# Mobility of platinum and gold in the environment and the roles of microbes

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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# Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

**Sahar Shar**

**5th September 2017**

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## List of abbrevations

| | |
|---|---|
| µg | Microgram |
| µg | Microgram |
| µL | Microlitre |
| µL | Microlitre |
| µM | Micromolar |
| µM | Micromolar |
| µm | Micron or micrometre |
| AAS | Atomic Absorption Spectrometry |
| Ag | Silver |
| AG | α-D-glucosidase |
| Al | Aluminum |
| As | Arsenic |
| Au | Gold |
| AuNPs | Gold nanoparticles |
| BG | β-D-glucosidase |
| BGR | BrnGrounds soil |
| BSE | Back-scattered electrons |
| C | Carbon |
| *C. metallidurans* | *Cupriavidus metallidurans* |
| Ca | Calcium |
| CB | β-D-cellobiohydrolase |
| Cd | Cadmium |
| CFU | Cell forming units |

| | |
|---|---|
| CLPP | Community level physiological profiling |
| Co | Cobalt |
| Cr | Chromium |
| Cu | Copper |
| DDI | Distilled, de-ionised water |
| DGGE | Denaturing gradient gel electrophoresis |
| dH2O | MilliQ water |
| DNA | Deoxyribonucleic acid |
| EC | Electrical conductivity |
| EDAX | Energy dispersive x-ray |
| EM | Electron microscope |
| ENPs | Engineered nanoparticles |
| eV | Electron volt |
| FAME | Fatty acid methyl ester analysis |
| Fe | Iron |
| FIB | Focused ion beam |
| FLN | FoxLane soil |
| FTIR | Fourier-transform infrared spectroscopy |
| g | Gram |
| HA | Humic acid |
| Hg | Mercury |
| HMDS | Hexamethyldisilizane |
| HNO3 | Nitric acid |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| Ir | Iridium |

| | |
|---|---|
| JBR | Jamberoo soil |
| K2PtCl4 | Potassium tetrachloroplatinate (II) |
| keV | Kilo-electron volt |
| LMWOAs | Low- and high-molecular weight organic acids |
| Mg | Magnesium |
| mg | Milligram |
| min | Minutes |
| mL | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| Mn | Manganese |
| MNP | Minnipa soil |
| Mo | Molybdenum |
| N | Nitrogen |
| Na | Sodium |
| NAG | N-acetyl glucosaminidase |
| ng | Nano-gram |
| NGS | Next Generation Sequencing |
| Ni | Nickel |
| nm | Nanometre |
| NP | Nanoparticles |
| $^{o}$C | Degrees Celsius |
| OD | Optical density |
| OM | Optical microscopy |
| Os | Osmium |

| | |
|---|---|
| OTU | Operational taxonomic unit |
| Pb | Lead |
| PBS | Phosphate buffer saline |
| PCA | Principal component analysis |
| PCR | Polymerase-chain reaction |
| Pd | Palladium |
| PGE | Platinum group elements |
| PGM | Platinum group metals |
| PHOS | Phosphatase |
| PM | Particle matter |
| PPN | Pinpinio soil |
| Pt | Platinum |
| PtCl4 | Platinum (IV) chloride |
| PtNPs | Platinum nanoparticles |
| PVP | Polyvinylpyrrolidone |
| RTA | Real Time Analysis |
| Ru | Ruthenium |
| RYLS | Arylsulphatase |
| Sb | Antimony |
| SEM | Secondary electron microscopy |
| TC | Total carbon |
| Te | Tellurium |
| TEM | Transmission Electron Microscopy |
| TiO2 | Titanium oxide |
| TMM | Tris Minimal Media |

| | |
|---|---|
| TRFLP | Terminal restriction fragment length polymorphism |
| w/v | Weight/volume |
| wt .% | Weight precent |
| XYL | β-D-xylosidase |
| Zn | Zinc |

## Abstract

Platinum (Pt) and gold (Au) are precious heavy metals that are widely used in the jewellery, automobile, chemical and medical industries. This increased use has resulted in their introduction into the environment at elevated concentrations which can adversely affect both ecosystem and human health. Currently, there is limited information available on the behaviour of Pt and Au in terrestrial environments and their impact on soil microorganisms. There is therefore a need to assess the environmental impacts of increased amounts of Pt and Au contamination. The aims of this study are to study the fate, transport and microbial interactions associated with Pt and Au in Australian soils.

The first experimental investigation of this thesis was carried out using a bacterium *Cupriavidus metallidurans (C. metallidurans),* known to significantly biomineralize Au but with limited information available on its interactions with Pt. Therefore, the aim of this research was to assess the ability of *C. metallidurans* to biomineralize Pt. The results showed that both *C. metallidurans* and a control microorganism *Escherichia coli (E. coli)* tolerated platinum (IV) chloride ($PtCl_4$). However, *C. metallidurans* had a higher tolerance to Pt, with the number of cells surviving at high $PtCl_4$ concentration (10,000 µM) 300-fold more than that of *E. coli*. Both isolates formed Pt nanoparticles but *C. metallidurans* showed a higher Pt retention (87%) than *E. coli* (74%) in sand columns. *C. metallidurans* was tolerant of Pt and effective at its biomineralization, confirming its suitability for use for further studies on Pt and Au environmental behaviour.

The interaction of Pt and Au with the biotic and abiotic components of Australian soils is largely unstudied, although metal transformation may lead to the nanoparticles formation. Importantly, little is known about the transportation of Pt and Au nanoparticles in soil. The second scientific investigation evaluated the movement of Pt and Au nanoparticles

(AuNPs and PtNPs) in columns containing different matrices (sand only, sand-clay, sand-humic acids and sand-FeO) and soil types, including an organic rich soil, FoxLane (FLN) and an iron rich soil, Jamberoo (JBR). Nanoparticle aggregations with soil particles were observed and Transmission Electron Microscopy (TEM) showed that the nanoparticles formed both homo- and hetero-aggregates in columns. Gold NPs were more mobile (as shown by reduced retention in column) and more reactive (as determined by Fourier-transform infrared spectroscopy (FTIR) analysis) than PtNPs. The rate of movement of nanoparticles based on the breakthrough curves through the various matrices was, in decreasing order of mobility; sand (2 hours) > JBR soil (4 hours) > FLN soil (6 hours) > sand-clay (8-10 hours) >sand-FeO (8-12 hours) > sand-humic acids (12 hours).

The aim of the third experimental investigation was to assess the effects of different Pt and Au concentrations (1, 25, 100, 500 and 2,000 mg $kg^{-1}$) on soil respiration and activities of seven key soil enzymes (N-acetyl glucosaminidase, phosphatase, β-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, α-D-glucosidase and arylsulphatase) in a range of Australian soils. The findings from this study showed that the effects of Au and Pt application on the soil microbial activity were related to soil types; (1) a high pH (alkaline) soil (Minnipa, MNP), (2) a low pH (acidic) soil (BrnGrounds, BGR), (3) an iron rich soil (Jamberoo, JBR), (4) an organic matter rich soil (FxLane, FLN), and (5) a high metal/silt soil (Pinpinio, PPN). Platinum or Au at most of the concentrations evaluated (1-2,000 mg $kg^{-1}$), generally resulted in significant reductions in soil respiration rates in BGR, JBR, MNP and PPN soils. Some reduction in soil respiration was also observed in FLN soils when Pt was applied, while the application of Au increased soil respiration in FLN soils at all concentrations (except 2,000 mg $kg^{-1}$). The application of Pt inhibited the activities of most of or all the enzymes tested in BRG, FLN, JBR and PPN soils while enhancing the activities of most of the enzymes in MNP soils (1-100 mg $kg^{-1}$). In contrast, the addition of Au resulted in a significant reduction in most

enzyme activities in BGR, JBR and MNP soils while enhancing enzyme activities in FLN and PPN soils. The explanation for these differential results may be a combination of specific enzyme sensitivity, metal concentration and soil type.

The aim of the final results chapter was to evaluate changes in the bacterial community of four soils (BGR, FLN, PPN and MNP) at three Au and Pt concentrations (1, 25 and 100 mg $kg^{-1}$) using Next Generation Sequencing tools (NGS). The soil bacterial community was affected by both soil type and metal concentration. While no shift in the dominant groups (Class level) was observed in FLN and MNP soils following addition of Pt and Au with *Proteobacteria* and *Actinobacteria* being dominant, other bacterial Phyla/Classes such as Kazan-3B-28, *Firmicutes* and *Caldithrix* were selected in BGR and PPN soils. Amendments with Au significantly reduced bacterial community diversity in organic rich BRG soil while amendments with Pt substantially reduced bacterial diversity in the three other soil types. At the Family level, shifts in bacterial community structure were observed in all Pt- and Au-amended samples which were positively correlated with increasing metal concentrations. Bacteria that were selectively enriched only in Pt-amended samples (irrespective of soil type) were identified as belonging to the groups *Burkholderiales*, *Burkholderiaceae*, *Alicyclobacillaceae*, *Rubrobacteraceae*, *Cytophagaceae* and *Oxalobacteraceae*. In Au-amended samples, the bacterial groups that increased in Pt amended samples belonged to *Sphingomonadaceae* and *Rhodospirillaceae*. Generally, Au and Pt toxicity was concentration related with Au more toxic to soil bacterial communities at a lower concentration (25 mg $kg^{-1}$).

One key aim of this study was to assess the transportation of Pt complexes, Au- and Pt-NPs in different environmental media (soils). Overall, this research has demonstrated that Pt and Au have different rates of transportation in soils which affected their interactions with

soil microorganisms and components. This research's outcomes have important implications in the management (remediation) of Pt and Au-gold contaminated environments. Future research should include the application of NGS to assess Pt and Au effects on microbial function and assays to isolate metal resistant microorganisms for use in bioremediation.

# Chapter 1 : Literature Review

## 1.1 Introduction

The term “heavy metals” in the last three decades has been widely used in scientific literature and linked with environmental contamination and associated toxicity or ecotoxicity (Duffus, 2003). Toxic metallic elements with relatively high densities, such as cadmium, thallium, lead, copper, aluminium, arsenic, chromium and mercury are referred to as heavy metals (Sherameti and Varma, 2010). These heavy metals can be at least five times as dense as water, whereas light metals (for example: sodium, magnesium, and potassium) have densities lower than the density of water (Sherameti and Varma, 2010). They are released into different environments such as water, sediment and soil, naturally and via anthropogenic sources (Franco-Hernandez et al., 2010, Gonzalez et al., 2007, Grimalt et al., 1999, Abdul-Wahab and Marikar, 2012, Kim et al., 2001). For example, heavy metals in mine tailings can be transported to and dispersed into the environment by wind and water (Kim et al., 2001, Abdul-Wahab and Marikar, 2012).

Heavy metals occur naturally, although rarely at toxic levels. Most highly contaminated soils are often associated with old landfill sites (particularly those accepting industrial wastes), fields and lagoons previously containing wastewater or municipal sludge (Sherameti and Varma, 2010). They are non-biodegradable and can accumulate in living organisms, adversely affecting both human and ecosystem health (Lesmana et al., 2009, Boechat et al., 2016). Most heavy metals are toxic to microorganisms with this toxicity modulated by metal speciation, soil factors and the composition of the microbial community. The presence of heavy metals can reduce the number of microbial cells, decrease their metabolic activities and enzyme functions (respiratory activity of the microbial community)

(Qing et al., 2007, Dick et al., 2000, Trasar-Cepeda et al., 2000, Wyszkowska et al., 2007, Wyszkowska et al., 2013).

While exposure to heavy metals is lethal to some members of the microbial community, other microbial groups can tolerate or develop resistance against these toxic metals (Issazadeh et al., 2013). Some bacteria tolerate heavy metals by transforming them to less toxic forms (Dell'Amico et al., 2005, Qing et al., 2007),while tolerance in other bacterial groups are linked to specific metabolic functions. Such functions include the existence of specific transportation (influx and efflux) systems for metals and synthesis and excretion of metal chelating compounds into the environment (Wyszkowska et al., 2013, Ulberg, 1997, Binet et al., 2003). Other mechanisms include metal accumulation through sorption (binding) onto the cell wall and membrane complex (Ledin, 2000, Ulberg, 1997), and the presence (expression) of metal resistance genes on plasmids (Zhang et al., 2001, Meguro et al., 2005, Lakzian et al., 2002).

### 1.1.1 Platinum Group Metals:

The platinum group of elements (PGE) are composed of heavy metals such as platinum (Pt), iridium (Ir), osmium (Os), palladium (Pd), rhodium and ruthenium (Ru) (Figure 1.1 ; (Uysal et al., 2009)). They are rare, inert elements and share similar chemical properties. However, some of these metals can become reactive and solubilised under specific conditions such as high temperatures and pressures and in environments with high corrosion and oxidation potentials (Kalavrouziotis and Koukoulakis, 2009, Glaister and Mudd, 2010). Out of the six elements, only Pt and Pd are found in pure states naturally, the other members are found as alloys with Pt and gold (Au) (Hartley, 2013).One common theme for all of these metals is their association with Au, often in small amounts (Prior et al., 2012). In the environment, PGMs can be found in soil, dust, surface water, sediments and plants (Sobrova et al., 2012).

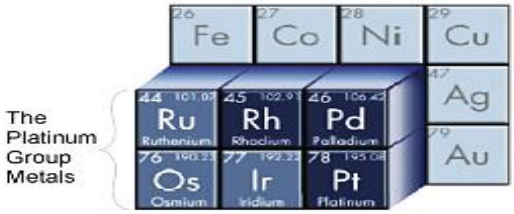


**Figure 1-1 :** Platium group metals. (from http://ambayagold.com/enhancer/tag/heavy-platinum-group/)

### 1.1.2 Distribution of PGM ores:

There are different types of PGM ores from which metal extraction can be carried out. Examples of these ores (suitable for potential commercial exploitation) include (i) Stratiform deposits - PGMs occur in large Precambrian mafic to ultramafic layered intrusions and (ii) Norite intrusions - PGM emplacement primarily due to meteoritic impact. Others include (iii)- The Ni–Cu bearing sills - related to rift structures, allowing concordant intrusive sheets and (iv)- Placer deposits - alluvial sedimentary deposits containing coarse PGMs (commonly Pt) mined along with alluvial Au (Gadd, 2010, Glaister and Mudd, 2010).

Despite an abundance of potential host rock in Australia and other countries in the world, only a few economic natural Pt deposits are known. In Australia, PGE production (Pt and Pd) in 2011 was very minor by world standards, amounting to 441 kg. This production was exclusively from nickel sulphide deposits found in Archean komatiitic rocks in the Yilgarn Craton of WA. A list of identified Resources of PGMs in Australia is presented in Table 1.1 (Sobrova et al., 2012).

**Table 1-1:** Australia's Platinum Resources (Sobrova et al., 2012).

| **Deposits** | **Pt g/t** |
|---|---|
| Munni Munni (WA) | 0.10 |

| | |
|---|---|
| Panton (WA) | 2.39 |
| Fifield (NSW) | 0.70 |
| Rosie (WA) | 0.80 |
| Nyngan lateritic nickel-cobalt-scandium-platinum deposit (NSW) | 0.22 |
| Syerston lateritic nickel-cobalt-platinum deposit (NSW) | 0.12 |
| Coronation Hill (NT) | 0.30 |
| Adamsfield(Tasmania) | 0.13 |
| Thomson River (Victoria) | 2.00 |

## 1.2 Gold and Platinum

Platinum and Au are metals of commercial value because of their widespread applications in the jewellery, medical and automobile industries. Previous studies have shown that the presence of Pt and Au in the environment has a significant impact on biotic and abiotic ecosystem components. This is particularly important in soil surfaces where they are preferentially deposited in their ionic forms, Au and Pt complexes (Rene et al., 2017, Akpor and Muchie, 2010). Platinum and Au complexes have substantial similarities in terms of their chemical composition and behaviour in the environment, although Au environmental mobility is higher than that of Pt; (Brugger et al., 2013), therefore Au and Pt are reviewed together in this study.

Several factors such as thermodynamics, solubility, microbial structure and activities influence the mobility of the complex forms of Au and Pt in the environment (Reith et al., 2009, Reith et al., 2014, Brugger et al., 2013). These are discussed in greater details later in

this chapter. However, Au is generally believed to be more mobile and reactive than Pt in the same state especially after microbial interactions (Brugger et al., 2013, Reith et al., 2014). The surface concentrations in many Pt-bearing soils are probably the result of physical enrichment (Reith et al., 2014, Cook and Fletcher, 1994, Fletcher et al., 1995, Sures et al., 2005), although changes in the mineral speciation of Pt can be associated with residual accumulation (Gray et al., 1996, Suarez et al., 2010, Brugger et al., 2013) . The maturation of Pt minerals in placer deposits (Cabri et al., 1996, Cousins and Kinloch, 1976, Brugger et al., 2013) further suggests the different behaviours of Pt and Au during weathering. In surface environments, the mobility of Au is associated with processes that do not necessarily apply to Pt (Brugger et al., 2013). Due to their widespread use as precious metals, Au and Pt are released in relatively high concentrations into the environment from anthropogenic sources (Kabata-Pendias, 2010, Zereini et al., 2004, Zereini et al., 2007). Their occurrence, speciation, uses and adverse impacts on the environment are explained in the following sections.

### 1.2.1 Occurrence and deposits of Au and Pt

#### 1.2.1.1 Gold

Gold, with an average concentration of 5 ng $g^{-1}$ (solid material) in the Earth's crust, and a concentration ranging from 19.5 to 197 ng $g^{-1}$ in natural waters, is among the 10 rarest elements in the world (Mchugh, 1988, Frimmel, 2008, Shuster, 2013, Nordberg et al., 2014). It is inert and a non-essential element but its free ions are unstable in aqueous solutions under surface conditions. As a complex, Au can be toxic to organisms (Boyles, 1979, Witkiewicz and Shaw, 1981, Karthikeyan and Beveridge, 2002, Checa and Soncini, 2011).

