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School of Molecular and Cell Biology

University of the Witwatersrand

Johannesburg

Defining the factors that influence the biosorption of lead by *Paenibacillus castaneae* and *Micrococcus luteus*

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in, Microbiology and Biotechnology

2017

by

Darshana Vallabh

453726

Supervisor: Dr K. Kondiah

Advisor: Dr D. Mavri-Damelin

Postgraduate Coordinator: Dr J. Mollett

Declaration

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15 Day of February 2017

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Abstract

Heavy metal contamination, of natural water resources, resulting from the large amounts of toxic waste generated by industrial practices is of great environmental concern. Lead (Pb) in particular is one of the most toxic heavy metals that leads to several health deficiencies upon human exposure. The reduction of heavy metals like Pb to acceptable levels in the water therefore becomes critical for potable and agricultural use.

Removal of heavy metals by conventional methods is expensive and results in secondary pollution. Bioremediation, a process that passively removes heavy metals from solution through microbial biosorption, is a much sought after alternative because it is more eco-friendly and cost-effective. *Micrococcus luteus* and *Paenibacillus castaneae* are two bacterial species reported to be highly resistant to Pb making them favourable as metal biosorbents. The present study aimed to further characterise these species as biosorbents by evaluating the influence of environmental conditions on their rate of biosorption of Pb. Each bacterial isolate was heat-killed and exposed to 0.5 mM (150 mg/L) Pb and the maximal rate of metal uptake calculated when the pH, temperature and biomass concentration were varied. Additionally, the initial metal concentration was increased from 0.005 to 1.25 mM to determine its effect on Pb uptake by each species. The influence of competing cations (Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+}) on the rate of Pb uptake by each isolate was also established.

Both bacterial isolates resulted in the biosorption of at least 50% of 0.5 mM Pb ions when used at a pH of 7, temperature of 25 ^{o}C, and a biomass concentration of 2 g/L. The rate of metal uptake for *M. luteus* at the above mentioned parameters was found to be 24.51 mg/g biomass, while the rate of metal uptake for *P. castaneae* was 15.63 mg/g biomass. These findings indicated that *M. luteus* took up more Pb at a faster rate in comparison to *P. castaneae*. The present study furthermore elucidated that as the metal concentration of Pb was increased, the amount of Pb biosorbed by *M. luteus* decreased from 84.76% to 46.10%. Similarly, *P. castaneae* yielded 81.39% biosorption from 0.005 mM Pb but only 34.29% of Pb was taken up when the concentration was increased to 1.25 mM.

When the bacteria were exposed to various competing cations an increase in the rate of Pb biosorption was observed for *P. castaneae* while the opposite effect was noted for *M. luteus*.

Findings from this study show that under high metal concentrations, both *M. luteus* and *P. castaneae* are capable of significantly reducing the level of Pb from pure solution. The results warrant further treatment of several industrial effluents using these biosorbents for subsequent application in wastewater treatment.

Keywords: Biosorption, heavy metals, lead, *Micrococcus luteus*, *Paenibacillus castaneae*, wastewater

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List of Abbreviations

AMD	Acid mine decant
BOD	Biological oxygen demand
COD	Chemical oxygen demand
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
EPs	Extracellular polymers
EPS	Extracellular polymeric substance
FTIR	Fourier transform infrared spectroscopy
ICPOES	Inductively coupled plasma optical emission spectroscopy
LB	Luria Bertani
LPS	Lipopolysaccharides
MIC	Minimum inhibitory concentration
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
NaOH	Sodium hydroxide
$NiCl_2.6H_2O$	Nickel chloride hexahydrate
$Pb(NO_2)_3$	Lead nitrate
Q	Rate of metal uptake
sp	Species
WHO	World health organisation
w/v	Weight per volume
x *g*	Times gravity
$ZnCl_2$	Zinc chloride

Chapter 1

Introduction

1.1. Background

The advancement of technology and industrialisation has led to the increase in water pollution due to the generation of large aqueous toxic effluents (Vijayaraghavan and Yun, 2008). These effluents are generated by many industries such as surface finishing, electroplating, metallurgical, tannery, chemical manufacturing, mining and battery manufacturing industries and are found to be the main source of heavy metal pollution (Meena et al., 2005).

Industrial effluents contain elevated amounts of toxic heavy metals such as mercury (Hg), copper (Cu), uranium (U), manganese (Mn), nickel (Ni), cobalt (Co), zinc (Zn) and lead (Pb). These metals have an adverse effect on the environment and health sectors upon exposure (Gavrilescu, 2004). Major concerns regarding the release of heavy metals into the environment is the drainage of these metals into rivers and dams which form the main source of drinking water for many human settlements downstream (Malik, 2004). Another concern regarding the disposal of heavy metals results from marine animals that readily adsorb them from wastewaters making a direct entry into human food chains, which presents a high health risk for consumers (Meena et al., 2005). Upon ingestion of these toxic metals, detrimental health impacts such as neurodegeneration, liver and bone damage as well as interference with the function of certain vital enzymes (Malik, 2004) can be experienced.

There have been many physicochemical methods employed for the removal of heavy metals from effluents. However, these methods are commercially impractical due to high operating costs or difficulty in treating solid wastes generated (Gavrilescu, 2004). Examples of such conventional technologies include chemical precipitation, reverse osmosis, evaporation recovery, ion exchange, sequestration and electrochemical treatment (Malik, 2004; Ahluwalia and Goyal, 2007). The increased amount of reagent requirement and the

unpredictable amount of metal ion removal are further disadvantages associated with these methods. Additionally, strong and contaminating reagents are used for desorption which results in toxic sludge and secondary environmental pollution (Malik, 2004).

An alternative and cost effective method for metal removal is through a biotechnological approach. This approach involves the use of microorganisms to adsorb and accumulate heavy metals (Zabochnicka-Świątek and Krzywonos, 2014). Microorganisms have the ability to capture, accumulate and bind heavy metals from water solutions; this procedure is usually referred to as biosorption or bioaccumulation.

Biosorption may be defined as the ability of certain types of microbial biomass to retain relatively high amounts of metal ions through a passive method of metal sorption (Volesky, 1990). Metal sorption can occur by complexation, coordination, ion exchange, adsorption and chelation (Volesky, 1990). The cell walls of microbial biomass are composed of polysaccharides, proteins and lipids that carry abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups (Nanda, Sharma and Kumar, 2011). These metal binding groups allow for metal cations to complex with negatively charged reaction sites adsorbing them onto the cell surface (Ahluwalia and Goyal, 2007). An important feature of biosorption is its ability to allow for binding and accumulation of metal species even once the cell is not metabolically active (Volesky, 1990).

Since heavy metals are natural elements and at a most basic level are just atoms, degradation and metabolism are not possible (Monachese et al., 2012). Instead microorganisms such as bacteria have evolved coping strategies to either transform the element to a less harmful form or they bind the metal intracellularly (bioaccumulation), thereby preventing any harmful interactions in the host cell (Monachese et al., 2012). It should also be noted that microorganisms have the ability to actively transport the metal out of the cell cytosol (Monachese et al., 2012).

The interaction between heavy metals and bacterial species, and the ability of bacterial species to remove heavy metals from solution is a unique process (Monachese et al., 2012) that can be exploited for our benefit. Biosorption appears to be more favourable than physicochemical processes and even bioaccumulation because it is an active process. Biosorbents are heat killed cells that are not metabolically active but still have the ability to bind heavy metal to their cell surface (Zabochnicka-Świątek and Krzywonos, 2014). This is more cost effective than using living biomass which would require additional energy supply and nutrients (Zabochnicka-Świątek and Krzywonos, 2014). Additionally, many biosorbents can be obtained from industrial waste (Vijayaraghavan and Yun, 2008) further alleviating the costs of culturing the biomass. Other advantages of this process include the rapid rate of metal removal and the relatively low energy demands (Vijayaraghavan and Yun, 2008).

Despite the benefits of biosorption as a treatment strategy for heavy metals, few biosorbents have been commercialised. One of the limitations involves the need to optimise the process itself. This is because the efficiency of metal biosorption is affected by several environmental conditions such as pH, temperature, biomass concentration and metal concentration (Ahalya, Ramachandra and Kanamadi, 2003). Several studies that have looked at biosorption kinetics have reported that at a lower pH, biosorption is decreased due to competition from H^+ ions (Chatterjee, Bhattacharjee and Chandra, 2010; Çolak et al., 2011; Abbas et al., 2014). Temperature affects the viscosity and solubility of metal ions which would influence the mobility and therefore the rate of metal uptake (Aksu, Sag and Kutsal, 1992; Zouboulis, Lokidou and Matis, 2004; Congeevaram et al., 2007; Fan et al., 2008). The biomass concentration is important because it represents the number of active binding sites therefore providing enough binding sites would enable maximal biosorption but too much biomass would lead to spatial interference reducing biosorption (Al-Asheh and Duvnjak, 1995; Fan et al., 2008).

In this regard a study conducted by Puranik and Paknikar (1999) on the biosorption of Pb, Cd and Zn by *Citrobacter* illustrated that the biosorption rate increased as the pH was increased. The same trend was reported when the initial

metal concentration was increased. However, as the biomass concentration was increased the rate of biosorption of Pb, Cd and Ni was found to decrease.

The examples stated above are just a few that indicate how the rates of biosorption may be affected by several environmental conditions. Furthermore, the range within which commercial biosorbents can function under such conditions requires optimisation on a case to case basis. The present study sought to study the performance of two bacterial biosorbents, *Micrococcus luteus* and *Paenibacillus castaneae*, in the uptake of Pb under the influence of pH, temperature, biomass concentration, initial metal concentration and competing cations. Both microorganisms were selected for the study as they were isolated from acid mine decant (AMD) on the West Rand of Johannesburg and were previously shown to be heavy metal resistant.

Pb is a toxic, mutagenic and non-degradable heavy metal that leads to Pb poisoning, neurological diseases and damages and disruptions to systems such as cardiovascular, renal and reproductive systems (Brochin et al., 2008). It is a prominent metal contaminant of water in Gauteng. Acid mine decant that is released into natural water sources can carry from 2 mg/L (West Rand) up to 60 mg/L Pb on the East Rand Basin (Personal communication, K. Kondiah) as a consequence of mining activities around the Witwatersrand Basin, Gauteng. This is between 200 – 6000 fold more as compared to the acceptable limit (0.05 – 0.10 mg/L) for drinking water (WHO, 2008). Other industries that contribute to Pb containing effluents in Gauteng include the paint and iron smelting industries.

Although these high concentrations are diluted within the natural rivers there is still a significant quantity of Pb that is reaching downstream human settlements. These communities use the water for personal consumption as well as agricultural purposes. The metal is thus directly or indirectly ingested through the food chain resulting in its accumulation within human tissues where it is detrimental to our health.

Bacterial biosorbents can be used as an eco-friendly and cost effective means of further reducing Pb concentrations from chemically treated wastewaters such as

AMD. Suitable biosorbents can be sourced from areas which are highly contaminated with heavy metals as these isolates may be attained with little/no additional cost therefore proving to be more economical (Vijayaraghavan and Yun, 2008).

The present study proposes to optimise *M. luteus* and *P. castaneae* as biosorbents for Pb. Both bacteria were isolated from AMD on the West Rand, Gauteng and are reported to tolerate up to 6000 mg/L of Pb (Vallabh, 2014). *M. luteus* is a common and known metal biosorbent that is reported to effectively accumulate Cu and Pb in an extracellular manner in layers of extracellular polymeric substances (EPS) (Maldonado et al., 2010). On the other hand, there are no literature reports documenting the same in *P. castaneae.* However, other species belonging to the genus *Paenibacillus* are known to biosorb heavy metals including Pb, Cu, Zn, Cd, Ni and Co by EPS (Pérez et al., 2008; Çolak et al., 2013) suggesting that *P. castaneae* may have similar capabilities.

1.2. Aim of the research

The aim of this particular research was to study the influence of external parameters on the rate of lead sorption by *M. luteus* and *P. castaneae*.

1.3. Objectives of the research

The specific objectives set out to fulfil the central aim were:

- To calculate the specific rate of Pb uptake by heat-killed *P. castaneae* and *M. luteus.*
- To calculate the changes in the specific rate of Pb uptake when temperature, pH, biomass concentration and initial metal concentration are varied in both bacterial strains.
- To establish whether competing cations such as Ni^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+} act synergistically or antagonistically on the uptake of Pb by each bacteria.
- To compare the efficiency of *P. castaneae* and *M. luteus* in the biosorption of Pb.

