for

biosorption of Zn by *P. castaneae* determined in the study is similar to what has been reported in literature for other biosorbents.

Özer & Özer (2003) reported that the biosorption of Ni by *S. cerevisiae* was best at pH 5 with specific metal uptake of 23 mg Ni/g biosorbent. Fourest & Roux (1992) found that the amount of Ni bound on the surface of fungi increased with increase in pH in the range between 5 and 8 with pH 8 recording the highest specific metal uptake. Altering the pH did not have any effect on the biosorption of Ni as there was no uptake of metal. These results further emphasise the need to establish the MIC as explained in section **4.2** as it would appear that by changing the pH to improve the bioavailability of Ni, the cells still remained impervious to Ni biosorption.

Thus far the results indicated that *P. castaneae* biomass is unable to bind Ni. Subsequently, the effect of competing cations, Co and Mn, was observed only on the uptake of Zn. However, all three pHs were studied as Zn was biosorbed at a similar rate for pH 5, 6 and 7 and Co is highly soluble at pH 5 and Mn at pH 5.5.

4.4 Effect of cations

Wastewaters contain multiple metal ions in varying concentrations that could compete with or enhance the metal to be removed for binding to biosorbents (Modak et al., 1996; Paknikar et al., 2003). For this reason, it is important to assess the effect of other cations on the rate of metal uptake for each metal of interest. This study focused on the effect of Co and Mn on the biosorption of Zn. These metals together with Pb, Zn and Ni commonly occur in wastewaters and acid mine decant in Gauteng. Therefore understanding their influence on the biosorption of Zn would allow for the development of a "designer" model for wastewater bioremediation.

The concentrations of metal biosorbed by *P. castaneae* from the binary systems are represented in Figure 4. The results indicate that both Co and Mn act synergistically and enhance the biosorption of Zn at pH 7. The percentage of Zn that remained in the presence of these metals was 75 and 74, respectively. Slight biosorption of Zn in the presence of Co occurred at pH 5 and 6 but none was observed in the presence of Mn at the same pH. However, at each pH there was low biosorption of both Co and Mn indicating that *P. castaneae* may also be able to biosorb these metals. Although the amount of Co and Mn biosorbed was almost negligible (between 2.67-8% for Co and 4.23-6.67% for Mn) in this study, it would be prudent to determine their specific rate of uptake under different

experimental conditions. This could include individual biosorption assays, increased contact time and the influence of other external factors mentioned earlier.

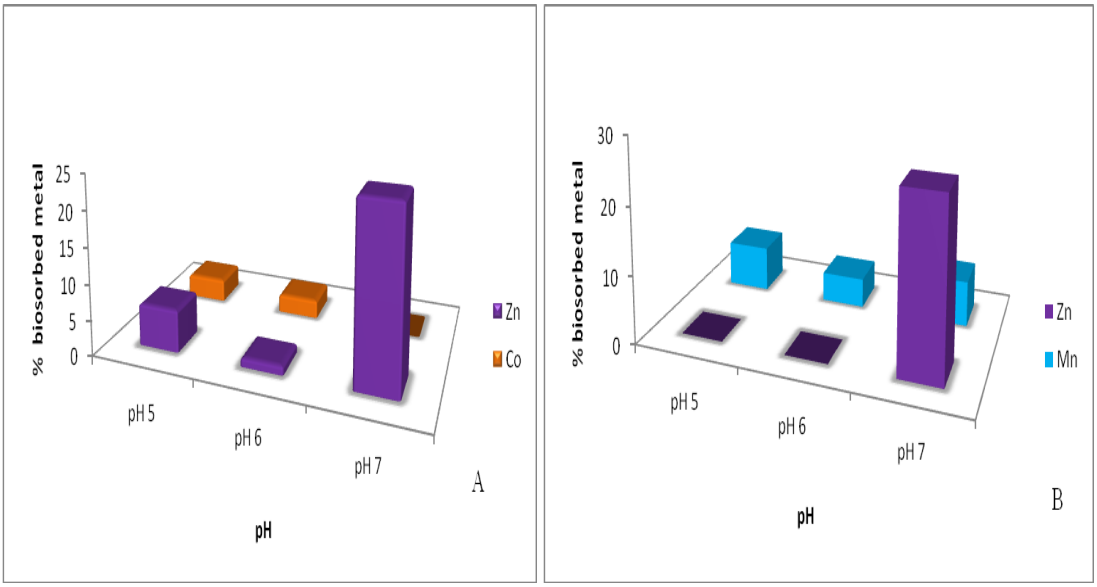


Figure 4: Percentage of metal biosorbed after exposing *P. castaneae* to binary metal solutions of (A) Zn and Co and (B) Zn and Mn. The highest amount of Zn biosorbed in the presence of Co or Mn was at pH 7. Control samples consisting of each binary metal solution without added biosorbent were included and showed no metal reduction. The statistical data is represented in Appendix A.