Gold is usually found in very low concentrations in different parts of the world in its native form as a metal. It generally forms alloys with Ag and can contain traces of Cu. The

tellurides, typically petzite ($(AuAg)_2Te$), calaverite ($AuTe_2$), sylvanite ($(AuAg)Te_2$) are some of the Au compounds naturally found in the environment. However in lode and alluvial deposits, Au is found in its native form. For example, in South Africa, Au is found as veins and stringers in a quartz matrix with pyrite and quartz sand in the largest Au reefs. Pyrite and pyrrhotite minerals also contain Au which is recovered during the extraction of Cu, Ag, Pb, Zn and Ni. Gold is also present in sea water (although in low concentrations) with 70 million tonnes of Au estimated to be present in oceans (Refİker, 2005).

#### 1.2.1.2 Platinum

Platinum group metals (PGMs) usually occur in a natural form associated with one or more of the other PGMs along with Au, Fe, Cu and Cr and generally in extremely low concentrations within the Earth's crust (Mcdonough and Sun, 1995, Wedepohl, 1995, Haus et al., 2007). They are found in sediments in higher anomalous concentrations due to several sources (e.g. volcanic and hydrothermal sources), and processes (e.g., continental weathering, eustatic fluctuations, biological processes, precipitation from seawater and redox-controlled enrichment) (Sawlowicz, 1993, Kramer et al., 2001). Platinum group metals also occur in placers originating from sulphide minerals. The host rock for PGMs is composed of basic or ultrabasic igneous rocks including peridotite, pyroxenites and dunites. In sedimentary rocks, PGM is usually present in complexes with quartz, Cu, Ni, Ag, etc., while in alluvial deposits they are associated with chromite, magnetite and ilmenite (Rao and Reddi, 2000). Platinum is present in Earth’s crust as concentrated primary deposits in the form of magmatic reef-type mineralisation in layered mafic and ultramafic intrusions (Koek et al., 2010).

The major deposits of PGMs are distributed around the world, with the most important mines being the Bushveld igneous complex (South Africa), the Ni–Cu–PGMs sulphide deposits of Nroil'sk in the Russian Arctic and placer deposits in the Ural Mountains (Russia),

Sudbury (Ontario, Canada), the Hartley mine (Zimbabwe), the Stillwater complex (Montana, USA), Northern Territory (Australia) and the Zechstein copper deposit in Poland. South Africa is the biggest producer of PGMs, contributing up to 85% of the total world production (Rao and Reddi, 2000, Glaister and Mudd, 2010). A more detailed picture can be obtained from Table 1.2 of the distribution of the production of the PGMs'.

**Table 1-2:** PGM production in tonnes (t) and resources in 2007 by country (Survey et al., 2009, Glaister and Mudd, 2010).

| **Country** | **Production** | | | **Reserves[b]** | **Reserve base[b]** |
|---|---|---|---|---|---|
| | **t Pt** | **t Pd** | **t PGM** | **t PGM** | **t PGM** |
| South Africa | 165.83 | 86.46 | 310.92 | 63,000 | 70,000 |
| Russia | 27.00 | 96.80 | 138.30 | 6200 | 6600 |
| Canada | 6.20 | 10.50 | 20.20 | 310 | 390 |
| Zimbabwe | 5.30 | 4.20 | 11.00 | – | – |
| United States | 3.86 | 12.80 | – | 900 | 2000 |
| Columbia | 1.40 | – | – | – | – |
| Australia | ~0.90[a] | ~0.73[a] | – | – | – |
| World | 212 | 219 | 509 | 71,000 | 80,000 |

a. Assuming Australia is credited for PGMs extracted from ores and concentrates exported to Japan.b. See USGS (2009) for detailed definitions, but they are broadly similar to reserves and resources as used in Australia, Canada, South Africa and elsewhere.

### 1.2.2 Applications of Au and Pt

#### 1.2.2.1 Gold

Gold has been a very popular precious natural material since ancient times. It is also generally used as a hedge investment and its physical properties such as high conductivity, ductility and high resistance to corrosion have led to continuous socio-economic and cultural demands for this precious metal (Frimmel, 2008, Shuster, 2013). Gold is also widely used in medical fields such as dentistry, dermatology and pharmacology(Ayesh et al., 1987, Evron et al., 1995, Bruze et al., 1994, Cederbrant et al., 1997, Svedman et al., 2005, Svedman et al., 2009, Sun et al., 2013, Nordberg et al., 2014).

#### 1.2.2.2 Platinum

Platinum also has significant industrial and commercial importance owing to its unique chemical and physical properties. It is widely used in many major industries including electronics, chemical, jewellery and glass manufacturing. Platinum is used in medicine and dentistry as alloys (Bencs et al., 2003) (Ravindra et al., 2004) as well as in certain cancer treatment drugs (Rao and Reddi, 2000). Platinum has an important role in the automobile industry as industrial catalysts due to its resistance to chemical corrosion over a wide range of temperatures and a high melting point (1772°C) (Bencs et al., 2003, Rajapaksha et al., 2004). The various Pt demands by use are shown in Figure 1.2. It can be observed that the demand for platinum decreased from 2007 to 2009 which was due to the global financial crisis but has since started recovering from its slump in demand (Sobrova et al., 2012, Prior et al., 2012).

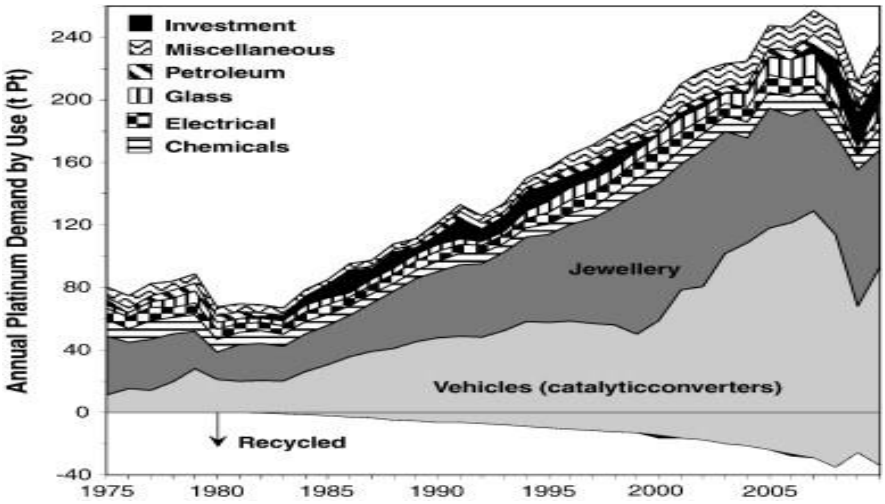


**Figure 1-2:** Global platinum demand for various applications (Prior et al., 2012, Sobrova et al., 2012).

### 1.2.3 Release of Au and Pt into the environment and their impacts

In recent years, Au and Pt have become more widely used in different fields which has led to accidental discharges through pollution events or "unintentional releases" as nanoparticles. Thus, the increasing use of these metals is one of the major contributors to the emission of nanoparticles and the pollution caused by Au and Pt (Fairbrother et al., 2013, Brugger et al., 2013, Reith et al., 2014, Reith et al., 2016).

#### 1.2.3.1 Gold

Gold mining is a major cause of heavy metal contamination in soils in different countries such as US (Straskraba and Moran, 1990), Korea (Lee et al., 2005), Sultanate of Oman (Abdul-Wahab and Marikar, 2012), Canada (Percival et al., 2014), South Africa (Gzik et al., 2003) and Ghana (Armah et al., 2014, Ngole-Jeme and Fantke, 2017, Fashola et al., 2016). The main source of metals and metalloids from gold mines are the processing of ores and disposal of mine tailings and heavy metal-rich wastewater (Donkor et al., 2005, Grimalt et al., 1999, Ngole-Jeme and Fantke, 2017). Gold is soluble in oxidized states as chloride complexes (Gammons and Williamsjones, 1995) under acidic conditions or as bisulfide

complexes under near neutral reducing conditions (Seward, 1973, Shenberger and Barnes, 1989, Benning and Seward, 1995, Mikucki, 1998).

Gold is transported as a bisulfide complex in low temperature and mesothermal hydrothermal systems. The deposition of Au is affected by the destabilization of bisulfide complexes caused by many processes and factors, including reduction, oxidation, changes in pH and temperature (Arehart, 1996). Aurous-hydrosulfide complexes (AuHSO) are dominant over a wide range of temperatures and pressures. They are thought to be largely responsible for the transportation and deposition of Au in the environment (Benning and Seward, 1995). The toxicity of Au to microorganisms (mainly bacteria) is strongly exhibited in Au nanoparticles (Zhou et al., 2012, Lima et al., 2013). However, Au mining can lead to the contamination of the environment due to the mobilization of other toxic heavy metals such as Cd, Cu, Cr and Zn which are sometimes associated with Au ore (Abdul-Wahab and Marikar, 2012). These heavy metals pose significantly higher health risk to humans than the Au being mined.

#### 1.2.3.2 Platinum

In terms of environmental contamination, the release of Pt from catalytic converters in automobiles is well documented (Ek et al., 2004). The emissions of Pt metals into the environment, enriched by anthropogenic industrial activities is shown in Figure 1.3 (Sobrova et al., 2012). An estimate of annual Pt emission from automobile catalytic converters is as high as 0.5-1.4 tonnes per year (Barbante et al., 2001, Knutsson, 2006). The edge of the motorway has the highest Pt soil concentrations which decrease with increasing distance from the highway (Kabata-Pendias and Mukherjee, 2007). Less than 10% of the total amount of Pt is solubilized in exhaust fumes of gasoline and diesel engines (Ek et al., 2004).

The continued increase of Pt emissions and other PGMs in certain forms have led to adverse health effects (Ek et al., 2004). Metallic Pt is biologically inert and must be in a soluble form to pose health risks (Ojeda et al., 2006). Pt in road dust from automobile exhausts gets solubilised and enters the aquatic system, soil and food chain. Also, consumption of food or drinks that contain Pt can be toxic to the gastrointestinal tract (Puls et al., 2012). A detailed list of diseases attributed to the toxic effects of Pt to humans is given in Table 1.3. Some of these Pt compounds are known to be cytotoxic and display mutagenic and carcinogenic effects (Gebel et al., 1997). Moreover, some Pt complexes can bind to N and S in proteins causing a reduction in the activity of enzymes such as glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and dihydrofolate reductase (Aull et al., 1979, Gebel et al., 1997). Platinum Group Metals pose a greater health risks due increased mobility and the ability of some the mobilized complexes to induce cellular damage in lungs, and cause a variety of ill-health in humans (Wiseman and Zereini, 2009). Thus, it is critical to understand the biogeochemical transformation pathways of highly reactive Pt to develop strategies for potential metal recovery and remediation to reduce human health risks.

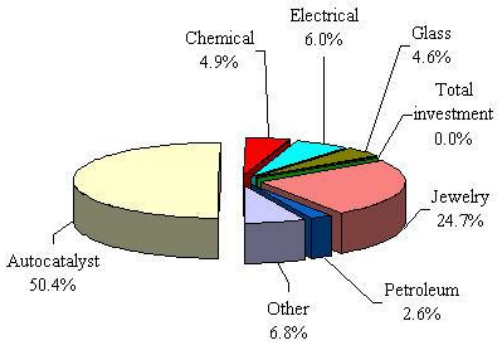


**Figure 1-3:** The main emission source of platinum metals in Europe in 2006 (from Sobrova et al., 2012).

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**Table 1-3:** Impact on platinum exposure to humans

| **Diseases** | **Effects** |
|---|---|
| Skin | Dermatitis, urticarial, skin sensitisation (Wiseman and Zereini, 2009). |
| Respiratory disorders | PGMs have also been found to be related to asthma. Asthma and dermatitis are caused by exposing workers to halogenated Pt salts such as ammonium tetrachloroplatinite and ammonium hexachloroplatinate (IV) used in the production of industrial catalysts (Ravindra et al., 2004). Rhinoconjunctivitis (Wiseman and Zereini, 2009). Allergic rhinitis (Wiseman and Zereini, 2009). |
| Digestion disorders | Nausea, increased spontaneous abortion (Kalam et al., 2009) |
| Optical allergy | Rhinoconjunctivities. Contact urticaria (Kalavrouziotis and Koukoulakis, 2009). |
| Others | DNA damage. Shrinkage of the glomeruli in the kidney. Induces the development of eosinophil inclusion bodies in the adrenal glands (Sobrova et al., 2012). |

## 1.3 Platinum occurrence on surface environments:

### 1.3.1 Platinum deposits

Platinum placer deposits are an important source of Pt and include alluvial, eluvial and Alaskan derived type deposits (Xiao and Laplante, 2004). Alluvial placers form the major proportion of placer Pt (Mertie, 1969). Economic PGE placer deposits occur in combination

with mafic-ultramafic Alaskan-, Uralian-, and Alpine-type intrusive complexes (Slansky et al., 1991). In the placers, Pt mainly occurs as Pt-metal alloys with 90 % of the alloys occurring as irregular shaped grains of Pt-Fe, in particular isoferroplatinum, and Os-Ir-Ru-Pt alloys (Tolstykh et al., 2002, Nixon et al., 1990).

## 1.4 Nanoparticles (NPs)

Nanoparticles (NPs) are 1-100 nm in size or at least one dimension less than 100 nm (Nel et al., 2006), which are widely used as catalysts, fillers, semiconductors in cosmetic and as drug carriers and in microelectronics. They are formed through the natural breakdown of metals or through human industrial activities. As previously stated, the increased use of Au and Pt has led to their release into the environment in nanoparticulate forms (Sperling et al., 2008, Shah et al., 2014, Saha et al., 2012). While anthropogenic activities (e.g. smoking, motor vehicle exhausts, industrial stack emissions) generate NPs, they can also be produced via natural sources such as volcanic dusts, natural bushfire products in air, colloids, aquatic systems and soils (Batley and McLaughlin, 2010, Shi et al., 2001).

The adverse effects of NPs (fine and ultrafine particles) on human health are well studied and there are legitimate concerns about the finer particles that can reach the deeper recesses of the lungs (Batley and McLaughlin, 2010, Ostiguy et al., 2006). Airborne particulate matter (PM) of Pt can affect the respiratory system depending on the size of particles. A range of particles of size 2.5-10 μm can be taken up by the pulmonary alveoli, tracheal and bronchial tract resulting in damage to the respiratory system during long term exposure. In addition to this exposure pathway, NPs can be ingested through the food chain; the intake of NPs in humans is therefore largely dependent on NP uptake by plants. For terrestrial and aquatic environments, there has been extensive research on natural colloids (Laabs et al., 2004, Buffle

and Leppard, 1995, Akkanen et al., 2012). However, one of the main drawbacks of current terrestrial ecotoxicological investigations of NPs is the lack of information on the transformations of the materials after addition to the test medium. Nanoparticles are designed to have radically different properties than macroscopic or bulk material. Given that the chemical and physical properties of the contaminant added to soil will have a large influence on its fate and effects in the terrestrial environment, the effects of NPs may be different from that of the bulk material (Korcak and Fanning, 1985, Voegelin et al., 2005, Klaine et al., 2008). While the dissolution equilibria and kinetics for NPs are closely related to the particle size, relationships at the nano-scale are not fully defined and are dependent on several factors (Magistad, 1925, Klaine et al., 2008). These factors include solute concentration, surface area, surface morphology, surface energy and charge, aggregation and adsorbing species (Magistad, 1925, Klaine et al., 2008).

Nanoparticles are found in water and soil and are usually treated as classical colloidal materials in investigations of NP in aquatic systems. In groundwater systems, NPs stabilization (suspension) in pore water may substantially reduce the retention rate of NPs in the aquifer and improve their removal from this environment (Li, 2015, Liu et al., 2012b). However, other factors such as the surface chemistry of the NPs, the nature of the porous media, the chemical properties of the pore water and environmental factors also affect the transportation of NPs (Chan, 2011). The transportation of NPs is also impacted by the properties of nanomaterials, solution ionic strength and pH as evidenced by studies on the transport of NPs such as carbonaceous nanomaterials, anatase ($TiO_2$) and zero-valent iron (ZVI) in porous media (Lecoanet et al., 2004, Fang et al., 2009, Petosa et al., 2010, Fang et al., 2011, Dunphy Guzman et al., 2006, Nowack and Bucheli, 2007, Keefe and Rome, 2007). The current literature suggests that aggregation and deposition mechanisms are primary factors that significantly limit the transport of ENPs (Jaisi and Elimelech, 2009, Solovitch et al., 2010).The introduction

of any NP into the environment is of scientific concern. Due to the increasing use of nano-products, the disposal of NP waste deserves more attention, especially in terms of the impact on the environment. Traditional waste treatment methods need to be reviewed and modified with regards to the detoxification of nanoscale materials.

Soil is described as being a “sink” and/or “source” of metallic and organic contaminants. Recent studies on the environmental risk of enhanced migration rates of contaminants have focused on the transport of contaminants by NPs in regular porous media (D'Williams, 2014, Jerez and Flury, 2006, El Badawy, 2011). However, such regular media are not representative of the complex soil systems and consequently, the understanding of the facilitated transport of contaminants by NPs in soils remains poor. The mobility and transport of heavy metals (and their nanoparticles) through soils may rely on dissolved organic matter (Weng et al., 2002, Hoffmann et al., 1998). Soil properties largely dictate the transportation of colloid-induced metal (Karathanasis, 1999). Electrostatic forces, porosity of media and the presence of organic matter are all known to limit the transport of Au NPs (D'Williams, 2014).

### 1.4.1 Gold and platinum nanoparticles- mobility and transport.

Gold and Pt NPs exist in various sizes and shapes and spherical particles of 5-100 nm diameter have been seen in microbial biofilms on Au or Pt grains from Australia, New Zealand and South America (Reith et al., 2012, Reith et al., 2016, Shuster et al., 2017). The mobility of NPs is affected by environmental, soil and microbial factors. For example, Au is concentrated in saprolite and ferruginous zones in humid regions and can be mobilised and precipitated with carbonates in more arid regions (Hough et al., 2011). Plant cells can play a role in the precipitation of Au NPs (Rico et al., 2011) (Zhai et al., 2014) while bacteria can also influence the morphology (and mobility) of Au NPs (Hough et al., 2011).