1.4. Chapter outline

This dissertation follows the structure outlined below.

Chapter 1 entails a brief introduction to the area of research and outline of the problem statement. The main aim/ research question and the specific objectives required in order to achieve and successfully address the problem in question are also discussed in this particular chapter.

Chapter 2 forms an in depth review on current literature discussing heavy metal contamination, its treatment and the optimisation of biosorption. This chapter also provides an explanation behind the feasibility and economic advantage of using a biological approach as compared to a chemical approach of metal removal.

Chapter 3 gives details of the materials used and methods followed in order to accurately and reproducibly conduct the experimental procedure required to address the aim.

Chapter 4 illustrates and discusses the results obtained from the research conducted. This chapter displays and discusses the results received for the following external parameters:

- Effect of pH
- Effect of temperature
- Effect of biomass concentration
- Effect of initial metal concentration
- The effect of competing cations on the biosorption of Pb by *M. luteus* and *P. castaneae*

Chapter 5 forms the final conclusion and future recommendations from the present study.

Chapter 2

Literature Review

2.1. Heavy metal contamination

Human industrial activities over the past decade have led to an increased release of inorganic and organic compounds into the environment. The release of these compounds has resulted in a drastic increase in heavy metal pollution (Lloyd, 2002). Industries such as electroplating, tannery, smelting and synthetic compound creation as well as mining operations, release aqueous effluents containing toxic heavy metals. Such heavy metals include: Cr, Cu, Pb, Hg, Mn, Cd, Ni, Zn and Fe (Meena et al., 2005; Ahluwalia and Goyal, 2007). Heavy metals are not degradable and are found to be persistent, toxic and mutagenic in nature (Gavrilescu, 2004).

Many mining operations and geochemical activities result in the creation of AMD. Acid mine drainage is produced when sulphide bearing material is exposed to oxygen and water (Oelofse et al., 2007). It is characterised by a low pH, high electrical conductivity and toxic heavy metals (Oelofse et al., 2007). The heavy metals are carried by acid water into rivers and dams downstream (Duruibe, Ogwuegbe and Egwurugwu, 2007) where they are harmful not only to the environment but also to humans upon exposure (Gavrilescu, 2004). Animals that drink water from these contaminated rivers and dams accumulate the heavy metals in their tissues (Duruibe, Ogwuegbe and Egwurugwu, 2007). Petukhova, 2013 reported an accumulation of 0.070 mg/kg of Pb in the muscle tissues of cattle that consumed such water. Humans are subsequently exposed to and accumulate the metal into their tissues by consuming the contaminated animals and plants where they may cause various biochemical disorders (Duruibe, Ogwuegbe and Egwurugwu, 2007).

While some heavy metals are toxic with no cellular role, others are important for life at low concentrations. However at elevated concentrations they become highly

toxic due to their inhibition of activities of sensitive enzymes such as oxidases, catalase and dismutase (Stern, 2010; Nanda, Sharma and Kumar, 2011). Heavy metals such as Cr, Hg, As and Pb are of major concern especially when found at non-permissible concentration levels in rivers and dams as they are known to be detrimental to human health upon exposure (Jarup, 2003). The present study focuses primarily on Pb contamination in water and its subsequent clean-up which will further be discussed in the sections to follow.

2.1.1. Pb toxicity and contamination

Pb is a metallic, malleable and ductile element. It is one of the most toxic heavy metals and is of no biological use (Jarup, 2003). It has a diversified use in petrol fuels, paints, food cans, ceramics, textile, mining industries and battery storage (Banik et al., 2014). Therefore Pb is present in air, soil, dust and water. Human exposure to Pb can occur through inhalation, digestion and dermal adsorption and thus may cause lead poisoning which leads to damages to the liver, kidney as well as mental disability and abnormalities in pregnancy (Meena et al., 2005; Duruibe, Ogwuegbe and Egwurugwu, 2007).

Chronic Pb poisoning may cause gastrointestinal diseases, neuromuscular disorders as well as central nervous system effects (Meena et al., 2005). Accumulation of Pb in the nervous system is found to block a receptor known as N-methyl-D-aspartate which is responsible for the maturation of brain plasticity. Its blockage therefore leads to limitation and disruption of the permanent intake of newly learned knowledge (Brochin et al., 2008). A crucial point and most harmful aspect of Pb poisoning may occur during the in utero period, whereby the foetus is susceptible to toxins and disease as a result of still being in a developmental phase and therefore is unable to protect itself (Brochin et al., 2008). Pb exposure during this period may lead to crucial neurological disorders and developmental problems that may manifest later in the unborn child's life (Brochin et al., 2008).

In South Africa, the most common sources of Pb associated with water pollution are emanated from mining activities as well as paint industries, unleaded gasoline and lead soldered pipelines (Nriagu, Blankson and Ocran, 1996; Awofolu et al., 2005). There are many provinces like the Eastern Cape where such activities

contribute the main source of income for semi-skilled workers. Communities formed by these workers and their families generally lack access to potable water systems. Human settlements in these areas are therefore solely dependent upon ground and surface water for domestic, irrigation and livestock activities (Awofolu et al., 2005). However, due to loosely regulated release of industrial effluents, the ground and surface waters build up high levels of trace metals. Aquatic life and vegetable produce cultivated using such water accumulate the heavy metals (Awofolu et al., 2005; Bvenura and Afolayan, 2012). The study by Awofolu et al. 2005 indicated that between 0.011 to 0.022 mg/kg of trace metals (Pb, Co. Cu and Zn) was accumulated in vegetable produce (cabbage and spinach) and the continual intake of these vegetables will subsequently pose as a grave health risk to consumers due to the food chain transfer.

2.2. Methods used for the removal of heavy metals from contaminated waters

There are several conventional physicochemical methods employed to assist with the removal of heavy metals. These methods include chemical precipitation, ion exchange, membrane filtration and reverse osmosis. Table 1 below gives a brief description of each physicochemical method and their respective drawbacks.

Chemical precipitation of metals as hydroxide salts is commonly incorporated in the wastewater treatment process. This strategy is inefficient at treating high concentrations of metals. Additionally, once the metal salts are filtered out they still need to be disposed of as metal recovery is difficult and costly. The high operating costs, secondary pollution, generation of toxic sludge and the difficulty in treating solid wastes generated by physicochemical methods are indicative of their inefficiency to treat heavy metal contamination (Ahalya, Ramachandra and Kanamadi, 2003; Gaverilescu, 2004). Hosseini and Mirbagheri, 2005 evaluated a hydroxide precipitation method to remove Cu (II) and Cr (VI) from wastewaters. They found that the hydroxide precipitation generated large amounts of sludge which presents disposal problems and the complexing agents in the wastewater are found to inhibit metal hydroxide precipitation.

Biological treatment is suggested to be a more cost-effective and economic alternative. This method entails the usage of biological material that has the

ability to bind metals through processes such as biosorption and bioaccumulation (Zabochnicka-Świątek and Krzywonos, 2014).

Table 1. Physicochemical treatment methods used for the removal of heavy metals from contaminated water.

Physicochemical Treatment	Description	Disadvantages
Chemical Precipitation	One of the most widely used treatments for the removal of heavy metals. This treatment involves chemical reactions with heavy metal ions to form insoluble precipitates (Fu and Wang, 2011).	Excessive sludge production, slow metal precipitation and the long-term effects of environmental disposal (Parmar and Thakur, 2013).
Ion Exchange	A reversible chemical reaction wherein an ion from solution is exchanged for a similarly charged ion attached to an immobile solid particle (Parmar and Thakur, 2013).	This method cannot handle concentrated metal solutions as the matrix gets easily polluted with other organics and solids in wastewater (Parmar and Thakur, 2013).
Membrane Filtration	This method has received considerable attention as it not only removes suspended solids and organic compounds but inorganic contaminants as well. Porous membranes are used for the removal of heavy metals (Ahalya, Ramachandra and Kanamadi, 2003).	The generation of sludge and high costs (Parmar and Thakur, 2013).
Reverse Osmosis	Uses a semi-permeable membrane allowing the fluid being purified to pass through rejecting any contaminants (Fu and Wang, 2011).	It is expensive (Ahalya, Ramachandra and Kanamadi 2003; Parmar and Thakur, 2013).

Biosorption may be defined as the ability of certain types of microbial biomass to retain relatively high amounts of metal ions through a passive method of metal sorption or complexation (Volesky, 1990). An important feature of biosorption is its ability to allow for binding and accumulation of metal species even when the cell is not metabolically active (Volesky, 1990). Bioaccumulation is an active process which utilises metabolically active cells for the uptake and accumulation of heavy metals (Malik, 2004; Zabochnicka-Świątek and Krzywonos, 2014).

Biosorption and bioaccumulation differ in terms of the mechanism that allows for metal binding. Biosorption allows for the binding of metal contaminants mainly to the surface of the microbial cell. Thus is dependent on the composition and the kinetic equilibrium of the cell surface; it is an energy independent mechanism (Zabochnicka-Świątek and Krzywonos, 2014; Mosa et al., 2016). While bioaccumulation transports the metal contaminants into the microbial cell and is an energy dependent mechanism (Zabochnicka-Świątek and Krzywonos, 2014).

2.3. Biosorption vs. bioaccumulation as a bioremediation method for heavy metal contaminants

Hussein et al. (2004) reported that for large scale metal removal applications biosorptive processes are more feasible than bioaccumulative processes. This is because living systems (active uptake) usually require the addition of nutrients and hence increases the biological oxygen demand (BOD) or the chemical oxygen demand (COD) in effluents. Furthermore, the maintenance of healthy microbial populations is complex due to metal toxicity and other unsuitable environmental factors (Hussein et al., 2004) that can lead to metabolic stress. The potential for desorptive metal recovery is also restricted in bioaccumulative processes since metals may be intracellularly bound and metabolic products may form complexes with metals to retain them in solution (Hussein et al., 2004). Feasibility studies indicated that biosorptive processes are more applicable as compared to bioaccumalative processes as bioaccumalative processes require additional nutrients (Hussein et al., 2004).

Table 2. Comparison of the biosorption and bioaccumulation processes (Vijayaraghavan and Yun, 2008).

Characteristics	Biosorption	Bioaccumulation
Cost	Usually low. Biomass can be obtained from industrial waste. Cost covers mostly transportation and production of biosorbent.	Usually high. The process occurs in the presence of living cells that have to be supported.
pH	pH of the solution strongly affects sorption capacity of heavy metals. However, the process can occur in a wide range of pH.	Significant change in pH can heavily affect living cells.
Selectivity	Poor. However can be increased by modifications/biomass transformation.	Better than in the case of biosorption.
Rate of removal	Most mechanisms occur at a fast rate.	Slower rate than in the case of biosorption. Intracellular accumulation takes a long time.
Regeneration and reuse	Biosorbents can be regenerated and reused in many cycles.	Due to intracellular accumulation reuse is rather limited.
Recovery of metals	With an adequate eluent the recovery of heavy metals is possible.	If it is even possible, biomass cannot be used for other purposes.
Energy demand	Usually low.	Energy is required for cell growth.

Biosorption does not require costly nutrients to maintain live cells in solution. Additional costs or problems associated with the disposal of unused nutrients or secondary metabolic products generated during bacterial growth are thus avoided. Furthermore, it is not governed by physiological constraints of living microbial biomass and since the cells are non-living, processing conditions are not restricted to those conducive for growth (Ahluwalia and Goyal, 2007). A comparison

between the major factors influencing biosorption and bioaccumulation and the feasibility of both methods is represented in Table 2. Using biosorption to bioremediate heavy metals is more favourable than bioaccumulation and was the focus of this study.

2.4. Biosorption mechanisms

Biosorption is a passive adsorption mechanism which is found to be fast and reversible. The biosorption process involves two phases, (I) a solid phase which is the biosorbent or biological material and (II) liquid phase which is the solvent containing the dissolved species to be adsorbed, usually metal ions (sorbate). The sorbate therefore binds to the biosorbent by the mechanisms mentioned afore until this process reaches an equilibrium state which is established between the amount of sorbate bound and the portion remaining in solution (Das, Vimala and Karthika, 2008). Equilibrium is reached once the available binding sites of the biosorbent have reached saturation, thereby causing an equilibrium shift. The affinity of the biosorbent and sorbate determines its distribution between the solid and liquid phases (Das, Vimala and Karthika, 2008).