Cations have been reported to have three effects on the binding of the metal of interest during biosorption. These are:

i. ability to increase the binding of the metal of interest,
ii. ability to decrease the binding of the metal of interest or
iii. may not affect the binding of the metal of interest (Sağ et al., 1995).

Pérez and co-workers (2008) found that *P. jamilae* bound lower concentrations of Pb when present as a single metal solution than when in a multi-metal system including Cd, Co, Ni, Cu and Zn. Plant derived biomass of *Hemidesmus indicus* have also been used in the removal of Pb, Zn and Cr. The presence of additional cations increased the concentration of Zn that the plant biosorbent removed. This study showed that 50.6% of Zn was removed in a single metal solution whilst 100% of the metal was removed in binary metal solutions of Pb and Cr (Sekhar et al., 2003). These results show that it is possible for cations to actually increase the amount of metal removed by the biosorbent as was observed from this research.

It is interesting to note that when Zn is the only metal present in solution, maximal biosorption occurred at pH 6 with a *Q* of 10.66 mg/g biomass (Table 4). However, in the presence of Co or Mn, maximal biosorption of Zn shifted to pH 7 with significantly higher *Q*

values of 25 and 26 mg metal/g biomass, respectively (Table 5). This showed that both cations had the ability to increase the amount of Zn biosorbed at pH 7.

Table 5: The specific rates of uptake for Zn, Co and Mn at pH 5, 6 and 7 by *P. castaneae* after 30 mins of exposure to 3 mM binary mixes of metal at 25 °C.

Metal	pH 5		pH 6		pH 7	
	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)
Controls[1]	3	0	3	0	3	0
Zn[C]	2.82	6	2.96	1.35	2.25	25
Co	2.9	2.95	2.92	2.35	2.75	7.35
Zn[M]	3	0	3	0	2.22	26
Mn	2.8	5.55	2.87	3.6	2.8	5.55

1 Controls for the binary solutions at a concentration of 3 mM were included and showed no decrease in concentration after the exposure period.

C Values recorded for Zn in the presence of Co

M Values recorded for Zn in the presence of Mn

Co and Mn are soluble within the pH range 5-8 and 5-8.8, respectively with maximum solubility of Co and Mn at pH 5 and 5.5 respectively (Fuerstenau & Palmer, 1976). Despite their pH preferences when it comes to the solubility of Zn, Mn and Co, all three metals can be biosorbed at a pH of 7 as confirmed in this study. Additionally, almost all biosorbents have been reported to work optimally within a pH range of 4-8 (Paknikar et al., 2003).

It would appear though that at a pH of 5 and 6, Mn could be exerting an antagonistic effect on the biosorption of Zn. Metal ions that belong to the same class share preferences for a specific binding site on a biosorbent. Therefore, should competing ions co-exist in a solution to be treated by a biosorbent, the *Q* of the targeted metal is expected to be lower than that corresponding to the single metal solution of the targeted element (Tsezos et al., 1996). All three metal ions used in the present study belong to the borderline class which could infer competition for binding sites on *P. castaneae* biomass hence the observations of low/no biosorption of Zn in the presence of Co or Mn. The increased uptake of Zn at a pH of 7 could be as a result of Mn and Co becoming less soluble as this tends toward their maximal pH beyond which these ions would precipitate while Zn is most soluble at a pH between 6.5 and 7.

The results from the binary metal studies indicate that at a pH of 7, *P. castaneae* has a preferential metal sorption in the order Zn>>Co>Mn. However, when all three metals are included in a multimetal solution and exposed to *P. castaneae* biomass at pH 7 under the same conditions, the order of preference changes to Co>>Zn with no biosorption of Mn. This is evident from Figure 5 and Table 6 below.