Bioprecipitation studies have shown that bacteria can mobilize and precipitate Au and Pt (and associated nanoparticles) and hence affect the transportation and formation or deposition of supergene deposits (Southam and Saunders, 2005, Lengke and Southam, 2006, Mossman et al., 1999, Konishi et al., 2007). Gold and Pt nanoparticles will interact with microbial groups in the deposits which can result in the microbial-mediated aggregation of nanoparticles outside the cells. Gold and Pt nanoparticles have also been found inside the cells of bacterium (Reith et al., 2009, Konishi et al., 2007) Some microorganisms such as *Thermomonospora* sp. (Mukherjee et al., 2002) and *Fusarium oxysporum* (Ahmad et al., 2003) were found to synthesize Au NPs in extracellular matrices. In addition to the microbial synthesis of NPs, the biosynthesis of Au NPs by plant species is known and is now drawing increasing scientific attention (Schrofel et al., 2011, Beattie and Haverkamp, 2011).

## 1.5 Biogeochemical cycling of heavy metals:

The biogeochemical cycling of metals involves both biotic (living) and abiotic (physical and chemical processes) components of the environment; however more emphasis is placed on microbial roles in this review. Microbes and heavy metals have a *quasi*-antagonistic relationship i.e. microbes can transport metal, change metal speciation and affect metal toxicity and mobility. In turn, the chemical and physical properties of heavy metals can influence microbial populations. However, some microorganisms are resistant to heavy metals; they can survive and grow in heavy metal contaminated conditions (Gadd, 2010). Therefore, microorganisms play key roles in determining the bioavailability, behaviour, speciation and toxicity of heavy metals through the biogeochemical cycle.

In general, microbial reactions lead to metal mobilization or immobilization through mechanisms such as redoxolysis, acidolysis, complexolysis, alkylation, biosorption,

bioaccumulation, redox reaction and complex formation. Redoxolysis, acidolysis and complexolysis increase the bioavailability of the metals and are the most effective processes for solubilizing metals carried out by bacteria and fungi (Brandl, 2002). Some of the other mechanisms immobilize the metals and thereby reduce their bioavailability. The balance between solubilisation and immobilization is dependent on factors such as the type of microorganism, their environment and the physiochemical properties of the environment (Gadd, 2010).

In the soil environment, the bioavailability of heavy metals is influenced by interactions with soil components such as iron oxide, humic acid and clays and other minerals. Some of these components, such as soil organic matter and humic acids can sequester heavy metals such as Pt-complexes and particles and make them unavailable. On the other hand, some interactions can lead to the release of heavy metals. For example, when ferric oxide Fe (III) is reduced to ferrous oxide Fe (II) by microbial action, contaminated metals that had been adsorbed to Fe (III) oxide get released and solubilized. This solubilisation in some instances is catalysed by humic acid and other compounds (Gadd, 2010). Humic acids occur naturally in soils and water and possess functional carboxyl groups which when dissolved in neutral waters become negatively charged. These charged molecules can react with metals such as Pt to form soluble Pt-organo complexes or stable Pt-organic colloids (Campbell, 2012). Soil type also influences the bioavailability of metals; clays are known for their ability to effectively remove heavy metals by specific adsorption and cation exchange.

### 1.5.1 Gold

#### 1.5.1.1 Biogeochemical cycling of Gold

Microorganisms are important in the biogeochemical cycling of Au as they are involved in Au mineralization (from complexes), solubilisation, dispersion and reconcentration

through different mechanisms (Reith et al., 2007) (Reith et al., 2013). These include mechanisms involved in detoxification and leaching processes which can lead to a reduction in metal toxicity (Kabata-Pendias, 2010). Some microorganisms are able to resist metal toxicity through adjustment of the ionic content of these metals and subsequently using then as terminal electron acceptors during anaerobic respiration process (Beazley et al., 2011). The following section will enumerate a few examples where microbial groups have interacted with Au, changing its speciation (forms) and affecting its toxicity in the process.

Bacteria and archaea are involved in the biogeochemical cycle of gold in soil, hydrothermal and deep subsurface systems converting it from one form to another (Reith and McPhail, 2006, Reith et al., 2007, Southam et al., 2009). Microbial reduction of ionic silver and Au species results in the production of elemental silver ($Ag^0$) and gold ($Au^0$) species (Holden and Adams, 2003, Kierans et al., 1991, Southam et al., 2009). Biomineralization of Au (in form of NPs) by the bacterium *Cupriavidus metallidurans* CH34 through Au-regulated gene expression leads to reductive precipitation of toxic Au(III) complexes from solution (Gadd, 2010). Cellular Au accumulation is associated with the formation of Au(I)–S complexes. This induces detoxification responses involving efflux systems, reduction and possible methylation of Au complexes, leading to the formation of Au(I)–C-compounds and AuNPs (Gadd, 2010). Similar particles have been observed in bacterial biofilms growing on Au grains (Reith et al., 2006). Iron- and sulfur-oxidising bacteria (e.g., *Acidithiobacillus ferrooxidans* and *A. thiooxidans*) are involved in the breakdown of Au-hosting sulfide minerals in zones of primary mineralization. These bacteria release the associated Au in the process. They, as well as other bacteria, produce thiosulfate, which is known to promote Au mobility by forming stable, water-soluble complexes with Au (Etschmann et al., 2011).

### 1.5.2 Platinum

#### 1.5.2.1 The biogeochemical cycling of platinum:

The geochemical behaviour of Pt in mineralized mafic and ultramarie rocks of the Stillwater Complex have been studied during the weathering of soils (Syed, 2012). The solubility of Pt in saline fluids at low pH and high oxygen concentration was found to be more than 10 ng/L (Azaroual et al., 2001, Zereini and Wiseman, 2015, Kummerer et al., 1999, Rauch and Morrison, 2008). Increased salinity, decreased pH and particle size of metallic Pt leads to an increase in the solubility of Pt. Metallic Pt is oxidised in soil because of its nanocrystalline particle size, with most Pt species formed in the oxidation processes being immobile (and probable less reactive). The lower toxicity of Pt-complexes means that the concentrations of dissolved Pt need to reach levels > 10 times higher than those of Au to drive a biogenic cycle similar to that of Au. It is worth noting that due to the capacity of Pt-complexes to irreversibly sorb to soil mineral phases, Pt toxicity may be reduced, as previously reported (Butt et al., 2001). In their experiments with ferruginous soils from Ora Banda, Mn-oxide-rich soils from Mt. Keith, Butt et al. found that Pt(II)-thiosulfate complexes were irreversibly adsorbed to ferruginous minerals (Butt et al., 2001). Secondary Fe-minerals were more efficient in sorbing Pt complexes than organic matter (Brugger et al., 2013). The end result of these processes was reduced toxicity as the complexes were no longer available for biological interactions. Solubilisation of PGM can be enhanced by the presence of natural organic acids such as humic acid and organic ligands (Eliopoulos et al., 2006).

Microorganisms can interact with Pt and play important roles in Pt biogeochemical cycle. Lengke in his study demonstrated the synthesis of nanoparticles of Pt organics and Pt metal by filamentous cyanobacteria from Pt (IV) chloride ($PtCl_4$) (Lengke and Southam, 2006). This was the first study reporting the synthesis of Pt nanoparticles by a biological method as

all previous studies involved chemical methods. It is believed that a similar process occurs in the natural environment. Another study (Dahlheimer et al., 2007) showed that siderophores, a class of organic ligands secreted by bacteria, fungi and plants under certain environmentally relevant conditions, significantly increase the solubility of Pt as well palladium and rhodium, two other PGE.

Sulphate-reducing bacteria have also been shown to reduce Pt (IV) to Pt (0) in aqueous solutions (Rashamuse and Whiteley, 2007) with a resting mixed culture of sulphate-reducing bacteria carrying out this reductive process. A pH-dependent rate of Pt removal was observed indicating that metal speciation was the main factor for its removal from solution. The role of the hydrogenase enzyme in the reduction process was also established, since Pt was seen to be precipitated in the periplasm, a major area of hydrogenase activity in the cells.

#### 1.5.2.2 Mechanisms of microbial mediated platinum and gold solubilisation

The study of the biogeochemical cycling and mobility of Pt is very recent and the part played by the biosphere is yet to be fully understood. The mechanisms associated with the interaction of bacteria with Pt are largely unknown with very limited published literature. Platinum and Au are known to share similar biogeochemical properties. They have very comparable reactivity in aqueous solutions and hence the knowledge and analysis of Au solubilisation by bacteria could be valuable in the study of Pt-microbe interactions.

The mechanisms of Au solubilisation are directly linked to the nature of the environment as well as the formation and excretion of various Au-complexing metabolites by microorganisms. In organic matter-poor environments, Au solubilisation takes place *via* the thiosulphate mechanism and is carried out by chemolithoautotrophic bacteria such as *A. ferrooxidans, A. thiooxidans* and archaea. In this mechanism, the bacteria excrete thiosulphate, which leads to Au oxidation and complexation in the presence of oxygen (Aylmore and Muir,

2001). In organic matter-rich environments like topsoil and rhizosphere soils, solubilisation occurs *via* amino acid and/or cyanide mechanisms and is carried out by heterotrophic microorganisms. Solubilisation occurs through the formation of Au-amino acid/cyanide complexes. The amino acid glycine is the metabolic precursor of cyanide. Cyanide producing microorganisms like *Pseudomonas fluorescens*, *P. aeruginosa*, and *Chromobacterium violaceum* are involved in the solubilisation of Au which occurs by a two-step process. Gold is oxidised and secondly complexed with cyanide to form a dicyanaurate complex (Faramarzi and Brandl, 2006, Faramarzi et al., 2004).

#### 1.5.2.3 Studying soil-metal-microbe interactions

Different methods have been used to study microbial interactions with heavy metals in the soil environment. Multiple studies have used techniques such as DNA extraction-PCR-Denaturing Gradient Gel Electrophoresis (DGGE), Fatty Acid Methyl Ester (FAME) profiling, Phospholipid Fatty Acid (PLFA) profiling and cloning and sequencing to evaluate the effect of heavy metals on soil microbial diversity (Qing et al., 2007) (Martinez-Inigo et al., 2009, Kozdroj and van Elsas, 2001, Chodak et al., 2013). Table 1.4 shows the advantages and disadvantages of selected traditional molecular methods with appropriate references for further reading.

**Table 1-4:** Advantages and disadvantages of selected traditional molecular methods (Kirk et al., 2004, Adzitey et al., 2013, Agrawal et al., 2015).

| **Methods** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|
| Fatty acid methyl ester analysis (FAME) | • Direct extraction from soil can be done.<br>• Specific organisms or communities are followed.<br>• No culturing of microorganisms required. | • Large amounts of raw material is required in case of fungal spores.<br>• Can be influenced by external factors. | (Gammons and Williamsjones, 1995).<br>(Siciliano et al., 1998, Zelles, 1999). |
| Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE) | Large number of samples can be analysed simultaneously.<br>Reliable, reproducible and rapid.<br>Separation of amplicons single base-pair differences. | PCR biases Dependent on lysing and extraction efficiency. Way of sample handling can influence community, i.e. the community can change if stored too long before extraction. One band can represent more than one species (co-migration) Only detects dominant species.<br>Technically difficult; subsequent sequencing required to identify bands; expensive and time-consuming; co-migration of similar sequences can complicate identification. | (Kirk et al., 2004)(Mills et al., 2002) (Prakitchaiwattana et al., 2004, Renouf et al., 2007, Renouf et al., 2006, Cocolin et al., 2000, Cocolin et al., 2001). |

| | | | |
|---|---|---|---|
| Community level physiological profiling (CLPP) | Fast.<br>• Relatively inexpensive.<br>• Highly reproducible.<br>• Differentiate between microbial communities.<br>• Site-specific carbon sources can be used for the study. | • Only represents the culturable fraction of community.<br>• Represents only those organisms capable of utilizing available carbon sources.<br>• Represents metabolic diversity rather than microbial diversity.<br>• More suitable for fast-growing organisms. | (Bokulich et al., 2012) (Bokulich et al., 2011, Bokulich and Mills, 2012) (Tiedje et al., 1999, Osborn et al., 2000, Dunbar et al., 2000). |
| Terminal restriction fragment length polymorphism (TRFLP) | Sensitive; rapid; inexpensive; technically easy.<br>Simpler banding patterns than RFLP. | Resolution compromised in highly diverse samples.<br>Dependent on extraction and lysing efficiency. | (Bokulich et al., 2012) (Bokulich et al., 2011, Bokulich and Mills, 2012) (Tiedje et al., 1999, Osborn et al., 2000, Dunbar et al., 2000). |

However, the development of Next Generation Sequencing (NGS) approaches which are high resolution molecular tools has provided accurate and more detailed information on microbial interactions with contaminants such as heavy metals than these traditional molecular approaches. Next-generation sequencing (NGS) or high throughput sequencing has transformed the field of microbial ecology and taken classical environmental studies to a higher level. This innovative technology has led to the development of metagenomics, a field where direct genetic analysis of genomes contained within an environmental sample is conducted, without the need for cultivating clonal cultures prior to the analysis.

Metagenomics or ecogenomics is a very important culture independent technique which utilizes genomic methods to analyse the collective genomes present in environmental samples. It permits the detection of a broad variety of microorganisms (taxonomy and phylogeny), genes of interest, operons and biological molecules suitable for biotechnological applications (Uhlik et al., 2012) and generates data of unrivalled depth, coverage and quality. While early metagenomic approaches involved cloning (Handelsman et al., 1998), recent approaches bypass cloning and has involved the use of sequencing equipment such as the Ion Torrent PGM and Proton (Life Technologies), Roche 454 FLX pyrosequencing, Illumina MiSeq and HiSeq, AB SOLiD, and PacBio SMRT systems. These next generation tools "embody an elegant interplay of chemistry, engineering, software and molecular biology" (Mardis, 2013) and have been extensively reviewed in terms of process and cost benefits, advantages and disadvantages and data analyses (Liu et al., 2012a, Mardis, 2013, Shokralla et al., 2012) .

As a result its high data yield and relatively low costs of data generation, NGS approaches have become the preferred method for the study of soil microorganisms. A substantial number of NGS studies have been amplicon based, targeting marker genes, such as

16S rRNA or *recA* genes (Weisburg et al., 1991) (Scholz et al., 2008) (Gołębiewski et al., 2014). To date, several reports on soil bacterial communities have been based on the use of 16S rRNA gene fragment NGS (Roesch et al., 2007) (Fulthorpe et al., 2008, Nacke et al., 2011, Will et al., 2010, Gołębiewski et al., 2014). However of these comparatively few have focussed on metal-contaminated soils (Chodak et al., 2013, Hur et al., 2011, Gołębiewski et al., 2014, Sheik et al., 2012). Therefore, in this study the impact of Au and Pt will be assessed via metagenomics, targeting 16S rRNA gene fragments.

### 1.6 Microorganism used in this study

Given that a focus of this review and planned experiments is on microbial interactions with Au and Pt, it is important to select microorganisms that have demonstrated the capability to mobilize these metals in previous studies. The bacterium, *Cupriavidus metallidurans* has been widely used in the study of Au biomineralization (Monsieurs et al., 2011). The biomineralization of Au by *C. metallidurans* is known to be due to Au-regulated gene expression which leads to the reductive precipitation of Au complexes which are toxic to some organisms (Reith et al., 2009). A recent study by Fairbrother and his colleagues has shown that *C. metallidurans* plays an important role in the detoxification of Au complexes and accumulation of pure Au in environmental surfaces (Fairbrother et al., 2013).

In a previous study (Brugger et al., 2013), Au and Pt foils were incubated with two different bacteria, *C. metallidurans* and *Chromobacterium violaceum* for 56 days. Biofilms were observed by Scanning Electron Microscopy (SEM) on both Au and Pt foils after 56 days with the *C. metallidurans* treated samples. There was a visible change in the surface morphology of the foils, implying reactivity of the bacteria with the metals. The other bacteria, *C. violaceum* had no effect on the foils. *C. metallidurans* was able to immobilize 5,000 μM

platinum (+IV) chloride ($PtCl_4$) and 5,000 μM $K_2PtCl_4$. Transmission electron microscopy (TEM) analysis revealed that *C. metallidurans* cells produced Pt nanoparticles which were observed within the cell walls (Campbell, 2012). *C. metallidurans* should therefore serve as an ideal candidate to study Pt solubilisation and determine bacterial roles in the bioaccumulation of Pt nanoparticles

### 1.7 Gaps in knowledge

*Cupriavidus. metallidurans* or *Chromobacterium violoaceum* have been reported growing on the surfaces of Au- or Pt-foils (Brugger et al., 2013, Fairbrother et al., 2009). This led to the suggestion that the differing mobility of Au vs. Pt results from the high turnover rates of Au due to differences in biotoxicity, passive uptake and biochemically active detoxification (Etschmann et al., 2016). The presence of biofilms which catalyze the biomineralization of secondary Au is thus attributed to the toxicity exerted by Au-complexes on microbiota (Reith et al., 2009). The biomineralization of Au occurred via the formation of intra- and extracellular spherical NPs. These NPs aggregated into spheroidal and framboidal micro-particles of up to 2 μm in diameter around cells. They eventually encapsulated and ultimately replaced them. These particles were analogous to Au particles commonly observed on natural Au grains. Bacterial cells were connected via exopolymer or nanowires, and extracellular gold-aggregates, which would intuitively improve the flow of electrons through the biofilm. This has provided experimental verification for the importance of biofilms of *C. metallidurans* for the biomineralization of Au and development of pure Au in surface environments (Reith et al., 2013). While the mechanism of microbial growth on Au-foil is reasonably well characterized, there is no report on the key mechanisms underpinning bacterial growth on Pt-foils. It is unknown whether the mechanisms for growth on Pt-foils would be the same as observed in Au-foils. More insights into the impact of Au and Pt on soil microorganisms and activities are

needed to better understand the toxicity of Au and Pt, and develop strategies to reduce their toxicity.

It is also well known that though Au and Pt have similar chemical properties, Pt behaves differently to Au in most surface environments due to the reduced mobility and lower toxicity of Pt complexes. A recent study directly compared Au and Pt mobility in groundwaters, soils, sediments and Pt/Au-grains from the Fifield Au/Pt-field (New South Wales, Australia). They found that Au was more mobile than Pt (Brugger et al., 2013). This suggested that Au was continuously mobilized on the surface of Au grains leading to a highly toxic microenvironment. Gold-complexes seem to be toxic to bacteria; once inside the cell, they may produce oxidative stress and inhibit enzymatic function. However, there is limited information available on microbial interactions with Pt complexes and Pt nanoparticles with respect to its mobility. Gaps remain in our knowledge of microbially-mediated Au and Pt mobility in a soil medium. Are the mechanisms the same or different for each heavy metal? With regards to the soil, there is very little information available on the impact of soil types on Au and Pt mobility.