Microorganisms for instance fungi, algae and bacteria have evolved many mechanisms in response to metal uptake (Volesky, 1990). They have the ability to biosorb metals through processes such as complexation, ion exchange, adsorption and chelation (Volesky, 1990). Complexation involves binding of ions of heavy metals to functional groups present in the cell membrane. Ion exchange is a reversible chemical reaction of the exchange of mobile ions for other ions of the same charge occurring on solids that contain relevant functional groups (Zabochnicka-Świątek and Krzywonos, 2014). Adsorption is triggered by an intermolecular interaction of Van der Waals forces (Figure 1) (Zabochnicka-Świątek and Krzywonos, 2014). However Aksu, Sag and Kutsal (1992) showed that electrostatic interactions can also play a role in adsorption and are responsible for Cu biosorption by the bacterium *Zoogloea ramigera*. Negatively charged groups such as carboxyl, hydroxyl and phosphoryl groups of bacterial cell walls adsorbs metal cations which are in turn retained by mineral nucleation; this process is known as chelation (Wase and Forster, 1997).

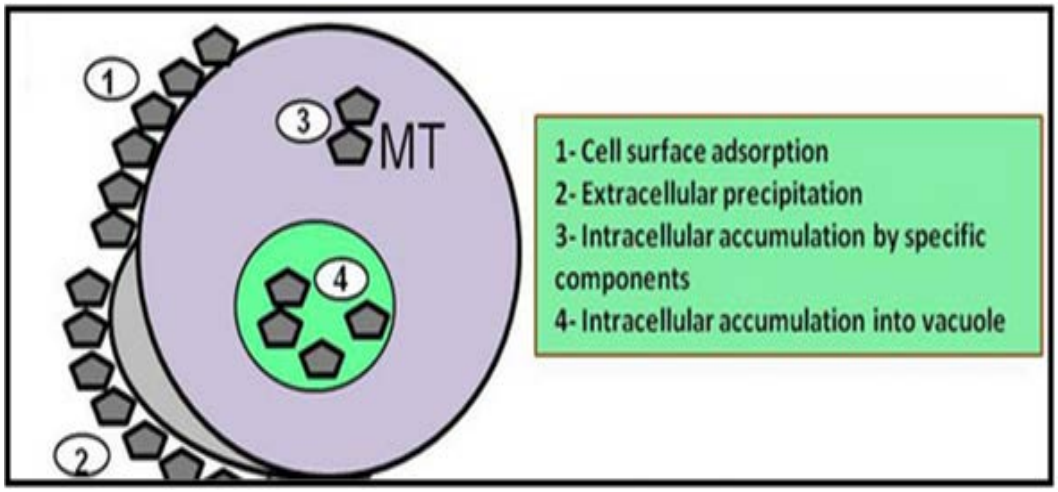


Figure 1. **Biosorption mechanisms utilised by the microbial cell during the uptake of heavy metals**. **Adapted from Banik et al. (2014).**

The chemical makeup of the microbial cell plays a huge role in the binding and uptake of heavy metals, which therefore allows for the above mentioned mechanisms to function. An example is the chemical makeup of the bacterial cell. Bacteria are seen as good biosorbents for the uptake of heavy metals due to their high surface to volume ratios and the presence of potential chemosorption sites on their cell walls (Beveridge, 1989). The bacterial cell wall is a well defined polymeric matrix located just outside the cell membrane and is known to provide mechanical strength and support to the cell (Prescott, Harley and Klein, 2002). During the uptake of heavy metals the cell wall is one of the first cellular structures that comes into contact with soluble metal species (Prescott, Harley and Klein, 2002). A Gram negative bacterial cell wall contains a thin peptidoglycan monolayer, lipopolysaccharides (LPS), phospholipids and surface proteins (Beveridge, 1989). The phosphate groups found within the cell wall constituents have been established to be the primary sites for metal interactions during heavy metal uptake by bacterial cells (Beveridge, 1989) as shown in Figure 2.

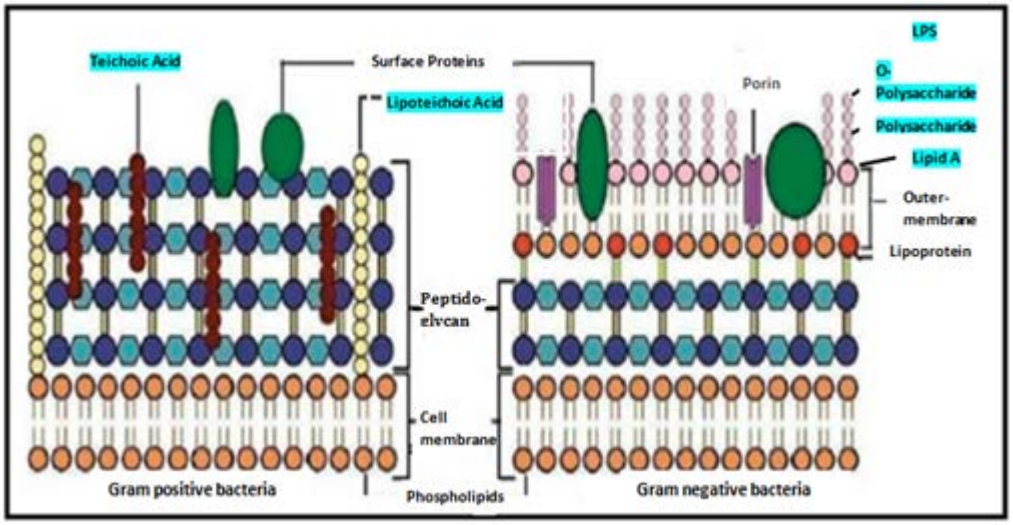


Figure 2. **Cell wall structure of a Gram positive and negative cell wall. The highlighted areas in blue indicate metal binding groups**. **Adapted from Hansda et al. (2015).**

In comparison a Gram positive bacterial cell is surrounded by a thick peptidoglycan layer containing teichoic acids and lipoteichoic acids (Figure 2). The negatively charged teichoic acids appear to extend to the peptidoglycan surface providing the gram positive bacterial cell a negative charge (Prescott, Harley and Klein, 2002). The phosphoryl groups as well as the carboxyl groups of peptide chains in the Gram-positive cell wall are able to partake in sequestration of metals during their uptake by the microbial cell (Prescott, Harley and Klein, 2002).

Pb is bound by both Gram positive and negative bacteria. However, the functional groups involved in binding differ based on the composition of the cell wall and other external layers such as the EPS found in some microorganisms. Binding of Pb (II) in *M. luteus* and *Aztobacter sp.* is reported to mostly occur in the cell wall and cell membrane (Jarosławiecka and Piotrowska-Seget, 2014). Çabuk and co-workers (2006) demonstrated that hydroxyl and carboxyl groups were involved in metal binding by *Bacillus sp.* which was able to bind 91.7% of Pb (II) added to growth medium. It should also be noted that each bacterial strain is unique in the way they bind heavy metals. For example in *Saccharomyces cerevisiae*, amide

and phosphate groups were involved in the immobilisation of Pb (II) in the cell wall, whereas for *Pseudomonas aeruginosa* the carbonyl, phosphate and amino groups were involved (Çabuk et al., 2007).

Microorganisms furthermore have the ability to synthesise extracellular polymers (EPs) to which cations of toxic metals bind (Jarosławiecka and Piotrowska-Seget, 2014). EPs are composed of proteins, humic acids, polysaccharides and nucleic acids and therefore chelate metals with a different specificity and affinity (Pal and Paul, 2008; Jarosławiecka and Piotrowska-Seget, 2014). Binding of Pb (II) by EPs has been reported for a number of bacterial strains including *Paenibacillus jamilae* which has a higher affinity for Pb compared to other heavy metals such as Cd, Cu, Zn and Ni (Jarosławiecka and Piotrowska-Seget, 2014). The EPs of *P. jamilae* has a high content of uronic acids which plays a vital role in the binding of Pb ions (Jarosławiecka and Piotrowska-Seget, 2014).

2.5. Factors that influence biosorption

Many environmental factors influence the chemical nature of bacterial binding sites and consequently biosorption. These factors include pH, temperature, biomass concentration and the competition of other cations within solution (Ahalya, Ramachandra and Kanamadi, 2003).

2.5.1. pH

pH seems to be the most important parameter in the biosorption process as it affects the solution chemistry of metals, the activity of the functional groups in the biomass as well as the competition of metallic ions (Ahalya, Ramachandra and Kanamadi, 2003). The availability of binding sites varies depending on the pH; at a lower pH, binding sites are partially protonated thereby preventing accessibility of positively charged ions (Babák et al., 2012). At a higher pH the solubility of the metal is significantly reduced and the increase in pH contributes to the formation of hydroxides which collides and impedes biosorption (Babák et al., 2012). The uptake of Pb from industrial wastewater by *Geobacillus thermodenitrificans* was reported to increase to 32.26 mg/g of Pb ions as the pH increased (3 – 4.5) (Chatterjee, Bhattacharjee and Chandra, 2010). A study by Çolak and co-workers (2011) showed that as the pH of a solution was increased from 1.2 – 6 for *Bacillus*

pumilus the biosorption capacity for Pb increased and consequently an increase in the metal uptake rate was observed from 4.57 – 28.06 mg/g. This was due to the change in the surface charge from positive to negative allowing the binding of cations.

2.5.2. Temperature

Temperature influences the rate of biosorption because of its effect on: (I) the stability of the metal species in solution, (II) the stability of the biosorbent – metal complex dependent upon biosorption sites, (III) the cell wall configuration and (IV) the ionisation of chemical moieties on the cell wall (Sag and Kutsal, 2000). For endothermic reactions, higher temperatures enhance sorption due to the increase in surface activity and kinetic energy of the solute (Vijayaraghavan and Yun, 2008). In contrast, an increase in heat would cause a decrease in the biosorption capacity of the biosorbents in the system where binding of the metal ion is exothermic. Fan et al. (2008) demonstrated that when the temperature of a solution was increased between 20 – 40 °C, the rate of Pb uptake by *Penicillium simplicissimum* increased from 20 – 35 mg/g. However a study conducted by Bahadir and co-workers (2007) reported between 2 and 2.2 mg/g of Pb uptake by *Rhizopus arrhizus* regardless of the temperature in the range 20 – 45 °C.

2.5.3. Biomass concentration

The specific uptake of metals is influenced by biomass/biosorbent concentration. A lower concentration of biomass allows for an increase in metal uptake. Sufficient interaction between the metal ions and the biosorbent as a result of more intercellular space and less crowding allows for increased contact. Conversely, a higher concentration in biomass leads to cell agglomeration and less biosorption caused by the reduction in the intercellular distance and spatial interference of ion binding to the biosorbent (Fourest and Roux, 1992; Rani et al., 2010). For instance as the biomass concentration was increased from 1 – 5 g/L of *Citrobacter* strain MCM B-181 the uptake of Pb from a 1 mM solution decreased from 90 mg/g to about 40 mg/g (Puranik and Paknikar, 1999). For this reason, it is important to determine the adsorption equilibria that will dictate the ideal ratio of

biomass to metal for maximal uptake (Bahadir et al., 2007) when preparing a biosorbent.

2.5.4. Competing cations

Industrial wastewaters are a complex mix of various contaminants of varying nature and concentrations that include heavy metals. Using biosorption to bioremediate wastewaters means that the biosorbent is exposed to more than one metal ion at a particular time (Ahalya, Ramachandra and Kanamadi, 2003). These metal ions could (I) compete with each other for binding sites, (II) enhance the binding of another metal ion or (III) not affect the binding of other metal ions in any manner (Wase and Forster, 1997). Sar and D'Souza (2001) reported that the uptake of the heavy metal uranium (U) by *Pseudomonas* biomass was not affected by the presence of Cd, Ag and Pb. However, the rate of uptake of uranium by *Rhizopus arrhizus* increased in the presence of Fe^{2+} and Zn^{2+} (Tsezos and Volesky, 1982).