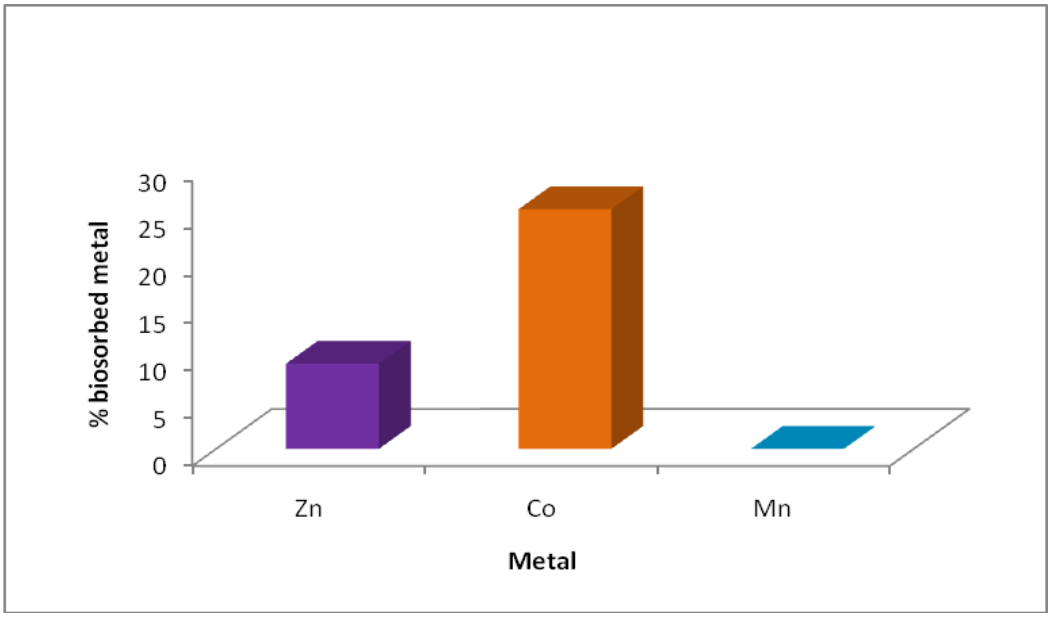


Figure 5: Percentage of metal biosorbed after exposing *P. castaneae* biomass to a multimetal solution of Zn, Co and Mn at pH 7. The biosorbent did not remove any Mn but had an increased affinity for Co followed by Zn. The statistical data is represented in Appendix A.

Table 6: The specific rates of uptake for Zn, Co and Mn at pH 7 by *P. castaneae* after 30 mins of exposure to a 3 mM mix of all three metals at 25 °C.

Metal	Final concentration (mM)	*Q* (mg metal/g biosorbent biomass)
Control[1]	3	0
Zn	2.73	9
Co	2.24	25.35
Mn	3	0

1 Control for multimetal solutions at a concentration of 3 mM was included and showed no decrease in concentration after the exposure period.

Since Zn, Co and Mn belong to the borderline class of metal ions, exposing *P. castaneae* to all three metals in a single solution increased the competition for binding sites on the biosorbent (Chojnacka, 2009). The percentage of Co, Zn and Mn that remained in the solution was 74.67, 91 and 100, respectively. These results suggest that Co behaves in a highly competitive manner and prevents the binding of the already low concentrations of soluble Mn due to the pH of the solution. Surprisingly, the biosorption of Zn was

significantly reduced approximately 3-fold from a *Q* of 25 mg/g biomass in the binary system containing Co to 9 mg/g biomass in the multimetal system. This is unexpected as Zn should be more soluble than Co at pH 7 and would have likely been the metal ion preferentially bound by *P. castaneae*.

The biosorption capacity of a biosorbent when introduced to a multimetal solution is usually affected by electrode potential, ionic charge and radii of the metals that are to be removed (Marcus & Kertes, 1969; Mattuschka & Straube, 1993). Therefore it is very important to consider all these three factors when interpreting results from a multi metal solution. Metals with a greater electrode potential have an increased affinity for the biosorbent sites when compared to those with a smaller electrode potential (Sekhar et al., 2003).

A linear relationship exists between ionic radii and metal uptake whereby the larger the ionic radii the more metal can be taken up. The ionic radius of Mn, Zn and Co is 0.46 Å, 0.74 Å and 0.745 Å respectively (Ruben, 1985) which could further explain the metal preferential order obtained for *P. castaneae*. Sekhar et al. (2003) showed that the biosorption of Pb, Zn and Cr in a multimetal solution increased with increase in the ionic radius of each metal.