## 1.8 Research questions, hypotheses and aim

The aim of this PhD project was to assess the mobility of Pt and Au in different environmental media, assessing their impacts on soil microbial community function and structure. This aim was addressed by completing the listed studies which are described in detail in subsequent chapters.

**Study 1:** Assessment of the role of selected micro-organisms, *Cupriavidus metallidurans* (a known heavy metal resistant microorganism) and *Escherichia coli* (a control microorganism) in the biomineralization of secondary platinum complexes and accumulation of Pt complexes in sand columns.

**Hypothesis**: A selected microorganism known to be resistant to heavy metal toxicity would mobilize more Pt from complexes and accumulate more Pt than the control microorganisms.

**Study 2:** Assessment of the influence of different soil types (clay, humic acid and iron oxide) on the movement of Pt and Au nanoparticles in the environment.

**Hypothesis:** Soils with high clay, humic acid and iron oxide content will reduce the movement and transformation of Pt and Au nanoparticles.

**Study 3**: Assessment of the impact of Au and Pt on soil microbial activity as assessed by soil respiration and enzyme activities.

**Hypothesis:** The presence of Au and Pt will cause stress and reduce soil respiration and enzymes activities.

**Study 4:** Assessment of the impact of Pt and Au on soil microbial diversity using next generation sequencing (MiSeq).

**Hypothesis**: The presence of Au and Pt in soil will result in significant changes in the soil microbial community leading to a reduction in microbial diversity.

These hypotheses were tested using a variety of approaches. For each research chapter (2-6) the approach is summarized below. These chapters contained a brief introduction outlining the aims of the work.

Chapter 2: The growth and uptake of Pt by heavy metal resistant and control microorganisms in liquid medium was assessed to determine microbial uptake and tolerance of introduced platinum complex. This allowed the hypothesis in study 1 which stated that "a selected microorganism known to be resistant to heavy metal toxicity would mobilize more Pt from complexes and accumulate more Pt than the control microorganisms" to be tested.

Chapter 3: A series of sand based columns were prepared in which Pt and Au were introduced to assess the movement of these metals through the column and further assess the interaction of Pt and Au on the two selected bacteria. The hypothesis from study 2 which stated that soils with high clay, humic acid and iron oxide content will reduce the movement and transformation of Pt and Au nanoparticles was evaluated in this chapter.

Chapter 4: The overall effects of the amendment of five different Australian soil types wit Pt and Au complexes on soil respiration and enzyme activities were assessed. These was carried out using different Pt and Au concentrations. The hypothesis that the presence of Pt and Au will cause stress and reduce soil respiration and enzymes activities from study 3 was subsequently evaluated.

Chapter 5: Finally, the impact of Pt and Au addition on microbial community structure in selected Australian soils was investigated using a next generation sequencing approach and an array of bioinformatic tools. This allowed for qualitative and quantitative assessment of the

impact Pt and Au introduction on different soil microbial groups. The hypothesis in study 4 on the presence of Au and Pt in soil resulting in significant changes in the soil microbial community leading to a reduction in microbial diversity was tested.

# Chapter 2 The biomineralization of platinum by *Cupriavidus metallidurans* and *Escherichia coli* in sand columns.

## 2.1 Introduction:

Platinum (Pt) is one of the six Pt group elements. In recent years, Pt emissions in the environment have increased due to the use of Pt in the automobile industry as exhaust catalysts (Wang et al., 2009), the chemical industry and hospitals in a variety of implanted biomedical devices and neuromodulators (Helmers and Mergel, 1998, Pawlak et al., 2014, Maes et al., 2017, Reith et al., 2014). The transformation of Pt in the environment is affected by a number of parameters such as pH, redox potential, soil salinity, organic matter content, soil structure, the presence of complexing agents and the activity of macro- and micro-biota (Wiseman and Zereini, 2009, Reith et al., 2014, Sobrova et al., 2012). In aqueous solutions, Pt ions are unstable and can be transported under extremely acidic and oxidizing conditions. Platinum can be transported in the environment as nanoparticles (NPs) and as complexes such as Pt-thiosulfate, chloride and complexes with low- and high-molecular weight organic acids (LMWOAs) (Reith et al., 2014).

Microorganisms can influence chemical and physical factors such as redox reactions and pH changes through the secretion of complex ligands which affect Pt mobility in soils, sediments, surface- and ground-water (Southam and Saunders, 2005, Reith et al., 2008, Gadd, 2010). Hence, biological activities play a key role in the biogeochemical cycling of metals and particularly the movement and stabilization of Pt in the environment.

Many studies over the last past two decades have investigated the various reactions between Pt and microorganisms (e.g. bacteria) adsorb Pt through their cell walls (Lustig et al., 1996, Lustig et al., 1998, Plyusnina et al., 2000, Kalbitz et al., 2008, Ljubomirova et al., 2008,

Reith et al., 2014) (Yee and Fein, 2002). Bacteria can also modify Pt and Pt complexes rendering them mobile as observed in *Pseudomonas plecoglossicida* which mobilized Pt, as Pt(IV)-cyanide after 10 days of incubation (Brandl et al., 2008). Halophilic bacterial cultures have been used to biologically recover Pt(II) and Pt(IV) from dilute industrial process streams with flow cytometric membrane staining used to confirm cell viability during Pt recovery (Maes et al., 2016). In addition to Pt, bacteria can mobilize other heavy metals such as gold (Au). A recent study conducted by Brugger et al. (2013) compared the mobility of Au and Pt in groundwaters, soil sediments and Au/Pt grains from Fifield, New South Wales, Australia. Experimental results showed that Au was more mobile than Pt due to geomicrobial processes occurring in the environment. In another study, the mobility of Au and Pt was evaluated by growing biofilms of *Cupriavidus metallidurans* and *Chromobacterium violaceum* on the surface of Au and Pt foils. The results suggested that Au was more mobile than Pt due to its higher turnover rate which arises from differences in biotoxicity, passive uptake and biochemically active detoxification (Fairbrother et al., 2009, Brugger et al., 2013, Etschmann et al., 2016).

The soil bacterium *C. metallidurans* contains the highest number of known heavy metals resistance genes (Monsieurs et al., 2011) and has been widely used in gold biomineralisation studies. The biomineralisation of Au by *C. metallidurans* CH34 has, in part been attributed to Au-regulated gene expression and subsequent protein production, resulting in the reductive precipitation of Au complexes which are toxic to organisms (Reith et al., 2009). Furthermore, a study by Fairbrother et al. has shown that *C. metallidurans* was capable of detoxifying Au- complexes, forming pure Au (Fairbrother et al., 2013). In another study, *C. metallidurans* was shown toreduce toxicity through biological survival responses when exposed to low concentrations of $PtCl_4$ (Campbell, 2012). *C metallidurans* has also been shown to immobilize Pt complexes such as $K_2PtCl_4$ (at concentrations of up to 5,000 μM) (Campbell,

2012). Transmission electron microscopy (TEM)-based investigations have revealed that *C. metallidurans* produces Pt nanoparticles within its cell walls (Campbell, 2012).

The bacterium *Escherichia coli* is among the most widely studied bacteria in terms of its response to exposure to metals. This is mostly due to the availability of the complete genomic sequence of *E. coli*, its low cost and wide availability (McCloskey et al., 2013). *E. coli* can also be readily manipulated genetically (Rensing and Grass, 2003). The bacterium is sensitive to heavy metals such as Zn, Cd and Hg and studies have shown that *E. coli*'s exposure to Pt complexes (e.g. $(NH_4)_2[PtCl_6]$) results in the inhibition of cellular division (Rosenberg et al., 1967 #164). Heavy metals can be absorbed by *E. coli* resulting in a reduction in both the heavy metals' toxicity and bioavailability (Vijayadeep and Sastry, 2014). Although some *E. coli* strains have been shown to exhibit a range of tolerances to different concentrations of Zn(II), Cd(II), Co(II) ,Ni(II) (Brocklehurst and Morby, 2000), most reports suggest that it has limited tolerance to heavy metals (Brocklehurst and Morby, 2000, Nies, 1999, Giller et al., 1998). *E. coli* has been used as a control test organism in studies on evaluating the tolerance of bacteria isolated from sewage to Ag, Hg and Cr (Lima e Silva et al., 2012). Based on its limited tolerance to heavy metals and history of being used as control microorganisms in previous studies, *E. coli* was chosen as a reference candidate for use in this study.

An increasing concentration of Pt in the environment might be detrimental to ecosystem function and stability based on its toxicity to biota. Understanding bacterial interactions with Pt may offer considerable insights into the biochemical detoxification of Pt. Therefore, the aim of the work presented in this chapter was to examine the microbial interactions with $PtCl_4$ by (i) assessing the differences in uptake of Pt complexes between a known metal tolerant bacterium *C. metallidurans* CH34 and a well characterized microorganism with limited metal tolerance (*E. coli*), growing in biofilms, and (ii) exploring the involvement of *C. metallidurans* CH34 in the biomineralization of Pt complexes. This

chapter formed the basis of a contribution to a paper in Nature Geoscience (Volume 9, page 294), (Biological role in the transformation of platinum-group mineral grains).

## 2.2 Materials and Methods

### 2.2.1 Materials

Pt (IV)-chloride ($PtCl_4$, 99.99+% [metal basis], Pt, 55-58%) was obtained from Sigma-Aldrich. Sand was collected from Melbourne (Bundoora Sand and Building Supplies, VIC, Australia).

### 2.2.2 Microorganisms

*Cupriavidus metallidurans* strain CH34 was obtained from the culture collection of Associate Professor Frank Reith. *E. coli* strain W3110 was obtained from the RMIT Bundoora Culture Collection.

### 2.2.3 Methods

#### 2.2.3.1 Optimization of Pt concentration for column experiments

Bacterial species were grown in Tris Minimal Media (TMM, 30 mL) supplemented with sodium gluconate (4% w/v) for 3 days at 30$^{o}$C and 160 rpm (Mergeay et al., 1985). Bacteria were harvested by centrifugation at 4,000 *g* for 10 minutes, the pellet washed twice with $dH_2O$ and then resuspended in TMM (30 mL). The cell density was then measured spectrophotometrically at $OD_{600}$.

Prior to setting up column experiments, an initial short-term study was carried out to determine the optimal concentration of Pt for bacterial survival and the time-period for stabilization of growth. A stock solution (50 mM) of $PtCl_4$ was prepared (1.83 g of $PtCl_4$ dissolved in 100 mL of DDI water); 30 mL aliquots of TMM were then amended with a range of $PtCl_4$ concentrations (25, 50, 100, 150, 200, 250, 500, 1,000, 5,000 and 10,000 µM) together with bacterial cells. Samples were collected every 7 days for 21 days. Bacteria were counted using the plate counting method (Miles et al., 1938) and the Pt concentration in the outlet solution was determined using Atomic Absorption Spectroscopy (AAS) with a 0.02 mg/L detection limit. Experiments were carried out at 25$^{o}$C for 21 days, in triplicate.

#### 2.2.3.2 Column experiments

Following the selection of the most appropriate Pt concentration from the experiment described above, pre-sterilised glass columns of 10 mm diameter containing 10 g of acid washed sieved quartz sand were setup up and incubated at 25˚C in a constant temperature room

in the dark. The experiment was set up as detailed in Table 2.1. Columns were inoculated with washed cell concentrate (1 mL) harvested from a pre-culture (10 mL grown in TMM) (Mergeay et al., 1985). The initial cell concentrations in the columns were $2.5\times10^8$ ($1.04\times10^8$) and $5.3\times10^8$ ($2.3\times10^8$) cells $mL^{-1}$ for *C. metallidurans* and *E. coli*, respectively. Control columns were amended with sodium azide (0.15 mL, 1 % w/v) to help maintain sterility. Columns were amended throughout the incubation by addition of TMM (10 mL) containing Pt (1,000 µM) at time periods of 0, 14, 28, 42, 56, 70, 84, 98 and 112 days. At each sampling time, columns were completely drained prior to amendment with new medium and the bacterial viable count determined at each time point.

**Table 2.1:** Summary of column experimental set up.

| Column Ref. | Column amendments | Description |
|---|---|---|
| A | *C. metallidurans* with $PtCl_4$ | Viable biofilm |
| B | *C. metallidurans* without $PtCl_4$ | Growth control |
| C | *E. coli* with $PtCl_4$ | Viable biofilm |
| D | *E. coli* without $PtCl_4$ | Growth control |
| E | Control with $PtCl_4$ | Abiotic control |
| F | Control without $PtCl_4$ | Abiotic control |

#### 2.2.3.3 Analyses of the outlet solution

Following incubation, all samples were centrifuged at 4,000 *g* for 10 min. The pellet was then washed twice with water, resuspended in TMM (30 mL) and the $OD_{600}$ value was recorded spectrophotometrically. Cell enumeration was carried out by plating aliquots (10 µL) of the outlet solution on agar plate counting media (Acumedia nutrient agar 7145). The assay was carried out based on the counting method previously described (Miles et al., 1938). The pH of the eluted solution was measured using a Hanna Instruments H11134 pH-electrode equipped with a CyberScan pH 310 meter.

Platinum concentrations in the outlet solutions were determined after centrifugation at 3,000 *g* for 20 min, filtration (using 0.22 µm sterile syringe filters) and acidification to 2 % (w/v) using $HNO_3$. Pt concentrations were determined using fast sequential Atomic Absorption Spectrometry (AAS) (AA280FS, Varian, wavelength: 265.9 nm, slit width: 0.2 nm, lamp current: 10.0 mA, burner height: 20.0 mm, detection limit: 0.2 µg $L^{-1}$).

#### 2.2.3.4 Scanning Electron Microscopy (SEM)

Sand grains were fixed in electron microscope (EM) fixative (4% paraformaldehyde, 1.25% glutaraldehyde in phosphate buffer saline (PBS) and 4% sucrose at pH 7.2) for 30 minutes. Samples were then washed in washing buffer (PBS and 4% sucrose) for 5 minutes. Samples were dehydrated using a series of increasing ethanol concentrations 70%, 90%, 100%, twice for 10 minutes for each concentration. Samples were then fixed using HMDS 1:1 with ethanol for 10 minutes followed by 100% HMDS for 10 minutes, twice. HMDS was then removed, and the samples dried. Grains were carbon coated and analysed using focus ion beam-scanning electron microscopy (FIB-SEM; Helios NanoLab 600, FEI, University of Adelaide) and FEI Quanta 450 microscopy (High Resolution Field Emission Scanning Electron Microscope, University of Adelaide). SEM-energy-dispersive X-ray elemental analysis operating at 200 kV (XEDS) (RMIT University) was used to detect Pt sections at a 90° angle to the surfaces, with a Ga ion beam at 30 keV 21 $nA^{-1}$ used to cut the section. Surfaces were cleaned by decreasing the power of the ion beam to 30 keV 2.8 $nA^{-1}$ and 20 keV 0.34 $nA^{-1}$. Sections were element mapped by EDXA using a 10 $mm^2$ Sapphire Si(Li) EDXA-detector (Wirth, 2009).

#### 2.2.3.5 Column analyses

At the end of the incubation period (112 days), the columns were sectioned into 20 mm segments and approximately 0.2 g of sand per segment (in triplicate) was taken and digested in *aqua regia* (1:3 $HNO_3$: HCl). Following digestion, Pt concentrations were measured by AAS.

The numbers of cell in the column were also enumerated. An aliquot (0.2 g per segment, in triplicate) of sand was taken and vortexed for two minutes in 1 mL NaCl (0.9 M). An aliquot (10 µL) was then plated on agar plate counting media (Miles et al., 1938). DNA

was extracted from 0.25 g of sand from each segment using an UltraClean Microbial DNA kit by following the manufacturer's instructions. All columns including controls were subject to DNA extraction protocols (chapter 5, section 5.2.2 P 117) to assess the sterility of the controls.

## 2.3 Statistical approach

Statistical analyses were performed using Graphpad prism. Multiple t-tests were used to test the significance between the control and treatments representing the different concentrations of Pt. Differences at $p < 0.05$ level were considered to be significant.

## 2.4 Results

### 2.4.1 Optimization and screening of Pt concentration for column experiments

The optimum concentration of $PtCl_4$ that was used for the subsequent column experiment was determined by exposing both *C. metallidurans* and *E. coli* to varying concentrations of $PtCl_4$. Both species survived up to the highest concentration of $PtCl_4$ (10,000 µM). However, the cell numbers of *C. metallidurans* and *E. coli* decreased significantly with increasing concentration at 100, 150, 1,000 and 10,000 µM of Pt ($p< 0.05$) (Fig.2.1). The numbers of cells $mL^{-1}$ of *C. metallidurans* and *E. coli* observed at the highest $PtCl_4$ concentration (10,000 µM) were $6.1\times10^4$ ($\pm3.5\times10^3$) and $1.99\times10^2$ ($\pm3.0\times10^1$) respectively. This indicated the population of *C. metallidurans* was about 300 times more than that of *E. coli* in columns containing $PtCl_4$ at 10,000 µM concentration. This reflected the higher tolerance of *C. metallidurans* to Pt compared with *E. coli.* Pt was also detected in the supernatant solution at all $PtCl_4$ concentrations. Comparing the Pt in solution at the lowest amendment concentration (25 µM) with that in the highest amendment (10,000 µM), a 60.4% reduction in Pt in solution was observed with *C. metallidurans* compared to a 9.2% reduction in *E coli* across the same amendment range (Figure 2.2). Therefore, across the range of concentrations evaluated, *C. metallidurans* adsorbed more Pt than *E. coli* leading to decreasing levels of Pt in solution as $PtCl_4$ concentrations increased.