2.6. Rationale for the present study

Gauteng is a province in South Africa that contributes approximately 34% of the national economy. This economy was once driven mainly by the mining industry but it has diversified to include manufacturing industries. These industries are the core contributors to heavy metal pollution of natural water resources in the province (Masindi et al., 2015). When Pb-contaminated wastewater flows untreated or partially treated into natural rivers, the metal finds its way into living tissues through the practices of irrigation and animal husbandry (Volesky, 2001). Within living tissues, Pb poses various hazards including mutations, nervous impairment and many others (Nanda, Sharma and Kumar, 2011). The effective treatment of such wastewaters prior to release and consumption is imperative.

The benefits of complementing chemical treatment with biosorption have been discussed above, yet the commercialisation of biosorbents has been slow (Atkinson, Bux and Kasan, 1998). One reason is due to a lack of understanding of the competing ions effect and metal selectivity of biosorbents. This is where a huge proportion of research has been directed. The discovery of new biosorbents

can be highly competitive and effective if the influences of external factors on biosorption are elucidated (Wang and Chen, 2009).

Two bacterial species, *P. castaneae* and *M. luteus*, were previously isolated from AMD on the West Rand, Gauteng by the Environmental Biotechnology group (University of the Witwatersrand). The decant had a pH of 5.4 and a Pb concentration of 2 mg/L which is above the acceptable tolerance limit of 0.01 mg/L according to the World Health Organisation guidelines (WHO, 2008). Both isolates were shown to have a high tolerance for Pb with minimum inhibitory concentrations (MICs) for *M. luteus* and *P. castaneae* being 5800 mg/L and 5400 mg/L, respectively (Vallabh, 2014). These findings strongly suggest their potential to be good biosorbents for the metal and warrant further investigation.

2.6.1. *Micrococcus luteus*

M. luteus is a Gram positive spherical and saprotrophic bacterium which is commonly found in environments of soil, dust particles, air and water (Stolp, 1988). It has been reported to accumulate a variety of toxic substances that include petroleum, pesticides and many metals such as Cd, Cu and Pb. Rod and cocci shaped Gram positive bacteria have high metal sorption capacity (Cotoras et al., 2008) enabling them to have a high affinity to metal-contaminated environments. A study done by Maldonado et al. (2010) indicated that *M. luteus* has the ability to effectively accumulate Cu and Pb in an extracellular manner, in layers of EPS. Additionally, they reported its growth in 1.5 mM of Pb while also being able to withstand up to 3 mM metal. Silambarasan and Abraham (2014) found that *M. luteus* isolated from Palar River basin, Vellore was able to adsorb 60% of Pb ions as well as 58% of Cd ions clearly indicating its metal resistance.

2.6.2. *Paenibacillus castaneae*

P. castaneae was first isolated from the phyllosphere of chestnut trees (Valverde et al., 2008). It is a Gram variable spore-forming bacterium which can be found in the environments of soil, rhizosphere, vegetable matter and water (Chien and Han, 2009). There are a number of reports associating *Paenibacillus sp.* with resistance to heavy metals such as Ni, Co, Cu, Cd, Zn and Pb (Pérez et al., 2008; Chien and Han, 2009). In a study by Abou–Shanab, Van Berkum and Angle (2007),

Paenibacillus sp. was able to withstand between 5 – 10 mM of Pb ions demonstrating significance in heavy metal resistance capacity. Çolak and co-workers (2013) also reported a metal uptake of 49.8 mg/g and 35.02 mg/g for Cu and Ni respectively for *Paenibacillus sp.* Although there is no formal literature on the ability of *P. castaneae* to tolerate high amounts of Pb, these findings suggest its potential as a metal biosorbent in wastewater treatment.

As a result of the harsh environment (low pH and high Pb concentration) from which these bacteria were isolated and their association with heavy metal resistance, it is proposed that they would constitute good metal biosorbents for wastewaters in Gauteng. The present study attempted to elucidate the impact of parameters such as pH, temperature, biomass concentration, metal concentration and the competition of metal cations within solution on their rate of uptake of Pb. Subsequently, it is anticipated that an optimised bioremediation process for Pb removal from wastewater using *P. castaneae* and *M. luteus* biomass would be designed by implementing the findings from this study.

Chapter 3

Material and Methods

All media and chemicals used in the study were of Reagent grade and Trace metal grade, respectively (Sigma Aldrich, USA). Stock solutions of lead nitrate ($Pb(NO_3)_2$), nickel chloride hexahydrate ($NiCl_2.6H_2O$), cobalt chloride hexahydrate ($CoCl_2.6H_2O$), manganese chloride tetrahydrate ($MnCl_2.4H_2O$) and zinc chloride ($ZnCl_2$) were prepared by dissolving the metal salts in deionised water with precise concentrations, followed by filter sterilisation through a 0.22 µm membrane filter. The metal solutions served as a source of metal ions throughout the experimental work. All experimental work was performed in triplicate in the presence of 0.5 mM of the respective metal ions, apart from the instance where the effect of metal concentration on biosorption was tested; a range of concentrations of Pb ions were used for this assay.

3.1. Growth and preparation of bacterial biomass.

The *M. luteus* and *P. castaneae* strains used in this study were previously isolated from AMD on the West Rand, Johannesburg and identified to species level using the Biolog Microbial ID System. They were maintained at -20 °C as glycerol stocks (addition of 200 µl of glycerol to 800 µl of bacterial culture) at a pH of 7, until required for the accumulation of biomass.

Both bacterial strains were cultured by the addition of a 1 mL glycerol stock in a final volume of 200 mL to Luria Bertani (LB) broth (pH 7) for a period of 24 h, at 37 °C, on an orbital shaker (Labcon) at 200 rpm. The bacterial cells were harvested by centrifugation (Multifuge X1R centrifuge, Thermo Scientific) at 10 000 x *g* for 10 mins after which the cell pellet was collected and heat-killed by autoclaving at 121 °C for 20 mins. The heat-killed cells were washed twice in 0.85% (w/v) physiological saline by centrifugation at 5000 x *g* for 5 mins to remove any external contaminants and residual media coating the cells. Physiological saline was used as it is isotonic with the cell and therefore would not cause any disruptions to the cell structure. After the final wash, the bacterial

biomass was resuspended in sterile deionised water to a concentration of 10 mg/mL.

For the remainder of the methods, reference to biomass during an experimental assay implies that it was prepared according to the procedure described above, unless otherwise stated.

3.2. Pb biosorption study at pH 7

In order to determine the biosorption of Pb ions by *M. luteus* and *P. castaneae*, 2 g/L of biomass was added to 0.5 mM of Pb ions in a total volume of 25 mL deionised water. The suspension was incubated at 25 °C for 24 h on a rotary shaker at 150 rpm, after which the cell biomass was separated from the metal ions remaining in solution by centrifugation at 17 000 x *g* for 10 mins. The supernatant was nitrified to a pH of ≤ 2, using a 50:50 combination of nitric acid to nuclease free water and sent for inductively coupled plasma optical emission spectroscopy (ICPOES) analysis to the Department of Chemistry (University of the Witwatersrand). Inductively coupled plasma optical emission spectroscopy was used to measure the amount of residual metal ions left in the supernatant for the determination of the rate of specific metal uptake (Q) calculated according to Equation 1 (Puranik and Paknikar, 1999).

$$\mathbf{Q = V (C_i – C_f)/ 1000M} \qquad \mathbf{(1)}$$

Where: **Q** – Specific metal uptake (mg metal / g biosorption biomass)

V – Volume of metal solution (mL)

$\mathbf{C_i}$ – Initial concentration of metal in solution (mg metal/L)

$\mathbf{C_f}$ – Final concentration of metal in solution (mg metal/L)

M – Dry weight of biomass (g).

3.3. Effect of pH

The biosorption of Pb ions was tested at different pH conditions for the individual species in order to establish the optimal pH at which maximal biosorption occurs. Bacterial biomass was resuspended in deionised water that had been conditioned separately to a pH of 4, 5, 6 or 7 using a basic 20 pH meter (Crison instruments).

The pH was adjusted either using 0.1 M sodium hydroxide (NaOH) or 50% nitric acid. The conditioned biomass was then incubated with 0.5 mM of Pb ions at the corresponding pH. The suspensions were further treated as described in section 3.2 above.

3.4. Effect of temperature

To evaluate the effect of temperature on the biosorption rate of Pb ions by each species, the procedure outlined in section 3.2 above was followed with some modification. The pH conditions were adjusted to the optimal pH (pH 7) determined from section 3.3 above for each species and individual treatments were incubated at 4 °C, 37 °C and 55 °C respectively, for 24 h on a rotary shaker at 150 rpm. Thereafter, cell biomass was removed by centrifugation and the supernatant was nitrified and analysed by ICPOES as previously described.

3.5. Effect of biomass concentration

The metal to biosorbent ratio is an important factor to consider when optimising metal uptake. Too high a biomass concentration leads to spatial crowding and less access to metal binding sites (Abbas et al., 2014). Alternatively, too low a concentration may result in reduced uptake due to rapid saturation of the binding sites (Abbas et al., 2014). To derive the optimal biomass concentration that would result in the most uptake of Pb by *M. luteus* and *P. castaneae*, several biomass concentrations (1, 2, 3, 4 and 5 g/L) were exposed to 0.5 mM of Pb ions in a final volume of 25 mL deionised water. Each treatment was prepared at the optimal pH (pH 7) determined in this study (Section 3.3 above) and incubated at 25 °C for a period of 24 h, with shaking at 150 rpm. Residual Pb ion concentrations after individual treatments were determined by ICPOES as previously described.

3.6. Effect of initial metal concentration

Harvested biomass (2 g/L) from individual species were exposed to 0.005 mM (low concentration) and 1.25 mM (high concentration) of Pb ions separately in a final volume of 25 mL deionised water at the optimal pH (pH 7) as determined from section 3.3 above. In solutions where the initial concentration of metal is low, a given concentration of biosorbent is able to take up more metal as compared to the instance when the metal concentration is too high. This is

because at higher concentrations, the biomass attains equilibrium much faster resulting in the saturation of metal binding sites within a short period of time (Fourest and Roux, 1992). Cell suspensions were incubated at 25 °C for 24 h on an orbital shaker at 150 rpm. Supernatant containing unbound Pb ions was collected and the metal concentration was quantified using ICPOES as previously described.

3.7. Effect of competing cations

Aside from H^+ that compete for negatively charged binding sites on the biosorbent surface, other cations may act in a synergistic or antagonistic manner. Some cations may enhance the binding of Pb to the biosorbent while other ions are known to compete with Pb for similar binding sites (Ahalya, Ramachandra and Kanamadi, 2003). The effect of various cations on the biosorptive ability of *M. luteus* and *P. castaneae* was studied in both binary as well as multi-metal systems. The metals of subject included: Pb, Ni, Co, Mn and Zn and were selected based on data collected by the Environmental Biotechnology research group (University of the Witwatersrand) on metal contaminants commonly found in AMD in Gauteng. Fifty mg of cells (equivalent to a concentration of 2 g/L) from each species was added to a combination of equal concentrations (0.5 mM) of the metals in binary as well as in a solution containing all the metals at the optimum pH determined previously. Each treatment was incubated at 25 °C for 24 h with shaking at 150 rpm. Subsequently the supernatant was collected, nitrified and sent for ICPOES analysis as previously described.

3.7.1. Consortium study

To show the potential of using a mixed formulation of the biosorbents for future studies on Pb uptake, a preliminary assay was conducted using a 1:1 and 2:1 ratio of *P. castaneae* to *M. luteus* cells. Each combination was subjected to equal concentrations (0.5 mM) of all metals in solution at pH 7 similar to the method described in section 3.7 above. The samples were incubated at 25 °C for 24 h with shaking at 150 rpm. The supernatant was thereafter collected and sent for ICPOES analysis as previously described.

Controls were set up for each parameter investigated and for each individual species. A control consisted of sterile Pb ions in solution subjected to the same conditions as each experimental treatment.

3.8. Statistical analysis

All experimental procedures were performed in triplicate to ensure statistical accuracy. Data was analysed using the statistical program R, version 3.3 for Windows. A two - way ANOVA test was used to evaluate the significance at a 95% confidence interval of the parameters tested within a bacterial species and between the two bacterial species.

Chapter 4

Results and Discussion

The increase in heavy metal pollution as a result of industrial and mining activities in South Africa is of growing concern. Release of metal-bearing effluents into rivers and dams not only affects the quality of potable water but also agricultural soils as well as aquatic and plant life found within these waters. Natural biosorbents such as fungi, algae and bacteria have been receiving a great amount of interest due to their metal-sequestering ability, good performance, large available quantities as well as being cost effective (Wang and Chen, 2009).