The findings of this study have shown that *P. castaneae* is able to biosorb Zn, Co and Mn to a certain extent in addition to Pb. It further confirms that the rates of uptake for these metals are influenced by pH and competing cations in both binary and multimetal systems. The order of metal preference changed within a multimetal solution and further work is necessary to understand all the factors that play a role in the biosorption of metals when using *P. castaneae* as a biosorbent. This research further contributes to the evidence that *P. castaneae* can be used as a biosorbent for the removal of heavy metals specifically Zn from contaminated water sources. It can be applied at a neutral pH in pure metal solutions or in natural conditions where metals occur as mixed ions. However there are other factors such as metal concentration, biomass concentration, temperature and contact time that need to be optimised before it can be commercialised as a biosorbent for environmental pollution. Future work will be done for the determination of the optimum conditions for biosorption of Zn and other metals using *P. castaneae*.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

The primary focus of the present study was to establish if *P. castaneae* has the ability to take up a range of other heavy metals in addition to Pb. The heavy metals tested during the experimental work were chosen on the basis that they form common metal contaminants of South African wastewaters especially in Gauteng. This was a key point in the planning of the research as the long-term goal is to apply *P. castaneae* as a biosorbent for the treatment of effluent derived from industrial processes in Gauteng. Building up from the knowledge accumulated as a result of this study, a greener, more efficient and cost-effective bioremediation strategy may be designed targeting communities at risk of consuming metal-contaminated water from local water sources.

From the study, it can be confidently concluded that *P. castaneae* can indeed biosorb other metals apart from Pb that include Zn, Co and Mn. The binding of metal ions and the rate at which this occurs is dependent on pH and the presence of other cations that can act as competitors or enhancers. Maximal absorption of Zn under the experimental conditions occurred at pH 7 in the presence of Co or Mn with a comparable *Q* of 25 and 26 mg/g biomass in the order Zn>>Co>Mn. However, the order of preferential uptake changed to Co>>Zn when all three metals were present in a single system with Co having the highest *Q* of 25.35 mg/g biomass and Mn not being biosorbed at all.

These rates are comparable to existing literature for other bacterial species and indicate the potential for *P. castaneae* to be used as part of a bacterial consortium in the treatment of contaminated wastewaters in Gauteng. Specifically, *P. castaneae* would be used for the removal of heavy metals like Pb, Zn, Mn and Co. However, further optimisation of the process is vital to understand the extent to which other external factors that were not tested in this study can affect the rate of metal uptake in the bacterium. In so doing, some of the future work that will be done to develop *P. castaneae* as an industrial biosorbent is outlined below.

5.2 Recommendations

Based on the findings of this study the following future work is recommended:

- Determining the MIC of individual metals on *P. castaneae*. This will enable predictions in the form of metal concentrations that can be tolerated by the bacterium

and therefore concentrations at which it can effectively biosorb each metal. Such experimental work would be done in the context of the average concentration at which each metal is present in South African wastewaters as this is the target of a bioremediation strategy.

- Fine-tuning the optimal pH at which maximal uptake of the different metals biosorbed by *P. castaneae* occurs and its effect in complex multi-metal solutions. This information would enable a predictive model that could be used to determine whether majority of the contaminating metals could be biosorbed at a lower pH characteristic of wastewaters or if a neutralisation step would be necessary for treatment.

- Investigating the effect of contact time, initial metal concentration, biomass concentration and temperature on the biosorption of Zn and other metals taken up by *P. castaneae*. Subsequently, an optimised process for removal of metals from complex wastewaters using *P. castaneae* as a biosorbent could be derived. Further application would be in the way of enhancing existing biological and physicochemical strategies used in the treatment of metal-contaminated water.

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APPENDIX A

Table 7: Statistical analysis of results shown in Figure 2 based on student t-test.

	Standard deviation	p value
Pb/Zn	2.28	0
Pb/Ni	2.28	0
Zn/Ni	3.62	0.015

Table 8: Statistical analysis of results shown in Figure 3 based on student t-test.

	pH	Standard deviation	p value
Zn/Ni	5	2.64	0.004
	6	11.1	0.094
	7	3.62	0.015

Table 9: Statistical analysis of results shown in Figure 4 (A) based on student t-test.

	pH	Standard deviation	p value
Zn/Co binary	5	3.68	0.025
	6	3.27	0.023
	7	1.46	0

Table 10: Statistical analysis of results shown in Figure 4 (B) based on student t-test.

	pH	Standard deviation	p value
Zn/Mn binary	5	5.79	0.993
	6	3.82	0.801
	7	3.54	0.332

Table 11: Statistical analysis of results shown in Figure 5 based on student t-test.

Multi metal	Standard deviation	p value
Zn/Co	4.25	0.157
Zn/Mn	0	0.023
Mn/Co	0	0.04