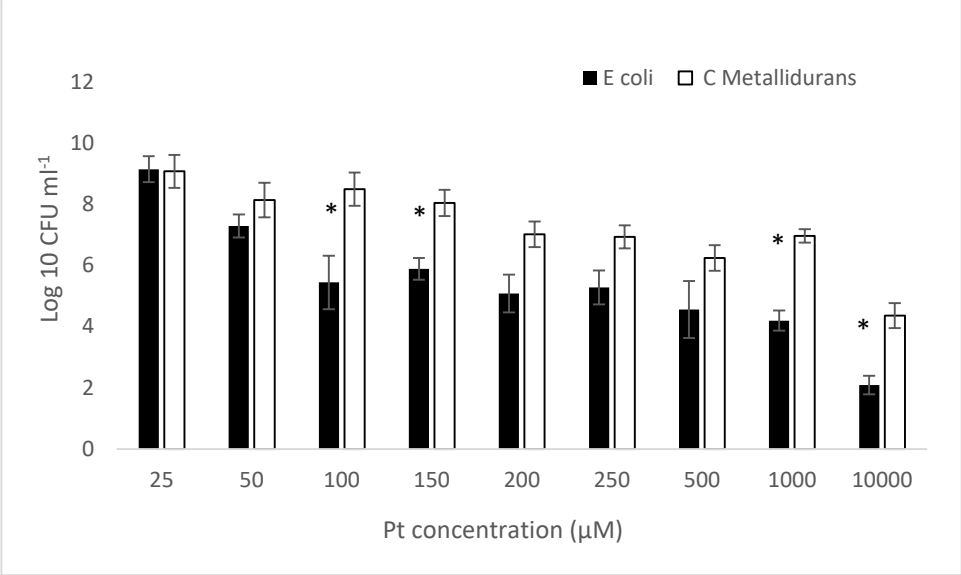


**Figure 2.1:** Survival of *C. metallidurans* and *E. coli* after 21 days of incubation with $PtCl_4$. Results shown are the means of three replicates. * indicates statistically significant difference between *C. metallidurans* and *E. coli* ($p < 0.05$).

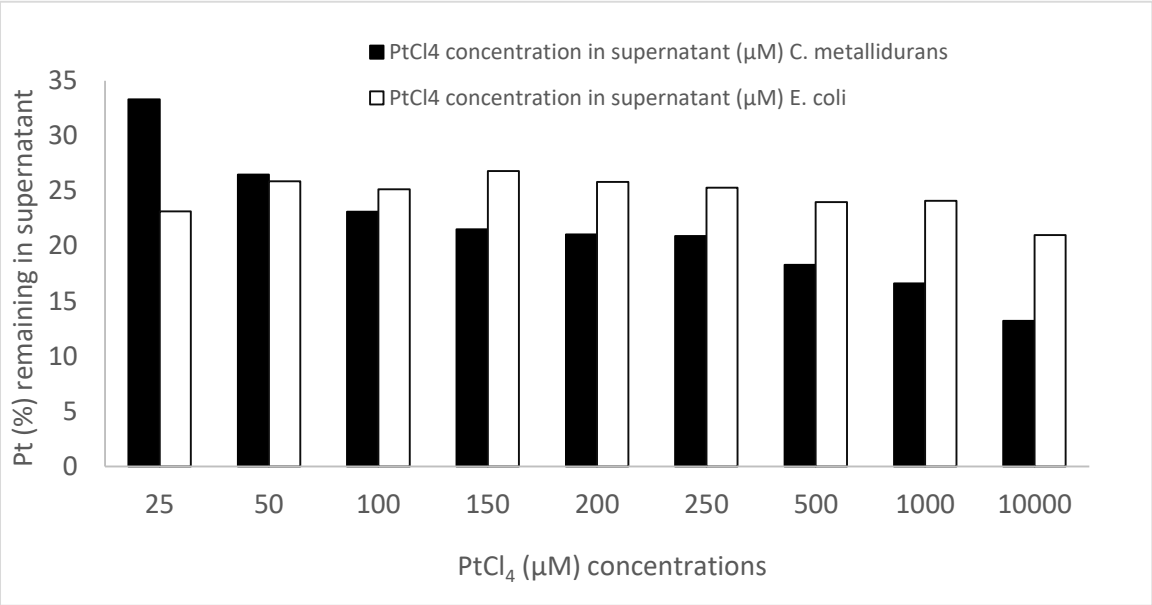


**Figure 2.2:** Percentage of Pt detected in the supernatant of *C. metallidurans* and *E. coli* after 21 days of incubation in different $PtCl_4$ concentrations.

### 2.4.2 Column experiments

#### 2.4.2.1 Cell viability

The impact of Pt concentrations on the viability of the two microbial strains, *C. metallidurans* CH34 and *E. coli* W3110 was investigated using a viable plate count method. At the start of the experiment, viable cell counts (CFU $mL^{-1}$) were $3\times10^{9}$ and $2.7\times10^{9}$ cells $mL^{-1}$ for *C. metallidurans* and *E. coli* respectively. Once $PtCl_4$ was added, the viability of

*Cupriavidus metallidurans* cells was maintained over the experimental time-period, although there was a slight decrease from the fourth amendment ($1.1x10^{9}$ ($\pm1.3x10^{8}$) $mL^{-1}$). In contrast, in the columns amended with *E. coli*, cell numbers started decreasing from the third amendment ($4.3x10^{8}$ ($\pm2.7x10^{8}$) $mL^{-1}$) (Fig. 2.3).

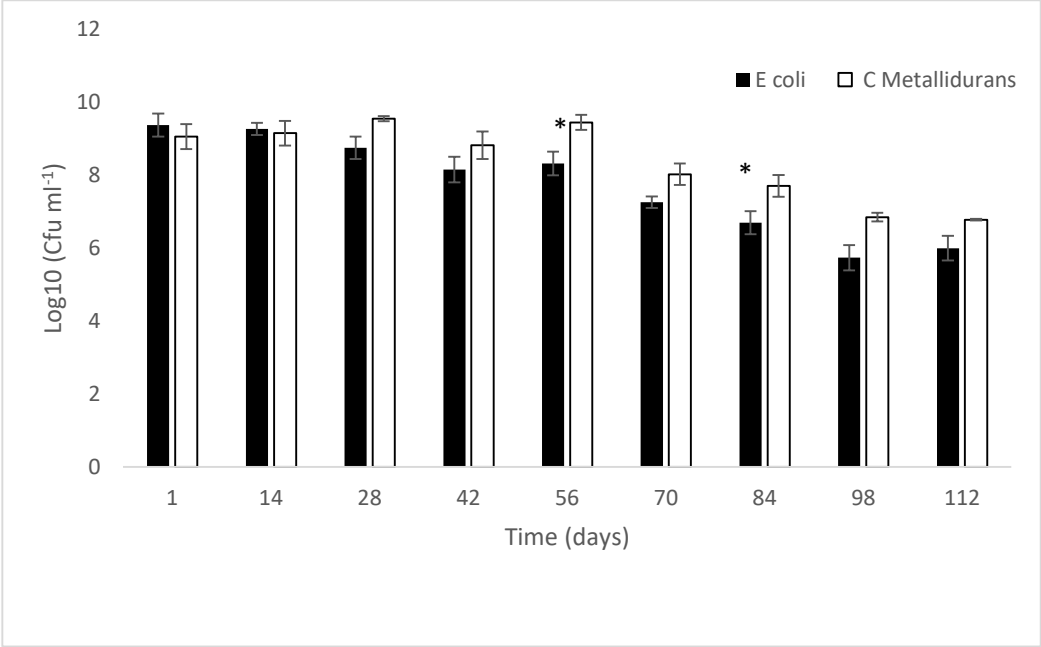


**Figure 2.3:** Cell number of *C. metallidurans* and *E. coli* during the amendments of $PtCl_4$ over 112 days. The results represent the means of triplicate values with standard deviations. * indicates a statistically significant difference ($p < 0.05$).

### 2.4.2.2 The effect of amendments with $PtCl_4$ on supernatant pH

The pH of the $PtCl_4$ (50 mM) stock was 6.8 and measurements during the experimental period showed that there was a substantial change in the pH of the supernatant compared to that of the control (Table 2.2). The pH value varied between 5.5 and 8.0 in all experimental samples. However, while the pH decreased in columns amended with *C. metallidurans* from 7.2 to 5.6, it fluctuated in columns amended with *E. coli.* The pH increased from 5.5 to 7.5 in the first two amendments then decreased to 6.6 by the end of the experiment.

**Table 2.2:** Changes in the pH values of the column outlet solution in different samples at various time points. Results represent the means and standard error of three replicates.

| Time (days) | columns with viable *CH34* biofilms | columns with viable *E. coli* biofilms | abiotic columns |
|---|---|---|---|
| | pH | pH | pH |
| 1 | 7.2 (± 0.2) | 5.5 (± 0.2) | 7.6 (± 0.3) |
| 14 | 7.6 (± 0.2) | 7.5 (± 0.2) | 7.6 (± 0.1) |
| 28 | 7.5 (± 0.3) | 7.5 (± 0.2) | 7.5 (± 0.3) |
| 42 | 7.7 ± (0.3) | 6.7 (± 0.1) | 7.1 (± 0.3) |
| 56 | 7.5 (± 0.1) | 6.5 (± 0.3) | 7.7 (± 0.2) |
| 70 | 7.4 (± 0.1) | 6.5 (± 0.1) | 7.7 (± 0.2) |
| 84 | 6.7 (± 0.2) | 6.5 (± 0.1) | 7.4 (± 0.1) |
| 98 | 6.6 (± 0.3) | 6.7 (± 0.1) | 7.6 (± 0.1) |
| 112 | 5.6 (± 0.2) | 6.6 (± 0.2) | 7.5 (± 0.3) |

### 2.4.2.3 Formation of Pt biominerals by *C. metallidurans* and *E. coli*

The retention percentages of Pt in the column were 87%, 75% and 50% for CH34, *E coli* and the control respectively (data not shown). SEM was utilised to observe the appearance of bacterial cells in the presence and absence of Pt. *E. coli* (Fig 2.4, A) and *C. metallidurans* (Fig 2.4, B) both grew in biofilm form in control columns. However, when treated with $PtCl_4$, *E. coli* (Fig 2.4, C) grew in single *cells,* while *C. metallidurans* (Fig 2.4, C) continued to grow in biofilm form. Analysis by SEM of both bacteria showed that Pt was associated with

extracellular substances (Fig 2.4, E, F) as shown in the back-scattered electrons (BSE) mode of C and D.

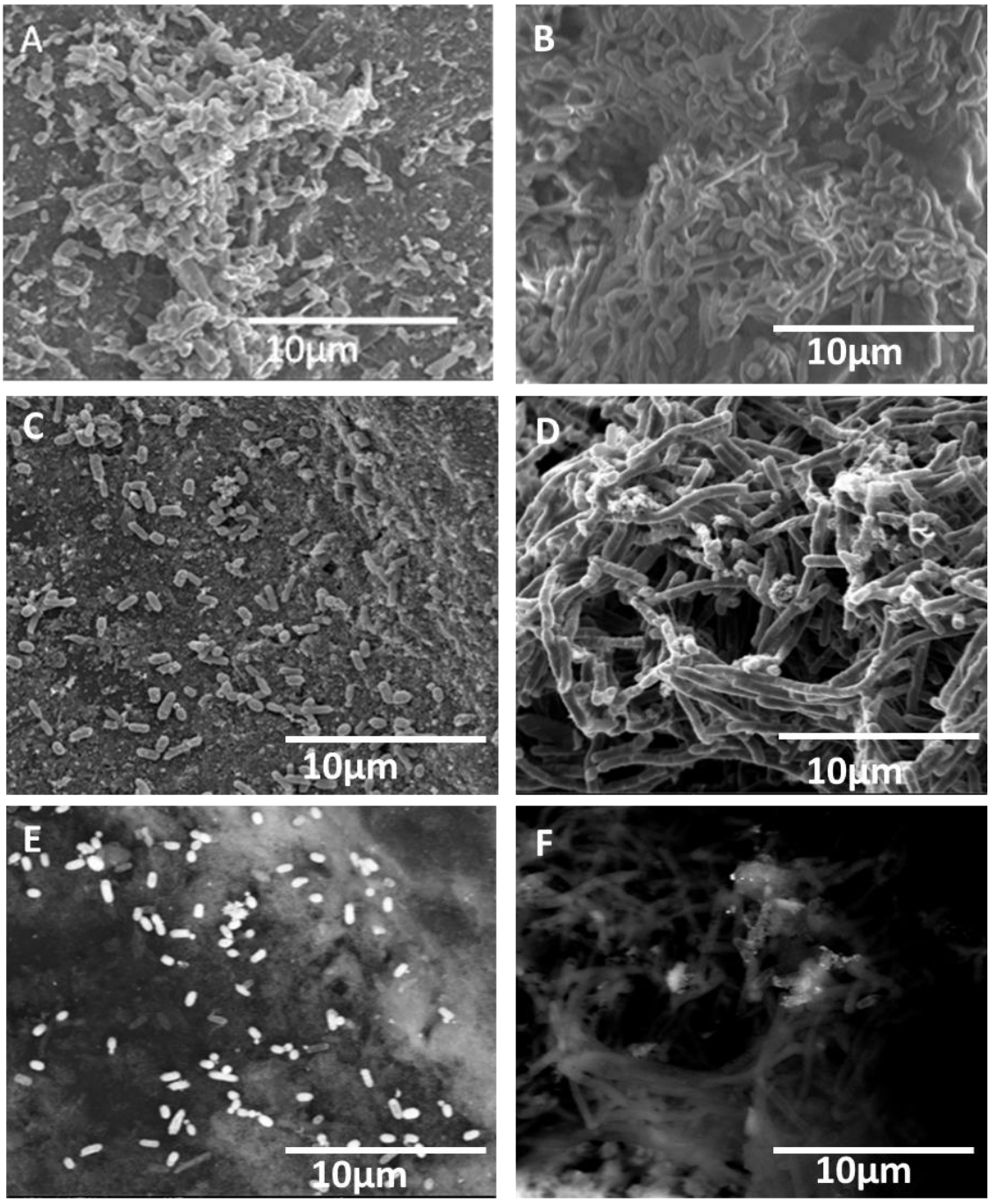


**Figure 2.4:** Pt nanoparticles formed by the bacterial activity of *E. coli* and *C. metallidurans* biofilms in column experiments. A, B = Scanning electron micrographs of *E. coli* (A) and *C. metallidurans* (B) when no $PtCl_4$ was added; C, D =Scanning electron micrographs of *E. coli* (C) and *C. metallidurans* (D) when $PtCl_4$ was added; E, F= Pt nanoparticles associated with *E. coli* (E) and *C. metallidurans* (F) biofilms (BSE mode of *E. coli* and *C. metallidurans*

#### 2.4.2.4 Distribution of Pt in Bacterial Biofilms

##### 2.4.2.4.1 FIB-Scanning Electron Microscopy

The distribution of Pt in the columns was examined using FIB-SEM and showed differences based on the species examined. In the columns amended with *E. coli*, Pt nanoparticles were formed within the cells (Fig 2.5 A). The FIB-SEM micrograph in Fig. 2.5 B shows a section through an *E. coli* cell. Energy-dispersive X-ray analysis (EDXA) analysis shows the peak of Pt, confirming its presence (Fig 2.5 C). In contrast, SEM analysis of *C. metallidurans* cells shows the formation of microcrystals aggregates, attached to the *C. metallidurans* biofilm (Fig 2.6 A and B). The presence of $PtCl_4$ appears to induce the production of nanowires in both bacterial columns as seen in Fig 2.7 (A and B). SEM analysis of the sterile control column showed no Pt in the sand column (Fig 2.8 A and B).

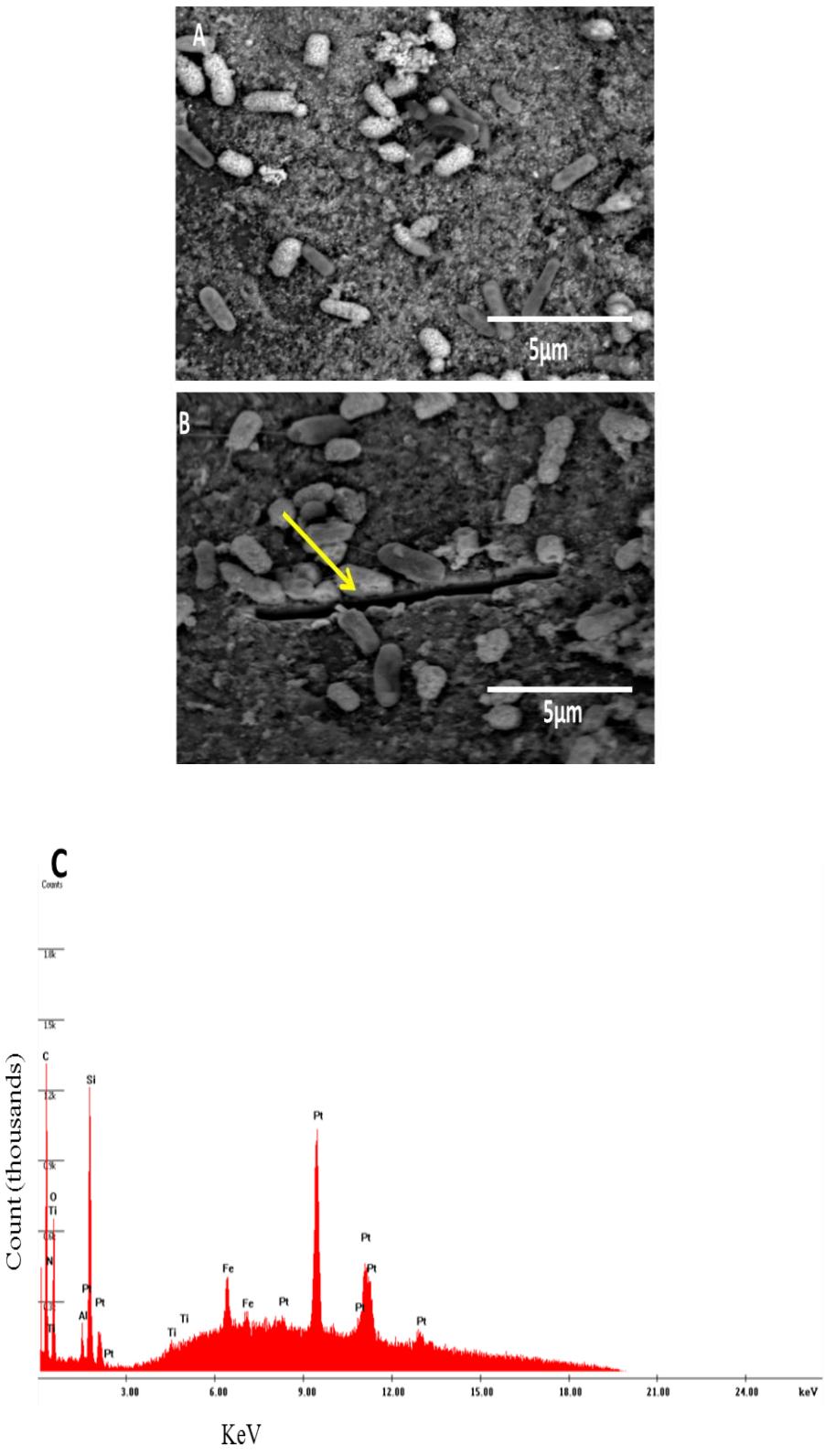


**Figure 2.5:** A=Scanning electron micrograph showing Pt nanoparticles replacing *E. coli* cells. B=FIB-SEM micrograph showing a section through *E.coli* cell. C=Pt analysis of the section assessed using energy-dispersive X-ray analysis (EDXA).