The current study therefore sought to optimise the conditions under which *M. luteus* and *P. castaneae* would best biosorb soluble metal ions with a focus on Pb. These bacteria were selected for their known resistance to Pb (GDARD, 2016). A number of external parameters outlined in the methodology (Chapter 3, pg 31) were analysed in order to establish a set of conditions under which *M. luteus* and *P. castaneae* would biosorp the highest amount of Pb ions in solution. The set of conditions reported here includes a pH of 7, temperature range between 25 – 37 °C and a biomass of 2 g/L of heat killed cells. The following chapter illustrates and discusses how these findings were reached. Furthermore, it elaborates on the influence of the initial Pb concentration and the presence of other cations on the rate of Pb uptake under these optimised conditions.

4.1. Effect of pH

pH is reported to be one of the most important parameters that affect the rate of biosorption of heavy metals (Hassan et al., 2010; Abbas et al., 2014). This is because it serves a dual purpose; pH influences metal binding sites on the biosorbent as well as the chemistry of the metal in solution (Hassan et al., 2010). At a lower pH the binding sites found on the surface of the cell are more closely linked with H^+ making them unavailable for binding by other cations (Hawari, Catherine and Mulligan, 2006; Uslu and Tanyol, 2006; Rani et al., 2010). On the other hand, at a higher pH there is an increase in ligands with negative charges

being exposed resulting in increased binding of cations (Ahuja, Gupta and Saxena, 1999).

This trend was observed in the present study when *M. luteus* was exposed to 0.5 mM of Pb ions for 24 h (Figure 3).The percentage of Pb ions biosorbed increased from 7.51% at a pH of 4 to 46.08% at a pH of 7 supporting the theory that biosorption of metal ions increases as pH is increased within limitation. It should be noted that too high a pH can lead to the precipitation of metal salts making them unavailable for biosorption. Filtration or sedimentation would then be necessary to remove the precipitates (Azizi, Colagar and Hafeziyan, 2012).

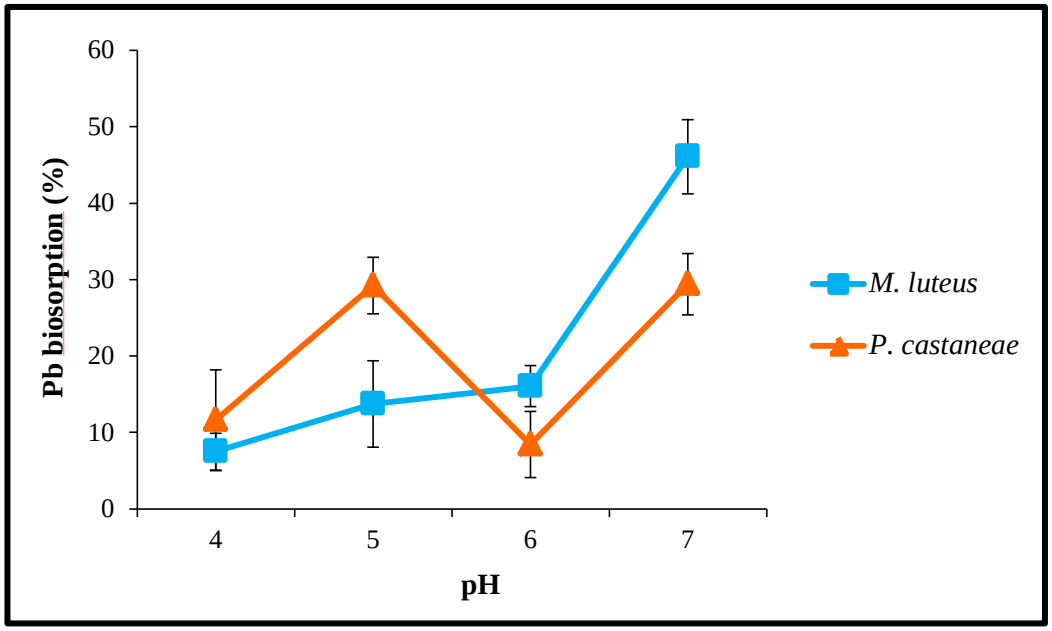


Figure 3. Graph showing % Pb biosorbed over 24 h when *M. luteus* and *P. castaneae* was exposed to 0.5 mM Pb at 25 °C under different pH conditions. Optimal uptake was achieved at pH 7 for both isolates.

Furthermore, these findings are similar to those reported for the biosorption of Pb ions by *Bacillus cereus* and *B. pumilus* (Çolak et al., 2011). Although these species do not belong to the same genus as *M. luteus*, they are all Gram positive bacterial strains. In their study, Çolak and co-workers (2011) reported an increase in the rate of Pb ion uptake from 4.57 – 28.06 mg/g for *B. pumilus* and 3.2 – 22.1 mg/g for *B. cereus* when the pH was increased from 1.2 – 6. Table 3 indicates that as *M. luteus* was exposed to Pb ions at a pH range of 4 - 7, the rate of metal

uptake increased from 4.00 – 24.51 mg/g. This increased rate is attributed to the acquisition of negative charges on the surface of biomass (due to a higher pH) leading to increased electrostatic attraction of the Pb ions (Çolak et al., 2011).

Table 3. Specific rate of Pb uptake at different pH by *M. luteus* and *P. castaneae*.

Biosorbent	pH	% Biosorption	Specific metal uptake (mg Pb/g biomass)
M. luteus	4	7.51	4.00
	5	13.74	7.31
	6	16.04	8.53
	7[(a)]	46.08	24.51
P. castaneae	4	11.62	6.18
	5[(b)]	29.23	15.53
	6	8.45	4.50
	7[(b)]	29.40	15.63

[(a)] Significant at $p < 0.005$ and [(b)] $p < 0.05$ (ANOVA)

In contrast to the trend seen for *M. luteus*, *P. castaneae* illustrated an increase in Pb biosorption from a pH of 4 - 5, followed by a decrease at a pH of 6 with a sharp increase again at pH of 7 (Figure 3 and Table 3). Both *M. luteus* and *Paenibacillus sp.* are reported to have EPS that contributes to metal sorption (Puyen et al., 2012; Jarosławiecka and Piotrowska-Seget, 2014; Liang and Wang, 2015). However the composition of the EPS between these two genera is likely to differ and consequently the trend in metal sorption is likely to differ. Both the content and composition of EPS in microorganisms are heterogenous (Wingender, Neu and Flemming, 2012) due to varying quantities of macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds. Subsequently changes in pH would affect the charges of the functional groups of proteins and carbohydrates differentially hence the observations made in the present study. A study by Çolak and co-workers (2013) on the heavy metal

resistance and biosorptive behaviours of *Paenibacillus polymyxa*, reported that as the pH was increased, an increase in the biosorption of Cu (II) and Ni (II) was observed. Although *P. castaneae* belongs to the same family a true comparison between these findings cannot be surmised because even within a family, the EPS composition can differ substantially between species (Sutherland, 2001).

The drop in metal uptake at a pH of 6 followed by the rapid increase at a pH of 7 is an uncommon occurrence. It could be as a result of a switch between competitive H^+ binding and the increase in negatively charged surface groups. Under more acidic conditions (high concentration of protons), active binding sites are closely linked with hydrogen ions and therefore limits binding of other cations. Under more neutral conditions there is an increase in ligands with a negative charge which results in an increase in binding of other cations (Hassan et al., 2010; Çolak et al., 2011). A similar observation was reported by Azizi, Colagar and Hafeziyan (2012) while testing the effect of pH on the biosorption of Cd (II) utilising biomass from a fungal species (*Oscillatoria sp*.). Additionally it was also found that at a pH of 5 and 7, phosphate groups of phospholipids present in the cell membrane began to deprotonate allowing for the binding of other cations (Azizi, Colagar and Hafeziyan, 2012).

Both *M. luteus* and *P. castaneae* were found to take up the most Pb under the present conditions at a pH of 7. Hence it was selected as the optimal pH to use in subsequent experimental work. Additionally, due to the similarity and vast amount of metal uptake (15.53 mg/g) that occurred at a pH of 5 using *P. castaneae* biomass, this pH was also selected for further experimentation. If a high rate of uptake is maintained at pH 5 it could result in lowered cost of treatment as the need to neutralise acidic wastewaters may be circumvented.

Although not a direct objective of this study, a comparison between the biosorption of Pb by *M. luteus* and *P. castaneae* can be inferred. At a pH of 7, *M. luteus* (46.08%) biosorbs more Pb ions from solution as compared to *P. castaneae* (29.40%). Statistical analysis using a two – way ANOVA test (see Table A.2 in Appendix A) indicated that when the rates of Pb uptake by *M. luteus* and *P.*

castaneae are compared, there is a significant difference between the two biosorbents at pH 5 (p = 0.041) and pH 7 (p = 0.008).

4.2. Effect of temperature

Another important factor that has an impact on the biosorption of heavy metals by bacterial biomass is temperature. A change in temperature has been reported to affect the stability of metal ion species placed into solution, the wall configuration of the microorganism cell and the ionization of chemical moieties on the cell wall (Özer and Özer, 2003).

From Figure 4 it can be seen that some biosorption of Pb occurs at 4 ^{o}C in all three treatments although at a slow rate (Table 4). Since biosorption is a passive process and is generally an electrostatic attraction, temperature would not affect binding of the metal ions. However, temperature can affect the viscosity of aqueous solutions whereby the lower the temperature the more viscous the solution (Huddleston et al., 2001). A more viscous metal solution will thereby reduce the mobility of metal ions and lead to a slower rate of uptake by the biomass.

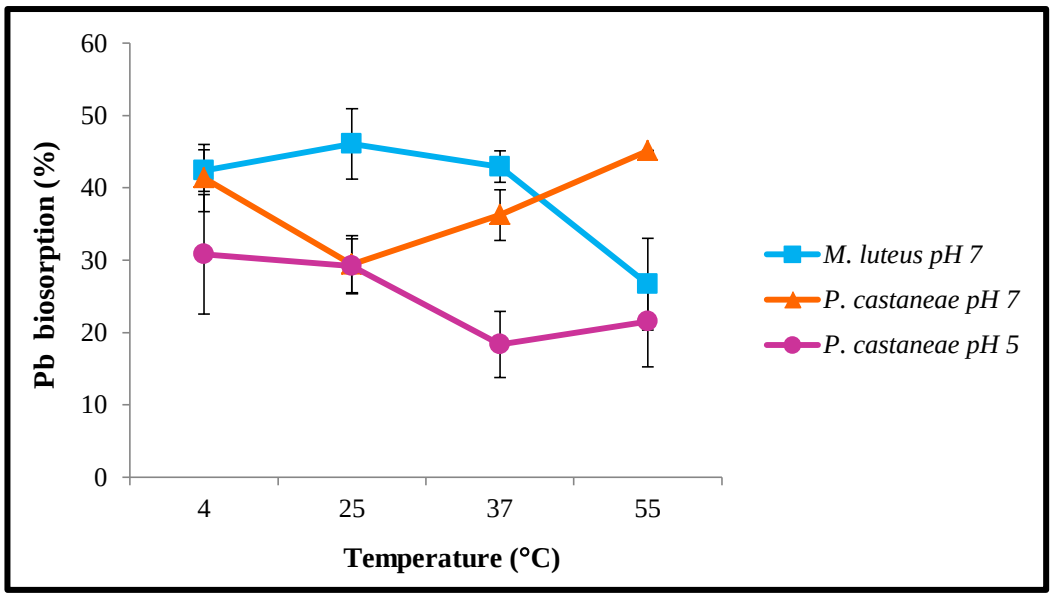


Figure 4. Graph showing the % Pb biosorbed over 24 h when *M. luteus* and *P. castaneae* was exposed to 0.5 mM Pb (pH 7) at different temperatures.

Opposing trends in Pb uptake by *M. luteus* and *P. castaneae* at pH 7 are observed between 25 - 55 ^{o}C. The percentage of Pb ion uptake decreases from 46.08% (25 ^{o}C) to 26.69% at 55 °C in the presence of *M. luteus*. This is characteristic of physical sorption which does not require energy and preferably occurs at lower temperatures (Lowell and Shields, 1984). However, using *P. castaneae* the % uptake increases from 29.40 to 45.15 in the same temperature range. Such increases are due to the endothermic nature of chemical sorption implying that the spontaneity of biosorption increases with increasing temperature (Babarinde, Babalola and Adetunji, 2008).