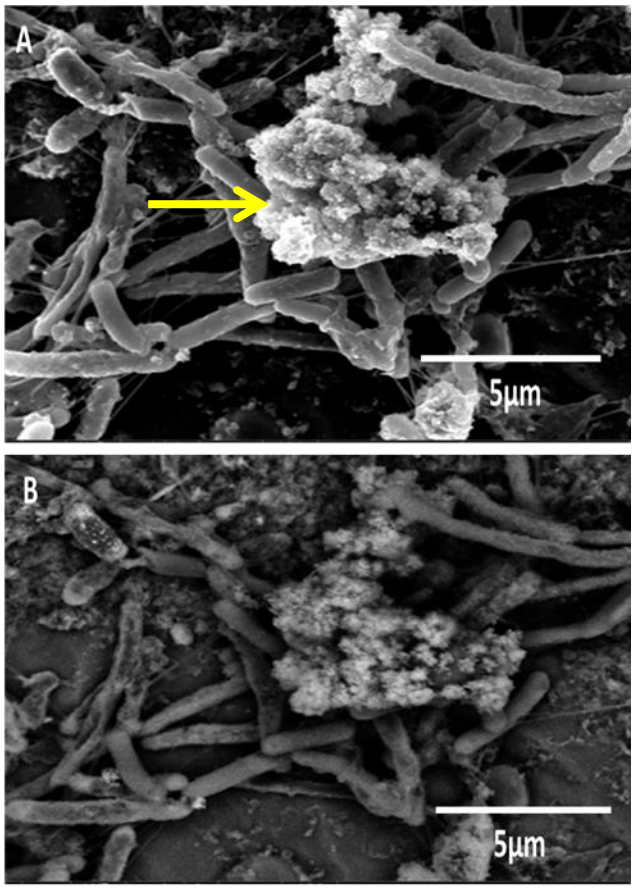


**Figure 2.6:** Scanning electron micrograph of Pt nanoparticles associated with *C. metallidurans* cells and exopolymeric biofilm substances, large Pt aggregates encapsulating biofilm sections; A=SE mode; B=BSE mode.

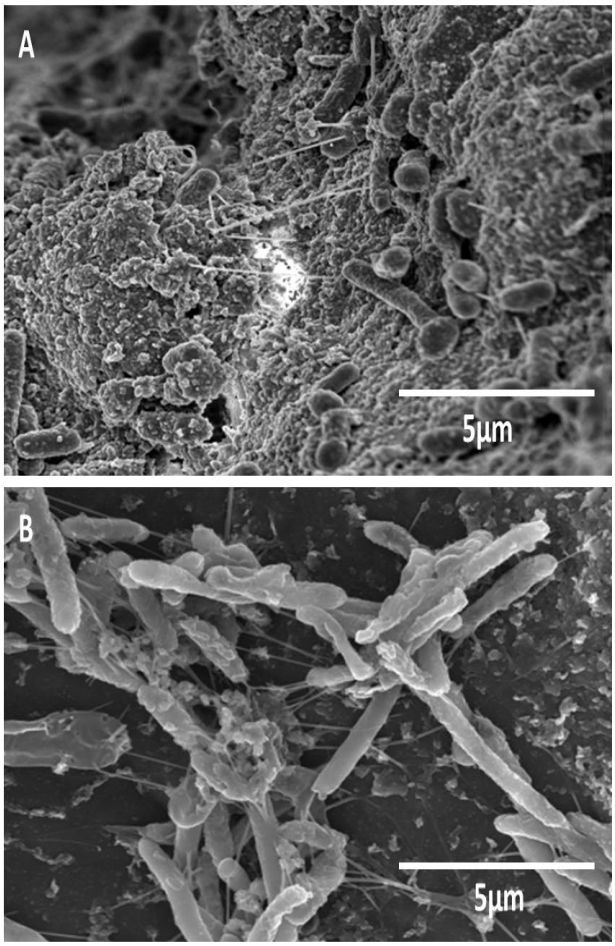


**Figure 2.7:** Scanning electron micrograph showing nanowires connecting cells to extracellular Pt aggregates *E. coli* (A) and *C. metallidurans* (B) cells.

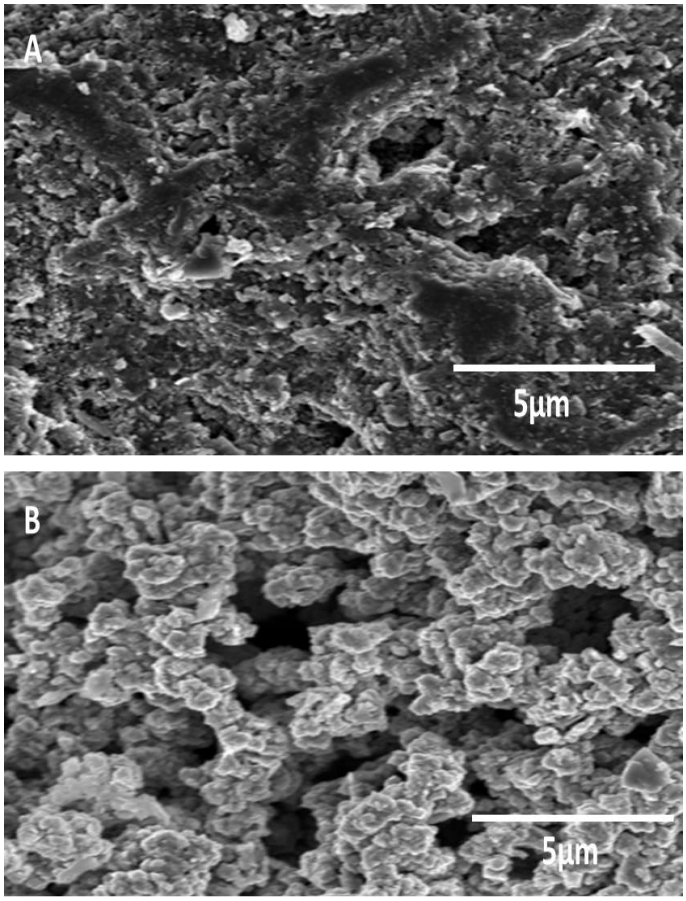


**Figure 2.8:** Scanning electron microscopy of (A) TMM (control column) with no $PtCl_4$ and (B), TMM containing $PtCl_4$ (control Pt).

### 2.4.3 Column extraction

To assess the locational preference of the two microbes within the sand column and the Pt concentration in the column, bacterial cell concentrations and Pt concentrations were examined throughout the entire column, from each 20-mm segment cut at different depths, with 100 mm representing the top of the column and 20 mm the bottom. Viable cell numbers were found to be $4.3\times10^{5}$ ($\pm2.6\times10^{4}$) CFU mL segment$^{-1}$ and $3.3\times10^{3}$ ($\pm3.2\times10^{3}$) CFU mL segment$^{-1}$, on the top and bottom segments respectively of the column amended with *C. metallidurans*. This compares with $2.2\times10^{4}$ ($\pm2.52\times10^{3}$) CFU mL segment$^{-1}$ and zero for the recovery of cells from columns amended with *E. coli* for the top and bottom respectively. Examination of the column for Pt revealed that most Pt was found at the top of both columns, 1.5 ± 0.2 µmol and 1.38 ± 0.07 µmol in columns amended with *C. metallidurans* and *E. coli* respectively Table 2. 3 and 2. 4.

**Table 2.3:** Number of cells extracted from each 20 mm segment cut at different depths 100 mm is the top of the column (n=3)

| **Column Depth (mm)** | ***C. metallidurans* (CFU ml$^{-1}$)** | ***E. coli* (CFU ml$^{-1}$)** |
|---|---|---|
| 100.00 | $4.30\times10^{5}\pm2.60\times10^{4}$ | $2.23\times10^{4}\pm2.52\times10^{3}$ |
| 80.00 | $3.50\times10^{4}\pm4.00\times10^{3}$ | $1.17\times10^{4}\pm1.53\times10^{3}$ |
| 60.00 | $1.20\times10^{4}\pm0.00$ | $7.00\times10^{3}\pm1.73\times10^{3}$ |
| 40.00 | $1.40\times10^{4}\pm0.00$ | $4.67\times10^{3}\pm1.15\times10^{3}$ |
| 20.00 | $3.33\times10^{3}\pm3.20\times10^{3}$ | 0.00 |

**Table 2.4:** Determination of Pt concentration associated with sand grains using *aqua regia* digestion. Pt was extracted from 20 mm segments at different depths as shown in the table where 100 represents the top of the column (n=3).

| Depth of the column (mm) | Pt in the segment of the column (µmol) | | |
|---|---|---|---|
| | ***C. metallidurans* column** | ***E. coli* column** | **Control column** |
| 100.00 | 1.50 ± 0.20 | 1.38 ± 0.07 | 1.30 ± 0.20 |
| 80.00 | 0.70 ± 0.00 | 0.33 ± 0.01 | 0.15 ± 0.01 |
| 60.00 | 0.10 ± 0.04 | 0.16 ± 0.01 | 0.22 ± 0.10 |
| 40.00 | 0.15 ± 0.04 | 0.16 ± 0.00 | 0.13 ± 0.01 |
| 20 | 0.60 ± 0.04 | 0.34 ± 0.01 | 0.35 ± 0.20 |

## 2.5 Discussion

This study examined the biomineralization of Pt by *C. metallidurans* and *E. coli*. In preliminary experiments aimed at determining the optimum concentration for the subsequent column experiment, a Pt concentration of 10,000 µM was selected for the long-term column experiment. This concentration was selected based on maximum Pt uptake by the cells seen in the preliminary experiment. *C. metallidurans* had lower Pt concentrations remaining in solution, perhaps reflecting the higher tolerance and absorbance of *C. metallidurans* to Pt compared with *E. coli*.

In the subsequent column experiment, *C. metallidurans*, grown as a biofilm maintained its cellular viability until the fifth amendment after which the viable cell numbers started decreasing, perhaps due to Pt toxicity, as has been previously reported (Monsieurs et al., 2011). In contrast, *E. coli* cells were found to grow as single cells when exposed to $PtCl_4$. This may indicate that Pt inhibited cell division of *E. coli* cells (Rosenberg et al., 1967, Rosenberg, 1971). The fluctuations observed in the growth patterns of *E. coli* might be attributed to changes in the thickness of the biofilm. An increase in biofilm thickness can reduce the growth of *E. coli* cells, whereas a decrease in thickness favours an increase in cell growth. When the thickness of the biofilm reaches its maximum, more planktonic cells are released. In this study, the biofilm thickness initially increased during incubation supressing the growth of *E. coli*. When the biofilm reached its maximum thickness, the number of planktonic cells increased, resulting in an increase in *E. coli* cells (Fairbrother et al., 2013). However, *E. coli* showed decreased cellular viability from the fourth amendment suggesting that *E. coli* was more sensitive to Pt toxicity than *C. metallidurans*. A previous study showed a reduction in *E. coli* growth when treated with several heavy metals including Cu, Cd, Zn and Hg (1-5 ppm) (Vijayadeep and Sastry, 2014). In contrast, *C. metallidurans* has been reported

to survive in extremely contaminated environments because of the complex transcriptional regulatory network that it has developed to resist heavy metal toxicity (Monsieurs et al., 2011).

In this study both *C. metallidurans* and *E. coli* were found to be involved in the biomineralization of Pt. This is in agreement with previous studies where an accumulation of $PtCl_4$ and subsequent formation of Pt NPs was observed in a range of bacteria (*Pseudomonas* spp., *E. coli* and sulfate reducing bacteria) and fungi (*Neurospora crassa* and *Fusarium oxysporum*) (Lengke and Southam, 2006, Ahemad, 2012) (Rashamuse and Whiteley, 2007). The concentrations of Pt detected in the outlet solutions of *C. metallidurans* and *E. coli* were very low compared to the control; this might be because Pt was absorbed into the bacterial biofilm. This is in agreement with a previous study on the biomineralization of Au (Fairbrother et al., 2013, Nies, 1999). These studies found that biomineralization of Au occurred in the presence of viable biofilms, forming intra- and extra-cellular NPs. The current study shows that *C. metallidurans* biofilms are not only involved in the biomineralization of Au but also in that of Pt, and that *E. coli* is also involved in the biomineralization of Pt, on the basis of their tolerance to Pt.

With regards to pH values, the pH in the control column did not significantly change but slightly decreased during the experiment. In columns amended with *C. metallidurans,* the pH decreased and this might have occurred because of the metabolic activities of *C. metallidurans*. Previous work had linked a pH reduction (to pH 5) to increased metal solubility. It was therefore possible that metal removal efficacy of *C. metallidurans* could be attributed to the reduction in pH (Macaskie et al., 1990). At low pH values, positively charged cell wall ligands adsorb negatively charged metal (Au) complexes onto cells (Nakajima, 2003; Mack et al., 2007). Platinum is mobile under acidic conditions (Barbante et al, 2001) and the transport of Pt in supergene environments primarily occurs in the form of $PtOH^+$ (90%) and $Pt(OH)_2$ (aq) (9.7%) (Azaroual et al., 2001). Given the acidic pH observed in this study, it is possible

that the $PtOH^+$ (90%) and $Pt(OH)_2(aq)$ were the dominant mobile forms of Pt with chloride complexes ($PtCl_4^{2-}$ and $PtCl_3^-$) accounting for less than 1% of the dissolved Pt.

Nano and micro-particles were observed through SEM around the cells of both bacterial species. In *C. metallidurans*, micro-nano-crystals were formed with the cells exopolymer. The Pt miro-crystal aggregates (>5 μm) formed in the biofilm may provide nucleation sites for the accretion and growth of grains due to biomineralization and electrochemical mechanisms (Reith et al., 2016). In contrast, NPs were found within and around the *E. coli* cells. Bacteria are capable of accumulating metals in amounts greater than their own weight due to their high surface area to volume ratio (Beveridge, 1989, Frankel and Bazylinski, 2003). FIB-SEM showed Pt particles in *E. coli* through sectioning of single cells, while in *C. metallidurans,* extracellular particles and crystals were seen growing on the biofilm. EDAX detected metallic Pt; the EDAX pattern showed strong signal energy peaks for Pt atoms in the range 9–13 keV. Furthermore, $PtCl_4$ seemed to enhance the production of nanowires in both *E. coli* and *C. metallidurans,* connecting cells with Pt NPs. These nanowires may be used to transfer excess electrons to remote electron acceptors (El-Naggar et al., 2010, Malvankar et al., 2011, Fairbrother et al., 2013).

In order to assess the distribution of *C. metallidurans* and *E. coli* in the columns, sectioning of the column in 20 mm segments was carried out. This resulted in the observation of dense cells in the top and bottom of the columns with *C. metallidurans;* Pt was also detected in the same areas. In segments with lower number of *C. metallidurans* cells, lower concentration of Pt was also found. This shows an association between the numbers of *C. metallidurans* cells and Pt concentration and biomineralization in this case. It is possible that increases in microbial population (activity) led to more Pt detoxification and biomineralization. This is supported by the findings of Fairbrother (2013), who reported a positive correlation between the microbial activity of *C. metallidurans*, metal (Au) detoxification and

biomineralization. This relationship however is not simple in *E. coli*. Increases in Pt concentrations did not necessarily reflect higher microbial number (potential activity) of *E. coli* since Pt was detected in column sections with no *E. coli*. Furthermore, similar concentrations of Pt were found in column sections with differing numbers of *E. coli* cells. This was perhaps due to the presence of Pt in dead *E. coli* cells. This result suggested that *E. coli* was less resistant to Pt than *C. metallidurans*. Overall the results confirm that microorganisms may facilitate Pt solubilisation, transport and precipitation thus affecting the dispersion and re-concentration of Pt in surface environments. Microbes can also influence redox and pH conditions, and are involved in the formation and secretion of ligands in soils and surface- and ground-waters (Southam and Saunders, 2005, Reith et al., 2008, Gadd, 2010, Reith et al., 2013).

## 2.6 Conclusion

The work reported in this chapter focused on understanding the interactions of Pt IV chloride ($PtCl_4$) with *C. metallidurans* CH34 and *E. coli* biofilms. A difference was found between *C. metallidurans* and *E. coli* in terms of their uptake of $PtCl_4$; *C. metallidurans* had a

greater uptake than *E. coli*. However, both species transformed $PtCl_4$ to micro and nanoparticles. The retention of Pt in *C. metallidurans* column experiments was 87% compared to 75% in *E. coli*. Both bacteria took up $PtCl_4$ at different rates and both were capable of transforming $PtCl_4$ to micro- and nano-particles. The NPs formed within the *E. coli* cells were large but in contrast, the Pt nanoparticles aggregated in the exopolymeric biofilm of the *C. metallidurans* cells. Although the retention of Pt in *C. metallidurans* column experiments was less than that reported for Au (99%) in a previous column study by Fairbrother (2013), it remained high at 87%. This shows that *C. metallidurans* cells are resistant to Pt and are more effective in terms of Pt biomineralization than *E. coli*. *C. metallidurans* may therefore represent a suitable candidate for use in Pt biomineralization studies such as those involving the detoxification of waste Pt complexes. However, the molecular basis of the observed Pt resistance in these microorganisms needs to be investigated in future studies.