Table 4. **Specific rate of Pb uptake at different temperatures by *M. luteus* and *P. castaneae***.

Biosorbent	Temperature (°C)	% Biosorption	Specific metal uptake (mg Pb/g biomass)
M. luteus **pH 7**	4	42.39	19.78
	25	46.08	24.51
	37[(a)]	42.93	19.98
	55	26.69	20.40
P. castaneae **pH 7**	4	41.36	19.19
	25	29.40	15.63
	37[(a)]	36.26	24.18
	55[(b)]	45.15	31.14
P. castaneae **pH 5**	4	30.83	17.94
	25	29.23	15.53
	37	18.37	10.94
	55	21.57	12.55

[(a)] Significant at $p < 0.05$ and [(b)] $p < 0.005$ (ANOVA)

An excessive increase in temperature can cause damage to the active binding sites of the biomass and thus results in a decrease in the rate of metal uptake observed (Puranik and Paknikar, 1999) as seen in the present study (Table 4). This is true when bacteria are exposed to higher than optimal growth temperatures; 25 - 37 ^{o}C for *M. luteus*.

While studying the biosorption of Pb, Cd and Zn by *Citrobacter* strain MCM B – 181, Puranik and Paknikar (1999) observed a similar trend in metal uptake. They reasoned that an increase in metal uptake from 4 - 25 °C was due to the higher affinity of sites for metal binding at these particular temperatures, whereas the decrease in metal uptake at increasing temperatures (37 - 55 °C) was as a result of the distortion of some metal binding sites due to an increasing temperature. Furthermore, Horsfall and Spiff (2005) reported that although the sorption of Pb^{2+} and Cd^{2+} from aqueous solution, by *Caladium biocolor* (Wild Cococyam) biomass increases with an increase in temperature (30 – 80 °C) a decline in the rate of sorption is observed. As the temperature is increased the attractive forces between biomass and surface metal ions are weakened and therefore the sorption rate decreases. The best rate of Pb ion uptake by *M. luteus* was observed at 25 °C, yielding a 46.08% of biosorption of metal ions and a metal uptake rate of 24.51 mg/g biomass (Table 4).

Contrary to these reports, other studies have found that an increase in temperature can lead to increased biosorption of Pb (II) and Zn (II) ions (Marandi, Ardejani and Afshar, 2010) and Cu ions (Al-Homaidan et al., 2014) as observed for *P. castaneae* (Figure 4) in this study. This could be due to a higher affinity of sites for metal on relevant biomass. In addition to the evident increase in the percentage biosorption, the overall rate of metal uptake as the temperature was increased also varied significantly ranging between 19.19 – 31.14 mg/g for *P. castaneae* (Table 4 and Table A.3 in Appendix A for statistical analysis). This is indicative of an endothermic based adsorption of Pb ions (Lowell and Shields, 1984; Babarinde, Babalola and Adetunji, 2008). The biomass contains more than one type of binding site for metal ions which react differently to any given temperature therefore influencing the overall metal adsorption process.

When *P. castaneae* biomass is used to biosorb Pb ions at a pH of 5, no obvious trend is observed (Figure 4). Pb ion uptake seems to increase and decrease unpredictably. This may be explained by the competition between excessive amounts of H^+ and other cations for binding sites that could possibly be distorted due to higher temperatures.

The highest percentage uptake of Pb by *M. luteus* was obtained at 25 ^oC at a rate of 24. 51 mg/g. On the other hand, at pH 7, *P. castaneae* has the highest rate of Pb uptake at 55 ^oC (31.14 mg/g biomass). Using higher temperatures for biosorption has energy implications that would increase the cost of the process especially if it were to be used as a commercial bioremediation strategy. Therefore, subsequent assays were maintained at 25 ^oC. The rates of uptake at this temperature were not significantly different ($p = 0.225$) between *M. luteus* and *P. castaneae* as indicated in Table A.4 in Appendix A. Furthermore when the pH is decreased to 5, the rate of Pb uptake by *P. castaneae* does not vary significantly from that at pH 7. The moderate rate of uptake at a pH of 5 has bearing on wastewaters of an acidic nature that may not require an additional neutralisation step for Pb removal.

4.3. Effect of biomass concentration

In the present study it was noted that the concentration of biomass can significantly affect the extent of Pb biosorption. The highest uptake of Pb by *M. luteus* at pH 7 and 25 °C over 24 h occurred when 2 g/L of biomass was used. Approximately 50% of the metal (Figure 5) was taken up at a rate of 24.51 mg/g biomass (Table 5). At a given equilibrium concentration, the biomass of subject will adsorb more metal ions at a lower density as opposed to a higher density (Fourest and Roux, 1992; Monteiro et al., 2009; Abbas et al., 2014). At this equilibrium, a lower biomass concentration results in a higher metal:biosorbent ratio leading to more metal retained by the sorbent unit (Al-Homaidan et al., 2014).

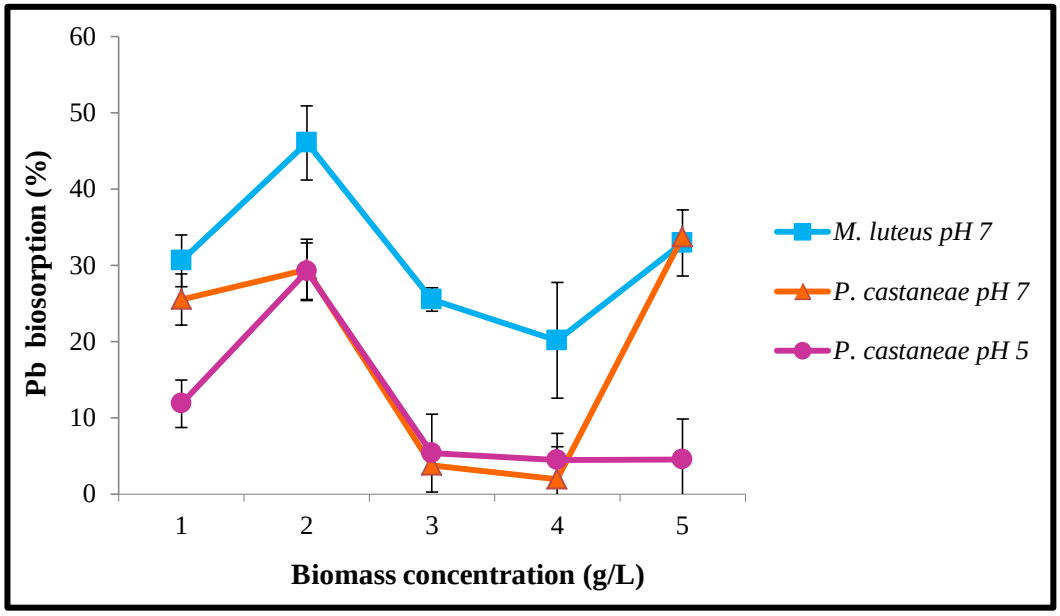


Figure 5. Graph showing the % Pb biosorbed over 24 h when *M. luteus* and *P. castaneae* was exposed to 0.5 mM Pb at 25 °C under different biomass concentrations.

Rani and co-workers (2010) reported 79.22% biosorption of Pb (initial concentration of 1.272 mg/L) by 200 mg/L of *Micrococcus sp.* biomass. It may be exaggeratedly suggested that in the ideal situation a ten-fold increase to 2 g/L biomass (as used in this study) would result in the almost total biosorption of 10 mg/L Pb. From the results of the present study, 2 g/L of *M. luteus* biomass resulted in an uptake of 76 mg/L Pb (46.08% of 0.5 mM Pb) suggesting that it is a highly efficient biosorbent. Other studies using *M. luteus* for Pb biosorption have been performed using live cells and report higher rates of uptake (Puyen et al., 2012) typical of active processes.

Similarly, *P. castaneae* biosorbed the most Pb ions (29.23%) when used at a concentration of 2 g/L at a pH of 5. On the other hand, under neutral conditions it appears that the most uptake of Pb occurred using 5 g/L *P. castaneae* biomass. This was observed as a sharp increase in Pb biosorption between pH 4 and 5. A similar trend was observed for *M. luteus* at pH 7 but did not result in the highest uptake of Pb. Al-Homaidan et al., 2014 reported a similar observation using *Spirulina platensis* biomass for the uptake of Cu ions, indicating that as the

biomass concentration was increased from 0.05 to 0.5 g the percentage of metal uptake decreased from 78.82 to 20%. Further work will be needed to explain the sudden increase in biosorption at higher biomass concentrations. Many biosorption studies that have investigated the influence of biomass concentration on metal uptake have reported on a general downward trend when an increase in the biomass is extended beyond the saturation threshold (Fourest and Roux, 1992; Puranik and Paknikar, 1999; Bahadir et al., 2007; Al-Homaidan et al., 2014).

The initial increase in biosorption at lower concentrations of biomass is due to the increase of the availability of sorption sites (Tewari, Vasudevan and Guha, 2005). Using too high biomass concentrations leads to cell agglomeration consequently reducing the inter-cellular distance (Rani et al., 2010). This causes the formation of a protective shell over active binding sites limiting their occupation by metal ions. Optimum electrostatic forces exist between cells at a lower concentration due to a larger inter-cellular space (Rani et al., 2010); consequently allowing more metal to be biosorbed.

The overall decrease in specific metal uptake rate as biomass was increased beyond the equilibrium threshold (Table 5) is consistent with other reports (Puranik and Paknikar, 1999; Tewari, Vasudevan and Guha, 2005; Özdemir et al., 2009). The highest rate of uptake was observed when 2 g/L biomass was used for both *M. luteus* (24.51 mg/g) and *P. castaneae* (15.63 mg/g). As such, under the parameters outlined in the study, using this concentration of biomass would yield the most uptake of Pb ions. Furthermore, the difference between the rate of uptake at 2 g/L and the other concentrations of biomass for the individual species was significant as reported in Table A.5 in Appendix A.

Table 5. Specific rate of Pb uptake using various biomass concentrations of *M. luteus* and *P. castaneae*.

Biosorbent	Biomass concentration (g/L)	% Biosorption	Specific metal uptake (mg Pb/g biomass)
M. luteus **pH 7**	1	30.60	16.70
	2[(a)]	46.08	24.51
	3	25.51	11.30
	4	20.61	5.49
	5[(b)]	32.95	7.18
P. castaneae **pH 7**	1[(a)]	25.51	11.15
	2[(a)]	29.40	15.63
	3	3.78	1.38
	4	1.95	0.66
	5[(a)]	33.67	7.33
P. castaneae **pH 5**	1	11.85	6.45
	2	29.23	15.53
	3	5.37	2.70
	4	4.48	2.33
	5	4.52	2.46

[(a)] $p < 0.005$ (ANOVA) [(b)] Significant at $p < 0.05$

4.4. Effect of initial metal concentration

The initial metal concentration provides an important driving force to overcome all mass transfer resistance of metal between the aqueous (solution) and solid (biomass) phases. Determining the behaviour of the biosorbent when initial Pb concentrations are varied is necessary to predict whether it is adequately functional in natural wastewaters. Most contaminated wastewaters constitute a

"cocktail" of different metal ions present in different concentrations which would have a direct effect on the rate and extent of biosorption.

The present findings (Figure 6) show that both *M. luteus* and *P. castaneae* biosorb maximally when the initial concentration of Pb is low (1 mg/L equivalent of 0.005 mM) under neutral conditions. At this concentration, 84.76% of the metal was taken up by *M. luteus* and 81.39% by *P. castaneae*. As the metal concentration was exaggeratedly increased 100 and 200 fold (0.5 and 1.25 mM, respectively), the % biosorption decreased for both biosorbents as expected. This is due to saturation of binding sites above a certain number in relation to the biomass concentration. Similar findings were reported for the sorption of Ni (II) and Mn (II) as the initial metal ion concentration was increased from 100 to 300 mg/L (Akpomie, Dawodu and Kayode, 2015). The authors reported a decrease from 64.8 to 37.63% of Ni (II) and 54.8 to 30.57% of Mn (II) due to the saturation of the adsorbent's affixed number of active binding sites.

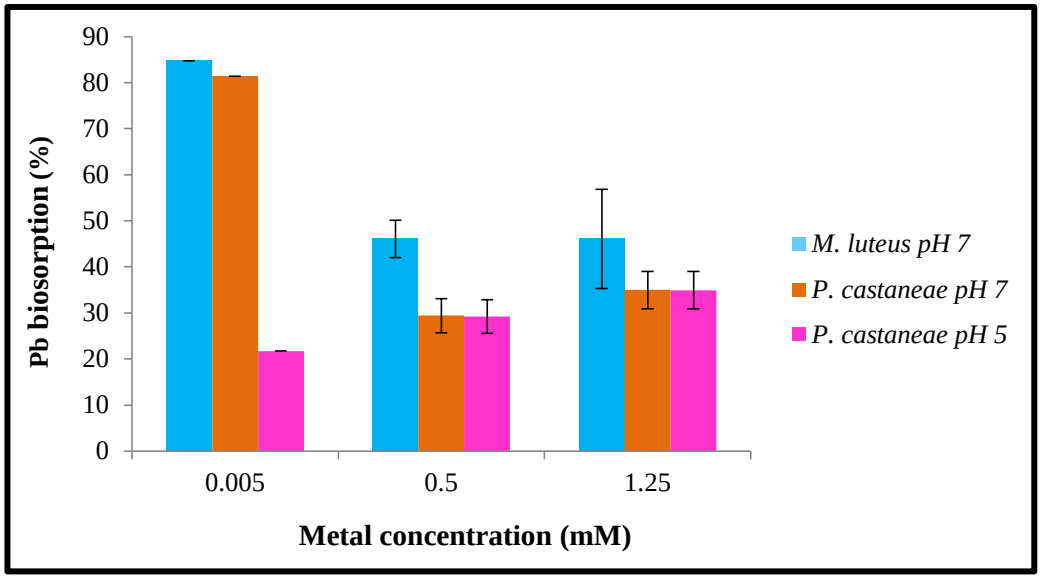


Figure 6. Graph showing the % Pb biosorbed over 24 h when *M. luteus* and *P. castaneae* was exposed to different metal concentrations at 25 °C.

Table 6 shows that as the Pb concentration increased, the rate of metal uptake for *M. luteus* increased from 0.42 mg/g biomass to 59.21 mg/g biomass. Similarly at 0.005 mM Pb, the specific rate of Pb uptake by *P. castaneae* (pH 7) was 0.40 mg/g biomass and increased to 44.85 mg/g biomass at a concentration of 1.25

mM. This could be as a result of increased electrostatic attractions due to an increase in number of metal ions to active binding sites. These findings are in agreement with previous studies that have looked at biosorption kinetics in various other bacterial species (Aksu, Sag and Kutsal, 1992; Puranik and Paknikar, 1999; Özdemir et al., 2009; Al-Homaidan et al., 2014).

Table 6. Specific rate of Pb uptake by *M. luteus* and *P. castaneae* when the initial metal concentration is varied.

Biosorbent	Metal concentration (mM)	% Biosorption	Specific metal uptake (mg Pb/g biomass)
M. luteus **pH 7**	0.005[(a)]	84.76	0.42
	0.5[(b)]	46.08	24.51
	1.25	46.10	59.21
P. castaneae **pH 7**	0.005[(a)]	81.39	0.4
	0.5[(b)]	29.40	15.63
	1.25	34.96	44.85
P. castaneae **pH 5**	0.005	21.73	0.10
	0.5	29.23	15.53
	1.25	34.92	43.92

(a) $p < 0.005$ (ANOVA) **(b)** Significant at $p < 0.05$

When comparing Pb uptake between *M. luteus* and *P. castaneae* at a pH of 7, the rates were significantly different at both 0.005 and 0.5 mM with $p = 0.002$ and $p = 0.005$, respectively (see Table A.7 in Appendix A). Once the initial metal concentration is too high, there is no significant difference in rate of uptake as saturation thresholds have been realised.

Unlike the decreasing trend in Pb biosorption under neutral conditions, *P. castaneae* showed a continued increase in Pb biosorption as the initial metal concentration increased at a pH of 5. The biosorption percentage increased from 21.73 (0.005 mM) to 34.92% (1.25 mM). Pb is more soluble at this pH which may result in a higher availability of metal ions. This could increase the driving force needed to overcome all mass transfer resistance of metal ions between the aqueous and solid phases resulting in higher probability of collisions between metal ions and sorbents (Tewari, Vasudevan and Guha, 2005) enabling more binding.

It is consistently challenging to compare these rates with existing literature since parameters such as the initial metal concentration, temperature and pH differ from study to study. Nonetheless it can be confidently stated that up to 46.08% metal can be taken up from 0.5 mM Pb using 2 g/L *M. luteus* at a pH of 7 and temperature of 25 ^{o}C while 29.40% can be taken up by *P. castaneae*. This is equivalent to 49.01 and 31.26 mg of Pb, respectively. Adding such a biosorption step after chemical precipitation of metals in the wastewater treatment process would make a significant reduction in Pb concentration of wastewaters likely down to acceptable limits.

4.5. Effect of competing cations

The complex nature of industrial wastewaters means that any commercial biosorbent would come into contact with more than one type of metal ion in varying concentrations. In addition to H^+ ions, other cations compete for the same non-specific binding sites (Puranik and Paknikar, 1999) that Pb ions are attracted to. The cations can either act synergistically to enhance the binding of Pb or antagonistically resulting in decreased biosorption of Pb in their presence. The cations Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} are reported in elevated concentrations in the AMD from which the bacteria were isolated (GDARD, 2016). Subsequently, they were included in the present study to determine their effect on Pb uptake by both biosorbents (Figure 7).

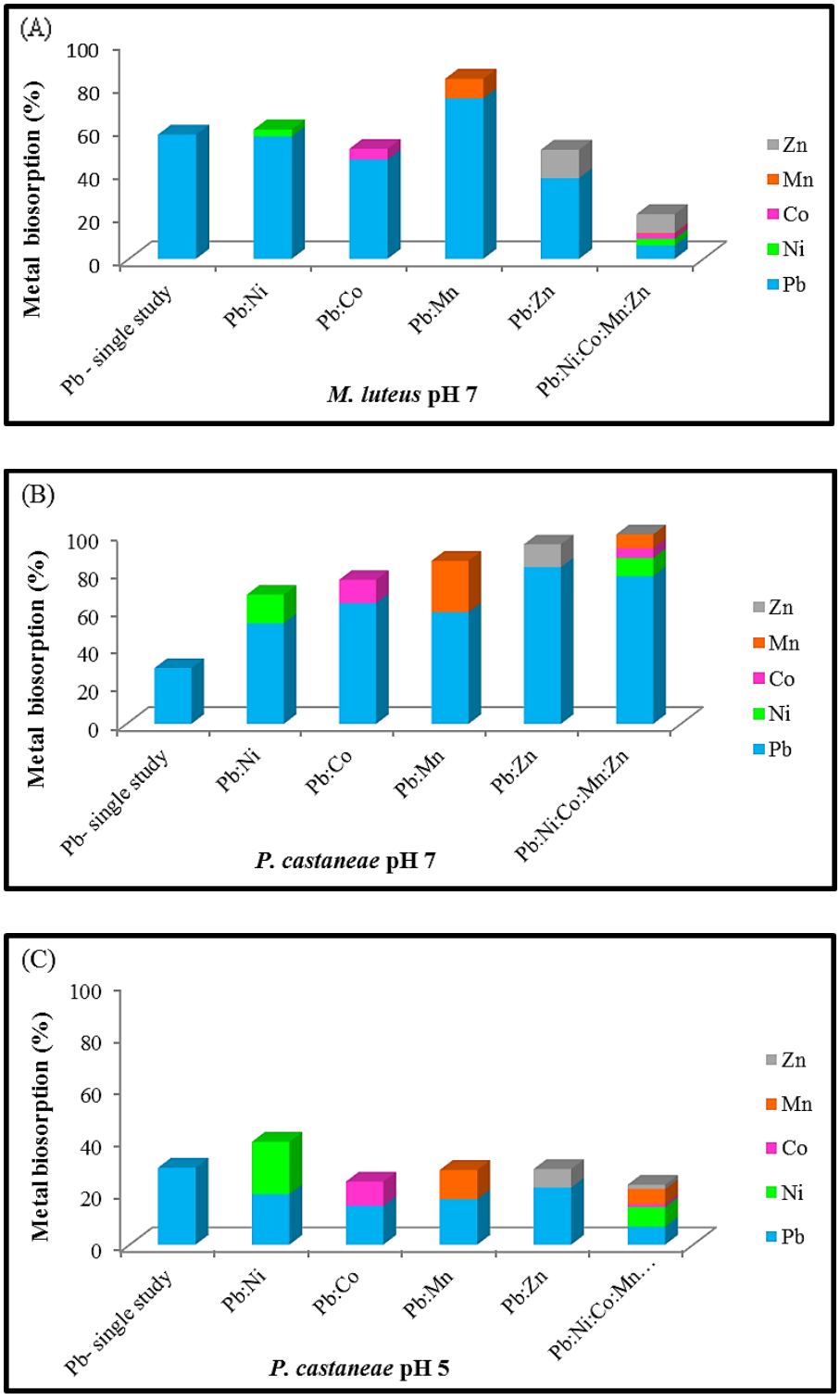


Figure 7. Effect of competing cations on the uptake of Pb by *M. luteus* and *P. castaneae* at a pH of 7 (A and B, respectively) and pH 5 (C). Individual metals were added at an initial concentration of 0.5 mM each and incubated with biomass at 25 °C for 24 h.

From Figure 7A it can be surmised that Pb uptake by *M. luteus* biomass is generally significantly decreased (Table A.8, Appendix A) when each of these ions is present either in binary or as a multimetal complex. Mn^{2+} was the only cation that enhanced the uptake of Pb by *M. luteus* biomass yielding 74.26% sorption. On the other hand, the biosorption of Pb by *P. castaneae* under similar conditions (pH 7, 25 ^{o}C, and 0.5 mM metal) significantly increased in the presence of the same cations both in binary and multi-mixes (Figure 7B). The highest % of Pb was taken up in the presence of Zn resulting in 82.62% biosorption.

Overall, the rate of uptake of Pb was always higher irrespective of whether a binary or multimetal complex was used (Table 7). In the presence of other cations, the uptake of metals is influenced by atomic weight and electronegativity as well as the ionic group to which they belong (Puranik and Paknikar, 1999). All five competing cations used in the present study belong to the same class of borderline ions while Pb ions are classified into the class b ions. The classes are based on the covalent index described by Nieboer and McBryde (1973) which is dependent on the electronegativity and crystal radius of cations. Generally, the higher the covalent index, the more potential to form covalent bonds with biological ligands (Puranik and Paknikar, 1999). Pb ions have the highest covalent index (Table B.1. in Appendix B) and are therefore more likely to bind to the biosorbent when compared to the other cations. Additionally, Pb has a higher atomic weight and the highest electronegativity amongst the cations used in the present study further supporting its preferential binding to the functional groups on the biosorbent. Hence, the trend in the rate of Pb uptake observed in this study.

According to the above reasoning, it is unexpected that Co^{2+}, Ni^{2+} and Zn^{2+} would compete with Pb for binding, yet this was observed for *M. luteus*. This could be explained by the difference in EPS and hence functional groups present between *P. castaneae* and *M. luteus*. It may be that the EPS of *M. luteus* carries more functional groups that are preferentially bound by the borderline class of ions as opposed to class b ions. Further research involving the extraction and determination of the composition of the EPS from both species would be

necessary to identify active binding sites. This can be achieved using Fourier transform infrared spectroscopy (FTIR).

Table 7. Specific rate of metal uptake by each biosorbent in binary and multimetal systems.