# Chapter 3 : Transport of platinum and gold nanoparticles in columns containing different matrices

## 3.1 Introduction

The widespread use of nanoparticles in many modern applications has contributed to their increased release into the environment with associated elevated risks to human health and other living organisms (Fischer and Chan, 2007) (Asharani et al., 2011). Once released into the environment, nanoparticles readily interact with water, soil, air and organisms including plants, algae, fungi and bacteria (Nur, 2013). The reactivity and mobility of nanoparticles is linked to their extremely small size (1-100 nm) and large surface-to-mass ratio (Hristozov and Malsch, 2009). These properties contribute to the observed environmental toxicity of nanoparticles (Auffan et al., 2009, Bhatt and Tripathi, 2011, Dinesh et al., 2012).

An understanding of the mechanisms by which these particles are transported is crucial to evaluating the potential effects of "nanoparticle waste" in different environments (air, water and soil) (Nur, 2013). The risk posed by these particles is related to their presence in the environment and ease of transportation through different environmental sectors e.g. groundwater (Whitley et al., 2013),(Rodrigues et al., 2016). Environmental factors such as temperature, ionic strength, pH, particle concentration and particle properties such as surface charge, size, coating agents, crystallinity, composition and shape affect the rate of transportation, aggregation, dissolution and accumulation of nanoparticles (Dunphy Guzman et al., 2006, Filella and Buffle, 1993, Lecoanet et al., 2004; (Nur, 2013). Soil properties such as pH, organic matter and clay content (Rodrigues et al., 2016), (Rodrigues et al., 2010, Batley et al., 2013, Fang et al., 2009, Duester et al., 2011) together with the characteristics of nanoparticles (size, charge, agglomeration rate and surface area) have been shown to affect the

rate of nanoparticle transportation in terrestrial environments (Darlington et al., 2009, Skebo et al., 2007, Brant et al., 2005) (Darlington et al., 2009).

Platinum nanoparticles (Pt NPs) are of great scientific interest because of their use in biomedical and industrial applications. Platinum derivatives are used in jewellery, as catalysts in automobile industries, in optical devices for the modification of electrodes and enzyme immobilization (Bhattacharyya et al., 2008), (Stepanov et al., 2014). They are expensive but very effective catalysts with higher catalytic rating than micro-particles, because of their increased surface area (Asztemborska et al., 2015). Furthermore, Pt NPs play important roles in reducing the emission of pollutant gases from automobile exhausts (Stepanov et al., 2014) (Bell, 2003, Cheung et al., 2004, Gopal et al., 2013). Platinum NPs can be released from automotive exhaust converters, allowing them potentially to be recovered for re-use (Sorensen et al., 2016). However, they can also be introduced to soil where they have been shown to increase the rate of decomposition of organic pollutants (Bystrzejewska-Piotrowska et al., 2009). In summary, the widespread applications of Pt NPs have contributed to an increase of these nanoparticles being released into the environment.

Similar to Pt NPs, gold nanoparticles (AuNPs) are used for a variety of industrial and biomedical applications in electronics, optical, catalytic and drug delivery systems (Khomutov et al., 2003), (Sharma and Gupta, 2005, Chau et al., 2006, Sau et al., 2001, Hutchings, 2005, Yang et al., 2005, Hong et al., 2006), (Lee et al., 2002), (Diegoli et al., 2008). Consequently, the use of Au NPs has increased exponentially in recent years (Sperling et al., 2008), (Sadowski and Maliszewska, 2011). Au NPs exhibit good catalytic activity due to their high surface area-to-volume ratio and their interface-dominated properties (Lim et al., 2016). Au NPs are considered to be great catalytic converters due to their property for oxidation of carbon monoxide (Welch and Compton, 2006), (Valden et al., 1998, Yu et al., 2003). As observed

with Pt NPs, both soil properties and environmental conditions affect the behaviour and movement AuNPs in the environment (Nur, 2013).

There have been specific scientific investigations of Au and Pt NP transportation in soil and aquatic environments. Gold and Pt NP capping agents, used for stabilizing the particles can influence their mobility and transportation in aquatic environments (Stankus et al., 2010), Stankus et al. 2010). Other factors such as organic matter content, ionic strength of the aqueous phase, grain size of soils and zeta potential limit or accelerate Au and Pt transportation in soil and aquatic media (Kulizhsky et al., 2013, D'Williams, 2014). However, to date there are limited reports on the impact of soil type on Au and Pt mobility. There exist significant gaps in our knowledge regarding Au and Pt NP transportation in natural soils rich in metals, metal complexes and organic matter.

Therefore, the aim of this chapter is to assess the transportation of Au and Pt NPs in different media and natural soils with a focus on determining the impact of soil component and type on their transportation. Gold and Pt NP transportation will be assessed in (i) sand and clay columns, (ii) sand and humic acids columns, (iii) sand and FeO columns and in three natural soils; (iv) sand (v) metal rich soil and (vi) organic matter rich soil. The stability, distribution and transportation rates of these NPs in these media will be determined using a combination of Transmission Electron Microscope (TEM), Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Fourier Transformed Infrared Spectroscopy (FTIR) based approaches.

## 3.2 Methods

### 3.2.1 Soils and columns set up

The two soil types used in this experiment were collected from two states in Australia (South Australia and New South Wales). The soil collected from South Australia was rich in organic matter, FxLane (FLN) soil while the second soil type collected from New South Wales was Jamberoo or JBR soil, an iron rich soil. The location and properties of these soils are provided in Chapter four of this thesis. The soils were air-dried, homogenized, sieved (<2 mm) and stored at room temperature until the desired experiments were performed. The sand particles, clay particles, humic acid powder and FeO compound which were used in the column set-up were provided as a gift from Dr Frank Reith's laboratory, CSIRO, Waite Campus, Adelaide, SA.

Six different media (10 g each) were used for column-based experiments designed to assess the transportation of Au and Pt NPs. The columns set-up were; (i) 90% sand and 10% clay, (ii) 90% sand and 10% humic acids, (iii) 90% sand and 10% FeO, (iv) 100% sand only (v) metal rich soil and (vi) organic matter rich soil. Each column was set up in triplicate and the experiments run for 14 hours.

### 3.2.2 Nanoparticles

The Au and Pt NPs used for the experiments were 50 nm diameter. Gold and Pt NPs suspended in 2 mM citrate solution were purchased from STREM Inc. and Nanocomposix Inc. (Newburyport, MA and San Diego, CA, USA).

### 3.2.3 Column preparation and nanoparticles application

Acid washed sands and soils were dry- packed into columns and washed by 10 pore volumes of Milli-Q water at a constant flow rate of 5 mL $min^{-1}$ (Darcy velocity 0.52 cm $min^{-1}$), further washed with 10 pore volumes of the background electrolyte solution NaCl (0.9 M)

before addition of the NPs (El Badawy et al., 2013). All experiments were carried out at a temperature of 25ºC and pH (5-7). Porosity was determined by dividing the volume of water that was poured into the material by the total volume of the material in column (Matko, 2003). Pore volume was measured using the saturation method, i.e. water was added until the saturation point and measuring the difference between the total volume and the matrix volume (Darlington et al., 2009). Flow rate was measured by amount of water added per minute for each column.

The columns were washed prior to the addition of NPs using an automated peristaltic pump to deliver a constant flow of 1 mL/min (Darcy velocity 0.10 cm/min). An aliquot (0.02 mL) (1 ppm) of AuNPs and PtNPs was added to the top of the appropriate columns followed by a constant background solution flow rate of 1 mL/min to ensure that the solution spread evenly over the soil and sand surface. Samples were collected (~10 mL) every 2 hours for 14 hours; every 2 hours was considered as one pore volume. Experiments were carried out in triplicate and lasted for 14 hours. pH measurements were carried out at the beginning and the end of the experiments for each column.

### 3.2.4 Zeta size and Potential Measurements

A zeta sizer (Malvern Instruments zetasizer_nanoseries Nano-ZS) analyser was used to measure size and zeta potentials of Au NP, Pt NP, sand, sand and clay, sand and humic acid, sand and iron oxide and soil particles prior to the start of the column experiments. During the experiments, the outlet solution collected from each column was analysed in terms of size and zeta charge of its particles. Briefly, the samples (either pre-experimental samples in solution or outlet solutions) were centrifuged at 4000 *g* for 10 minutes and an aliquot of supernatant (1 mL) added to a cuvette using a 1 mL syringe and the rest of the procedure as described by

(Sagee et al 2012) was followed using a zetasizer with Malvern software version 7.11 (Sagee et al., 2012).

### 3.2.5 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was carried out as described by (Ramanathan et al., 2012) . Briefly, samples for TEM were prepared by using a drop-casting technique onto a carbon support film. This was followed by TEM measurement using a JEOL 1010 TEM instrument operated at an accelerating voltage of 200 kV (Ramanathan et al., 2013). TEM was used to confirm the size of the NPs (Au and Pt) prior to the start of the experiments and in the solutions collected at selected points on the breakthrough curves during the experiments.

### 3.2.6 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Samples were collected and centrifuged at 4,000 *g* for 10 minutes and filtered using a 0.45 µm syringe filter before being acidified with 2% of nitric acid. ICP-MS standards were made with concentrations of 0, 10, 20, 50 and 100 ppb for both Au and Pt. Samples for ICP-MS (7700X, Agilent Technologies analysis) were prepared using Milli-Q water containing 2% $HNO_3$. Samples collected at pre-determined elution times from each column during the 14 hours experimental period were subject to ICP-MS to determine their Au and Pt NPs concentrations.

### 3.2.7 Fourier Transformed Infrared Spectroscopy

At the end of each column experiment, samples were taken from the top of the column, dried, ground and analysed using Fourier Transformed Infrared (FTIR) spectroscopy as previously described (Kannan et al., 2013). Measurements were carried out on a Perkin Elmer-Spectrum ATR instrument to identify interactions between NPs and various constituents of soils as previously described (Kannan et al., 2013). The FTIR analyses were carried out on

samples obtained from all the columns at the end of the experimental period. In addition, FTIR assays were performed on control sand samples which had not been spiked with nanoparticles.

## 3.3 Results:

### 3.3.1 Determination of breakthrough curve

Breakthrough curve analyses were carried out to determine the time point and pore volume at which the highest concentrations of the nanoparticles were recovered from the outlet solution. Different columns had different breakthrough curves, dependent on the interactions between the columns content (four different sand media and two soil media), Pt NPs and Au NPs. In the sand column Fig 3.1 (a, b), the highest breakthrough values of 55% (Pt NPs) and 70% (Au NPs) were observed in the 2-pore volume (2 Pv) at an elution time of 4 hours. In sand and clay columns (Fig 3.1 a, b), Pt NPs highest breakthrough values were detected at the 5 Pv (25%) (elution time of 10 hours) and Au NPs at the 4 Pv (30%) at an elution time of 8 hours. In sand and humic acid columns (Fig 3.1 a, b), Pt NPs and Au NPs highest breakthrough values were detected late at 6 Pv (40 and 60% respectively) or 12 hours elution time. In sand and iron oxide column Fig 3.1 (a, b), Pt NPs highest breakthrough values were detected at 5 Pv (20%) (10 hours elution time) and Au NPs at the 4 Pv (40%) or an elution time of 8 hours.

In the Jamberoo soil (JBR) column (Fig. 3.1 c, d), Pt NPs and Au NPs breakthrough points were detected at 3 Pv or an elution time of 6 hours (30 and 70%) respectively. In the FxLane soil (FLN) column (Fig 3.1 c, d), Pt NPs and Au NPs breakthrough points were detected at 4 hours elution time or 2 Pv (40 and 65 %, respectively).

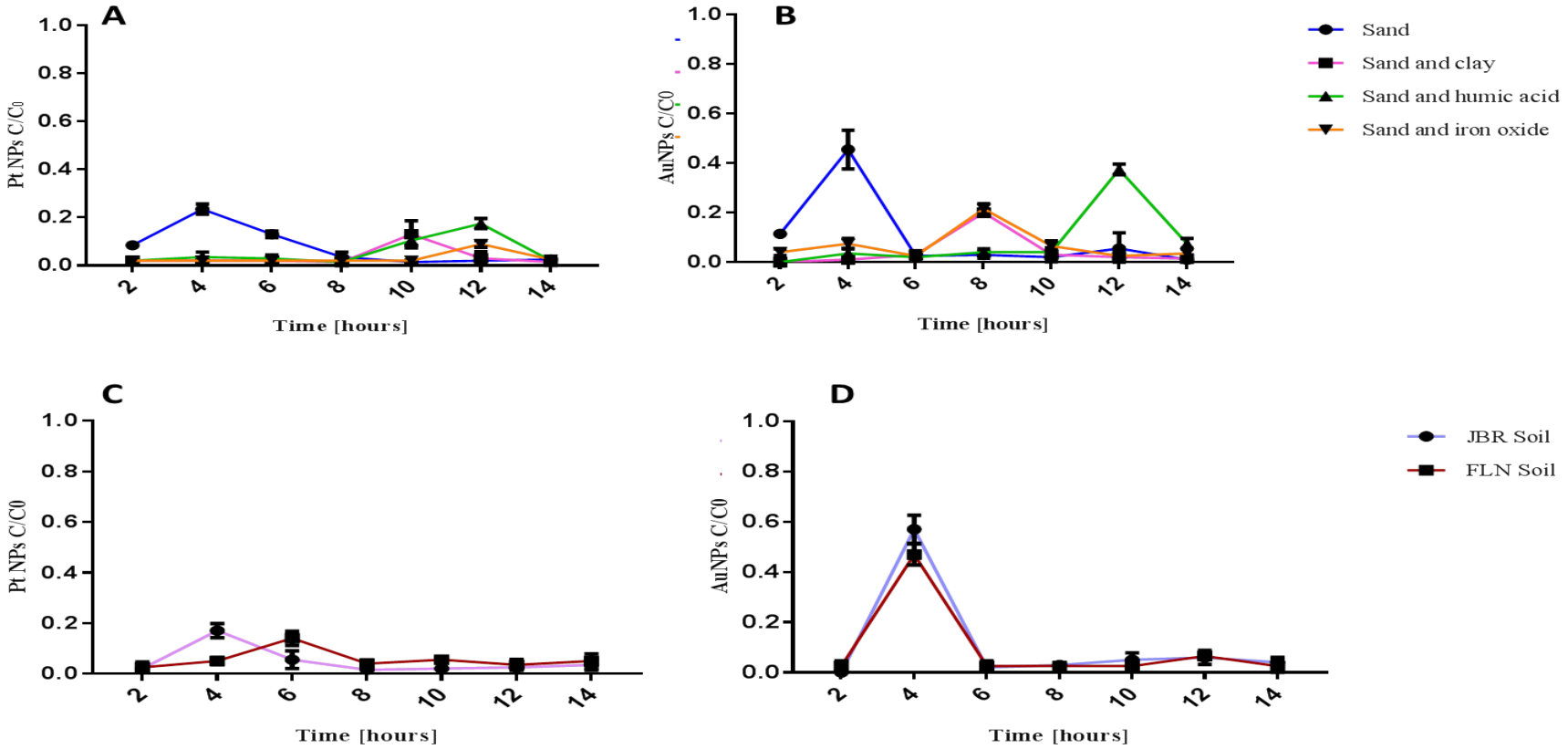


**Figure**
**3.1:** Breakthrough curves of the elution of outlet solution from each column over a 14 hour experimental period

Note A- Pt NP spiked Sand, sand and clay, sand and humic acid, sand and iron oxide columns, B- Au NP spiked Sand, sand and clay, sand and humic acid, sand and iron oxide columns), C- Pt NP spiked Jamberoo and FxLane soils and D- Au NP spiked Jamberoo and FxLane soils. Each pore volume was collected every 2 hours at 0.2 mL/min.

### 3.3.2 TEM and Size distribution

Prior to the addition of Au and Pt NPs at the start of the experiments, the size and Zeta potential of each NP (Au and Pt), sand-clay, sand-humic acid, sand-iron oxide, sand (only), JBR and FLN soil particles were determined (Table 3.1). This ranged in size from 49 nm in Pt NP to 871 nm in FLN soil (Table 3.1). Samples from the highest points at each breakthrough curve of the different columns were subjected to investigation under TEM to determine their particle size and zeta potential. As observed in TEM images, Pt NPs and Au NPs sizes were ~50 nm prior to being added to the columns (Fig.3.2 A, B) (Table 3.1) After adding the NPs to the columns, TEM showed there were significant changes in the size and shape of both Au and Pt NPs following transport through the column. However, the size of the individual nanoparticles could not be determined from the outlet solution as it contained residues formed from the materials of column. The overall size of the NP was at the maximum size at the highest points in each breakthrough curve.

At the conclusion of all the experiments, samples were subject to TEM analysis. In the sand column, TEM investigations showed aggregations of Pt NPs with the size (nm) and zeta potential values of 1090 ± 153, (-19 ± 0.7) and Au NPs 1138 ± 85, (-17 ± 0.7) (Fig 3.3 A, B) (Table 3.2). In the sand and clay columns, Pt NPs and Au NPs were found bound to clays and formed small aggregates (493 ± 38, 1683 ± 6.3 nm and zeta potential -17 ± 0.7-14 ± 0.7 respectively) (Fig 3.3 C, D) (Table 3.2). In sand and humic acid columns, Pt NPs bound to humic acid more than AuNPs (1066 ± 57 nm versus 647 ± 9 nm aggregates) and their charges were -22 ± 0.7, -26 ± 0.7 respectively (Fig 3.3 E, F) (Table 3.2). In sand and iron oxide columns, Pt NPs bound partially to iron oxide column materials whereas Au NPs form larger aggregates (1047 ± 4.2 nm, zeta potential -18 ± 0.5) compared with Pt NPs (882 ± 44 (nm, zeta potential -17 ± 0.7) (Fig 3.3 G,H) (Table 3.2).