(A)	**Rate of metal uptake (mg/g) for *M. luteus* pH 7**				
Sample	**Pb**	**Ni**	**Co**	**Mn**	**Zn**
Pb and Ni	38.03	7.475	-	-	-
Pb and Co	30.46	-	7.15	-	-
Pb and Mn	49.96	-	-	12.625	-
Pb and Zn	25.26	-	-	-	6.85
Pb and Ni, Co, Mn, Zn	9.22	7.46	4.87	4.97	3.31

(B)	**Rate of metal uptake (mg/g) for *P. castaneae* pH 7**				
Sample	**Pb**	**Ni**	**Co**	**Mn**	**Zn**
Pb and Ni	40.04	4.85	-	-	-
Pb and Co	43.41	-	7.45	-	-
Pb and Mn	40.03	-	-	21.93	-
Pb and Zn	56.07	-	-	-	11.43
Pb and Ni, Co, Mn, Zn	52.88	0.13	1.31	7.79	4.88

(C)	**Rate of metal uptake (mg/g) for *P. castaneae* pH 5**				
Sample	**Pb**	**Ni**	**Co**	**Mn**	**Zn**
Pb and Ni	13.3	12.45	-	-	-
Pb and Co	10.22	-	8.2	-	-
Pb and Mn	11.98	-	-	7.65	-
Pb and Zn	15	-	-	-	7.2
Pb and Ni, Co, Mn, Zn	4.88	1.70	1.20	2.83	1.33

Another interesting observation is that the rates of uptake of Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} all decreased when placed in a multimetal system as compared to the binary system (Table 7). The only exception was for the uptake of Ni for *M. luteus* which remained similar irrespective of a binary or muitmetal system. This is in agreement with literature that states that cations belonging to the same class

undergo significant ionic competition (Paknikar, Pethkar and Puranik, 2003) for binding to the same sites on biosorbents. This in turn will lead to decreased binding of any particular ion as reported in the present study.

When the pH was decreased to 5, the uptake of Pb by *P. castaneae* biomass was reduced both in binary and multimetal systems (Figure 7 and Table 7). This indicates that solution pH also plays a role in sorption in the presence of competing ions. At pH 5, Pb is less soluble than Co, Ni and Mn; consequently there would be less Pb ions available for binding compared to the other ions. Similar findings have been reported in other biosorption studies (Zhou and Robert, 1991; Paknikar, Pethkar and Puranik, 2003).

The results from Table 7 indicate that at pH 7, *M. luteus* exhibited a preferential order of sorption $Pb^{2+}>Ni^{2+}>Mn^{2+}>Co^{2+}>Zn^{2+}$ while *P. castaneae* exhibited a preferential order $Pb^{2+}>>Mn^{2+}>Zn^{2+}>Co^{2+}>Ni^{2+}$. Interestingly, the biosorption of Pb (77.90%) by *P. castaneae* was highest in the presence of other cations while it was the lowest when using *M. luteus* as a biosorbent (6.58% Pb biosorption).

4.6. Primary conclusions

Under the conditions presented in this study (pH 7, 25 ^{o}C, 0.5 mM Pb), using 2 g/L of biosorbent resulted in the greatest uptake of 69 mg/L and 44 mg/L Pb by *M. luteus* and *P. castaneae* respectively. When competing cations are introduced to the Pb solution, the specific rate of metal uptake for Pb by *P. castaneae* was increased while the opposite effect was observed for *M. luteus.* However, both biosorbents prefer to take up Pb and as such would make good biosorbents. Additionally, even at lowered pH levels or increasingly high temperatures both isolates are able to biosorb Pb ions. These findings suggest that both biosorbents could find use in the secondary stage of wastewater treatment. This can be done at no additional cost to the existing process with the added benefit of lowering the metal concentrations especially Pb down to acceptable levels, Subsequently, the water decanted into the natural environment would be of a higher quality than what would have resulted from chemical removal of the metal salts.

4.7. Combined uptake of heavy metals by *M. luteus* and *P. castaneae*

The findings from the present study indicate that both *M. luteus* and *P. castaneae* can be used as biosorbents for Pb from wastewaters. In nature, bacteria usually take on the formation of biofilms to withstand harsh conditions such as heavy metal toxicity (Harrison, Turner and Ceri, 2005). Mixed populations of sulphate reducing bacteria have been shown to reduce and lower concentrations of not only sulphate but also Zn, Cu and Ni in a short-term bench scale upflow anaerobic packed bed reactor (Jong and Parry, 2003).

Bacterial consortiums also play a pivotal role in the cycling of heavy metals in the environment (Harrison, Turner and Ceri, 2005) showing between 2 – 600 fold tolerance to metals like Cu, Pb and Zn (Teitzel and Parsek, 2003). Therefore it would be of interest to determine if enhanced biosorption of Pb and the other cations tested in the present study would be possible if both biosorbents were present as a mixture.

A preliminary study to evaluate the uptake of Pb from a multimetal system using a combination of *M. luteus* and *P. castaneae* was conducted according to the method described in Chapter 2, subsection 3.7.1 pg 34. Briefly, heat-killed biomass of *P. castaneae* and *M. luteus* were added in a 1:1 and 2:1 ratio to a multimetal system containing 0.5 mM Pb, Co, Ni, Mn and Zn ions in equimolar concentrations. After 24 h the supernatant was analysed for residual ions by ICPOES.

The results (Figure 8) show that less Pb is biosorbed (32.50%) by a mixture of equal concentrations of both biosorbents as compared to when *P. castaneae* is used on its own (77.95%). This is about a 40% reduction in biosorption. However, as the ratio of *P. castaneae* to *M. luteus* biomass is doubled, the % biosorption of Pb increases to 80.31% suggesting that effective biosorption may only be contributed from *P. castaneae*.

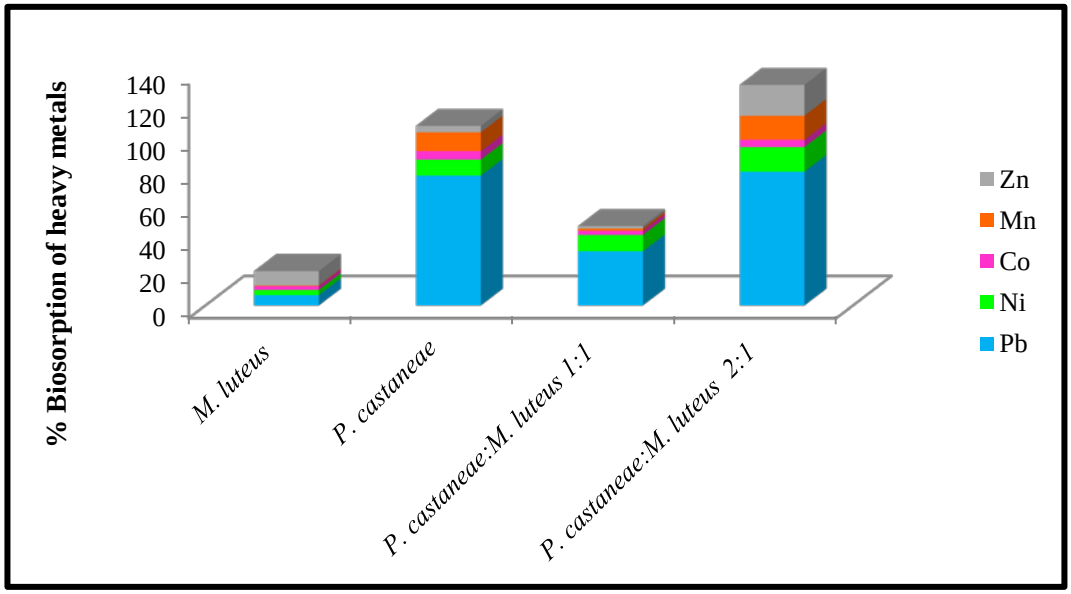


Figure 8: Graph showing the % metal biosorbed over 24 h when *M. luteus* and *P. castaneae* was added in a 1:1 and 2:1 ratio to 0.5 mM equal concentrations of Pb, Ni, Co, Mn and Zn (pH 7) at 25 °C.

These results further validate the decreased performance of *M. luteus* in the presence of competing cations when used on its own (6.58% biosorption). As more *P. castaneae* biomass is added to the mixture, the % biosorption increases. It may be necessary to test various combination ratios to conclusively decide if using a mixture or using *P. castaneae* biomass alone would result in more effective biosorption of Pb and the other metal cations.

Chapter 5

Conclusion and Recommendations

M. luteus and *P. castaneae* illustrated an optimal biosorption efficiency of up to 50% using 2 g/L of biomass when exposed to 0.5 mM of Pb ions at a pH of 7 at 25 ^{o}C. Although not the ideal conditions for Pb biosorption, both biosorbents were still able to take up metal at lowered pH levels and increasing temperatures. Furthermore, *P. castaneae* biomass performs better in the presence of other cations. This is an indication of their versatility for use in the treatment of complex industrial wastewaters under naturally fluctuating environmental conditions. The findings from this study support the further development of these isolates as commercial biosorbents for metal removal.

The benefits realised when integrated into existing wastewater treatment processes may include economic viability, biodegradability and efficient lowering of toxic heavy metal concentrations to acceptable levels for potable use. It is recommended to further pilot test the biosorbents in the treatment of actual effluent samples from various industries under the optimised parameters. It is also suggested to investigate various combinations of both biosorbents in wastewater treatment so as to achieve the highest uptake of heavy metals. The appropriate combination of the bacterial biomass could then be immobilised onto calcium alginate nanoparticles to improve the stability of the biosorbents and enhance metal uptake in a biodegradable and eco-friendly manner. Additionally, it would allow retrieval of the spent biosorbents for metal retrieval and recycling. In the long-term this system could be incorporated into a filtration module to treat small water volumes or included in the activated sludge process during wastewater treatment.

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Appendices

Appendix A

Tables showing the *p* values for each species within the parameters tested in the present study. A *p* value < 0.05 indicates a significant difference at a confidence interval of 95%.

Table A.1. Probabilities obtained for the pH study in individual species.

pH	*M. luteus* (*p* value)	*P. castaneae* (*p* value)
4 vs. 5	0.765	0.016
4 vs. 6	0.032	0.277
4 vs. 7	0.000	0.008
5 vs. 6	0.513	0.002
5 vs. 7	0.004	0.277
6 vs. 7	0.000	0.001

Table A.2. Probabilities obtained for the pH study between species.

pH	*p* value
4	0.341
5	0.041
6	0.052
7	0.008

Table A.3. Probabilities obtained for the temperature study in individual species.

Temperature (°C)	*M. luteus* (*p* value)	*P. castaneae* (*p* value)
4 vs. 25	0.019	0.305
4 vs. 37	0.001	0.027
4 vs. 55	0.742	0.001
25 vs. 37	0.163	0.008
25 vs. 55	0.075	0.001
37 vs. 55	0.014	0.004

Table A.4. Probabilities obtained for the temperature study between species.

Temperature (°C)	*p* value
4	0.031
25	0.225
37	0.129
55	0.002

Table A.5. Probabilities obtained for the biomass study in individual species.

Biomass concentration (g/L)	*M. luteus* (*p* value)	*P. castaneae* (*p* value)
1 vs. 2	0.06	0.041
1 vs. 3	0.059	0.002
1 vs. 4	0.0429	0.000
1 vs. 5	0.0477	0.001
2 vs. 5	0.025	0.313
3 vs. 4	0.971	0.865
3 vs. 5	0.038	0.000
4 vs. 5	0.291	0.000

Table A.6. Probabilities obtained for the biomass study between species.

Biomass (g/L)	*p* value
1	0.304
2	0.008
3	0.000
4	0.022
5	0.784

Table A.7. Probabilities obtained for the metal concentration study between species.

Metal concentration (mM)	*p* value
0.005	0.002
0.5	0.005
1.25	0.134

Table A.8. Probabilities obtained for the competing cation study with reference to Pb uptake by individual species in pure and mixed metal systems.

Competing cations	*M. luteus* (*p* value)	*P. castaneae* (*p* value)	
		pH 7	pH 5
Ni	0.888	0.387	0.001
Co	0.012	0.043	0.001
Mn	0.003	0.019	0.001
Zn	0.021	0.000	0.001
Multi-metal	0.004	0.000	0.000

Appendix B

Table B.1. Covalent index of competing cations

Element	Ionic crystal radius (r) [a]	Electronegativity (Xm) [b]	Cation index $(Xm)^2$ (r + 0.85) [c]
Pb	1.19	2.33	7.18
Ni	0.70	1.91	5.73
Co	0.70	1.88	5.61
Mn	0.70	1.55	3.96
Zn	0.74	1.65	4.54

(*a*) Ionic crystal values obtained from Barbalace (1995).

(*b*) Electronegativity, values obtained from Allred (1961).

(*c*) Cationic index values obtained from Nieboer and Mcbryde (1973).