In JBR and FLN soils, Pt NPs and Au NPs interacted with soil components forming aggregates with soil particles. In JBR soils, Pt formed smaller aggregates (1121 ± 26 nm) than those observed in Au (1531 ± 76 nm). Zeta potentials were -17 ± 0.5 and -18 ± 0.7 for Pt NPs and Au NPs in JBR soil column respectively (Fig 3.4 A, B) (Table 3.2). in FLN soil, Pt NPs bound to soil particles more than AuNPs (1130 ± 7 nm compared to 1000 ± 142 nm) and zeta potential increased (-10 ± 0.4, compared to -8 ± 0.7) (Fig 3.4 C, D) (Table 3.2). Homo and hetero-aggregation were observed in Fig 3.4. The retention percentages showed that Au NPs were more mobile (lower retention %) than Pt NPs in all columns (Table 3.3)

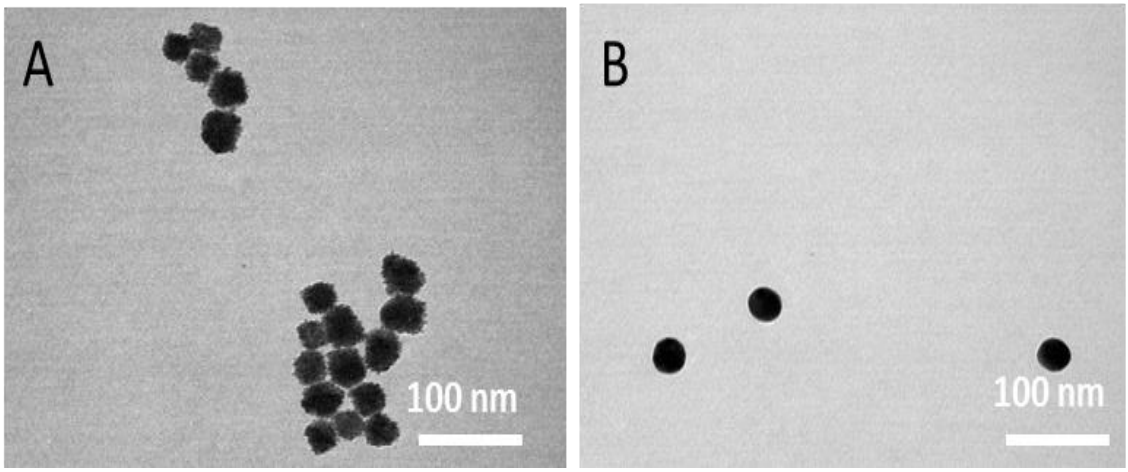


**Figure 3-2:** Transmission Electron Microscopy image of Pt NPs (A) and Au NPs (B) prior to addition to the columns**.**

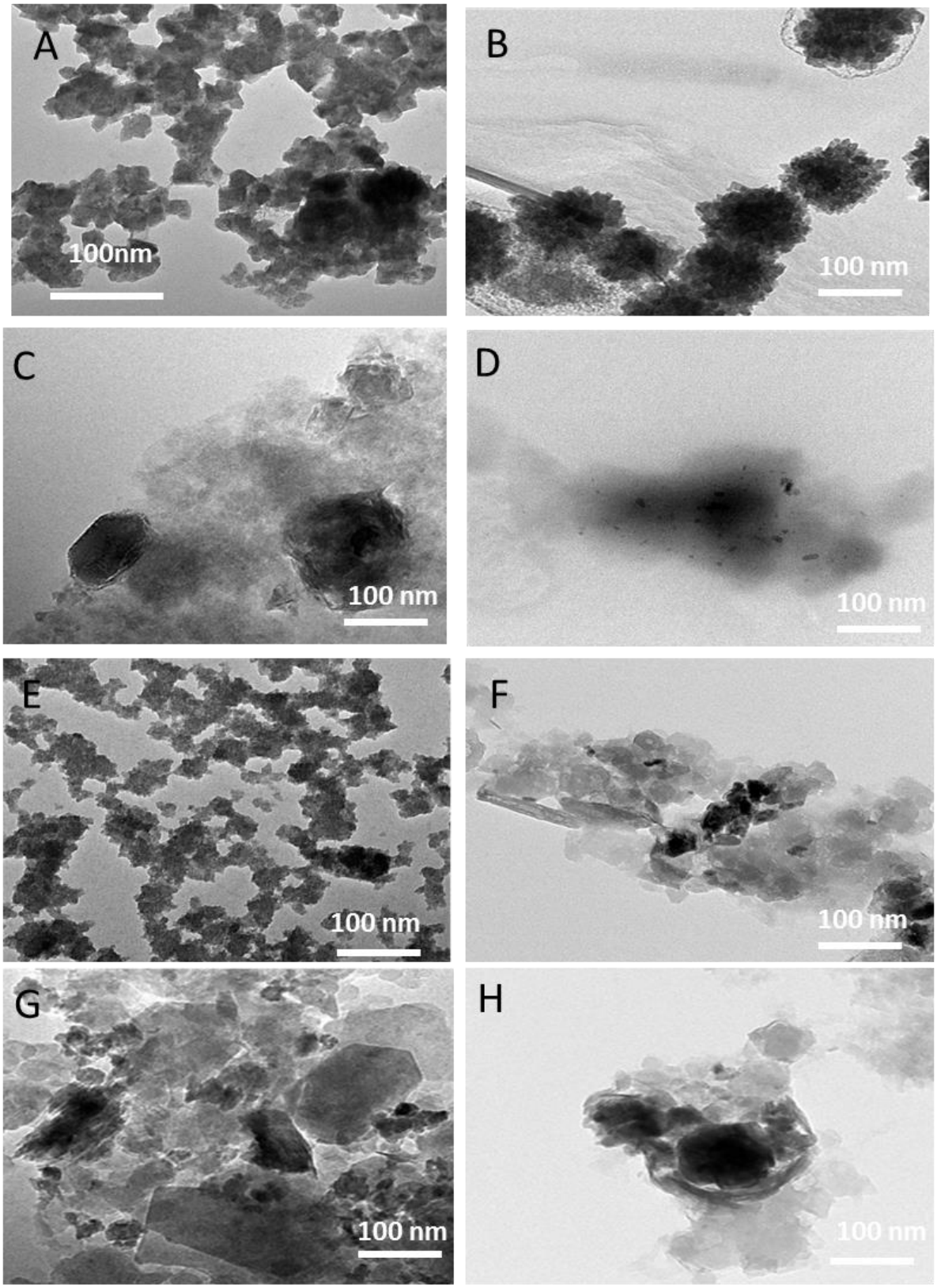


**Figure 3.3**: Transmission Electron Microscopy images of sand column (A- Pt NPs, B- Au NPs) sand and clay column (C- Pt NPs, D- Au NPs), sand and humic acid column (E- Pt NPs, F- Au NPs), sand and iron oxide column (G- Pt NPs and H- Au NPs).

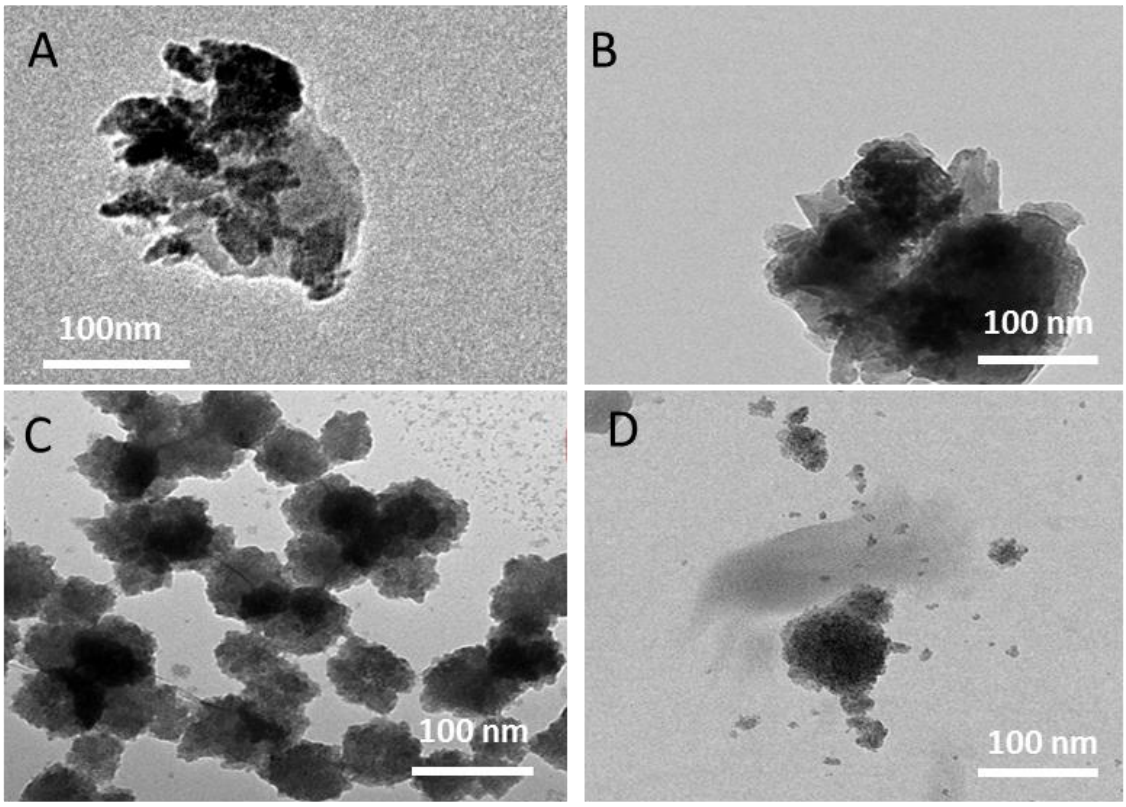


**Figure 3.4:** Transmission-electron microscopy image of JBR soil column (A- Pt NPs, B- Au NPs) and FLN soil column (C- Pt NPs and D-Au NPs).

**Table 3.1**: Size and zeta potential of different materials used in the column prior to the start of the experiments.

| **Materials** | **Size (nm)** | **Zeta potential(mV)** |
|---|---|---|
| PtNPs | 49±1.4 | -46±2 |
| AuNPs | 51±2.1 | -45±2 |
| Sand | 466±4 | -30±0.7 |
| Sand and clay | 354±25 | -34±0.7 |
| Sand and humic acid | 271±8 | -43±2 |
| Sand and iron oxide | 385±14 | -6±0.7 |
| JBR soil | 575±35 | -28±4.9 |
| FLN soil | 871±26 | -29±1.4 |

**Table 3-2:** Size and zeta potential of Pt NPs and Au NPs at the highest breakthrough values of each column.

| **Column types** | **Size (nm)** | | **Zeta potential (mV)** | |
|---|---|---|---|---|
| | PtNPs | AuNPs | PtNPs | AuNPs |
| Sand | 1090±153 | 1138±85 | -19±0.7 | -17±0.7 |
| Sand and clay | 493±38 | 1683±6.3 | -17±0.7 | -14±0.7 |
| Sand and humic acid | 1066±57 | 647±9 | -22±0.7 | -26±0.7 |
| Sand and iron oxide | 1047±4.2 | 882±44 | -18±0.5 | -17±0.7 |
| JBR soil | 1121±26 | 1531±76 | -17±0.5 | -18±0.7 |
| FLN soil | 1130±7 | 1000±142 | -10±0.4 | -8±0.7 |

### 3.3.3 Fourier transform infrared (FT-IR) spectroscopy

To elucidate the molecular interactions of various soils with the nanoparticles, FT-IR analysis was performed on the soil samples obtained from the top of the column and non-spiked sand. The main application of this technique is to detect the structure of chemical species and provide qualitative measurement, based on the adsorption and molecular vibration peaks. For ease of analysis, samples from the same column type (for example sand alone column) with Au NP (sand-Au) and with Pt (sand-Pt), Au NP, Pt NP and sand (control sample, no NP added) samples were combined to generate a composite spectrum. The same principle was applied to all other column types. Therefore, Figure 3.5A shows sand-Au samples, sand-Pt samples, sand (control) samples, Au NP samples and Pt NP samples in a composite spectrum.

The results of the FTIR test for pure sand before and after mixing with different components (clay, humic acid, FxLane soil and Jamberoo soil) and nanoparticles (gold, platinum and iron oxide) are presented in Figs. 3.5 (A)–(F). The infrared spectra of pure sand showed its characteristic adsorption peaks at ~1051 and 1161 $cm^{-1}$, due to the stretching vibration for SiO asymmetric bond vibration. Confirming that sand particles contained pure silica as its main composition, sand also showed peaks at 2851.85 $cm^{-1}$ and 2924.64 $cm^{-1}$. These two peaks indicated symmetric and asymmetric $–CH_2$ stretching. Furthermore, Au and Pt NPs did not show any peak in the infrared region.

No significant changes were observed in the FTIR spectra (Fig. 3.5) of sand, sand and clay, sand and humic acids, sand and iron oxide matrices following addition of Pt NPs; similar peaks were observed with and without the Pt NPs (Fig. 3.5). Platinum NPs following with interaction of FxLane and Jamberoo soils showed the similar spectra to the soils only FxLane and Jamberoo soils due to their reduced reactivity. Peaks for SiO asymmetric bond vibration

and symmetric and asymmetric $–CH_2$ stretching were clearly present with no change in the spectra.

However, when Au nanoparticles were mixed with sand, sand-clay, sand-iron oxide and sand-humic acid matrices, the characteristic peaks of sand disappeared due to the reactive nature of Au nanoparticles with sand particles. (Fig.3.5). The IR spectra of FxLane and Jamberoo soil (Fig. 3.5E, F) were similar to the sand spectra, however Au NPs following the interaction with these soils showed reduced peak intensely in the region of SiO asymmetric bond vibration region. This indicated that the Au NPs in these columns were less reactive than in the other columns and showed the peaks arising from these soils.

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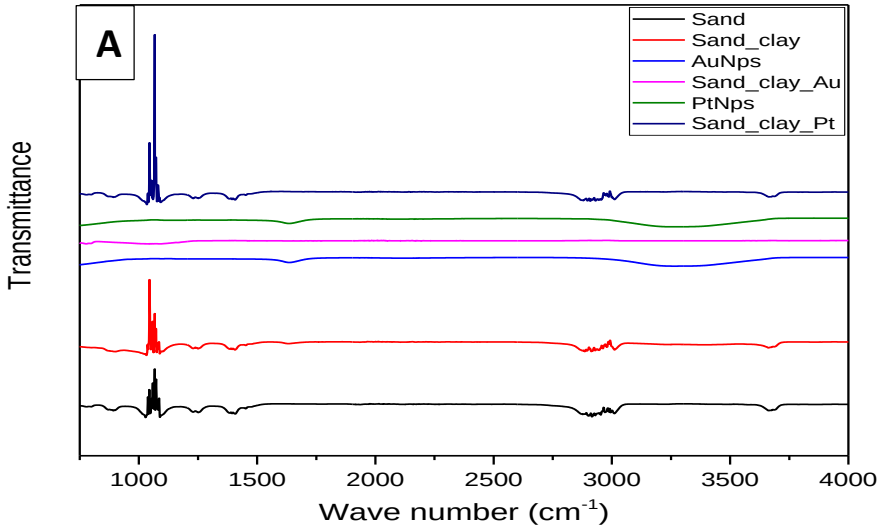
A
Sand
Sand_clay
AuNps
Sand_clay_Au
PtNps
Sand_clay_Pt
Transmittance
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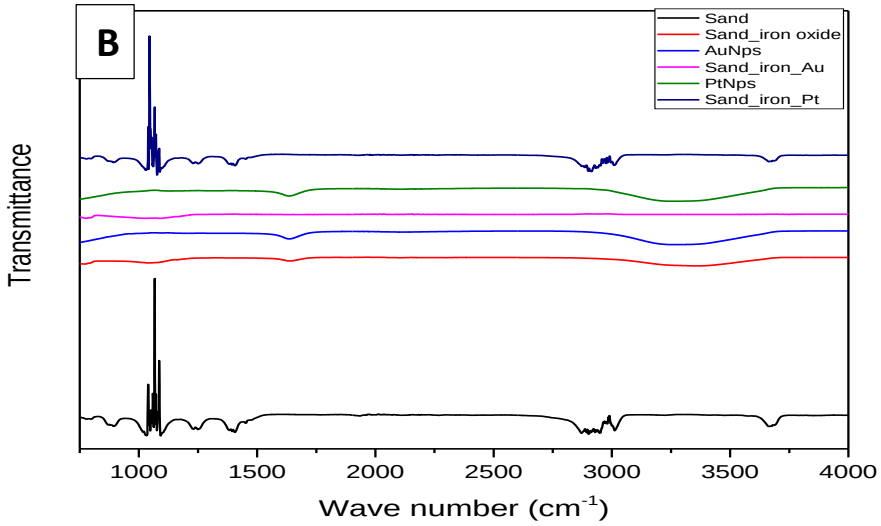
4000
Wave number (cm-1)


B
Sand
Sand_iron oxide
AuNps
Sand_iron_Au
PtNps
Sand_iron_Pt
Transmittance
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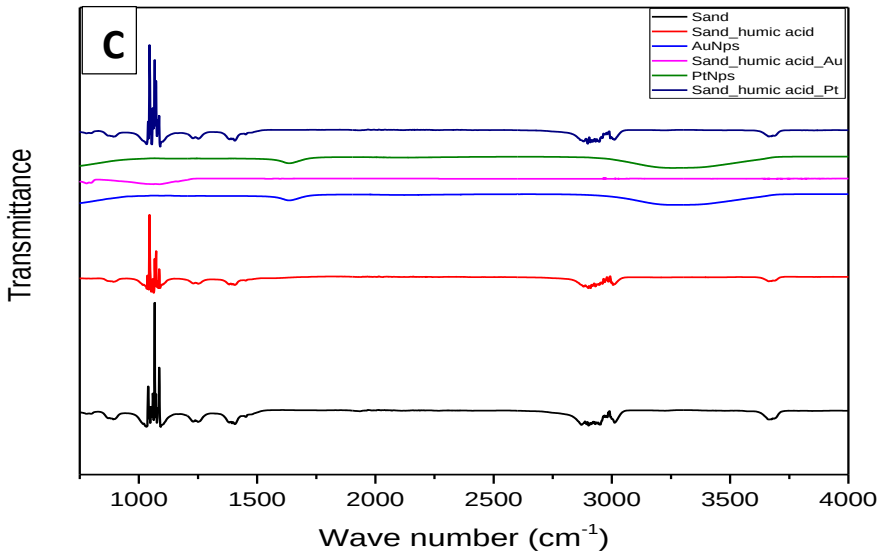
Wave number (cm-1)


C
Sand
Sand_humic acid
AuNps
Sand_humic acid_Au
PtNps
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Transmittance
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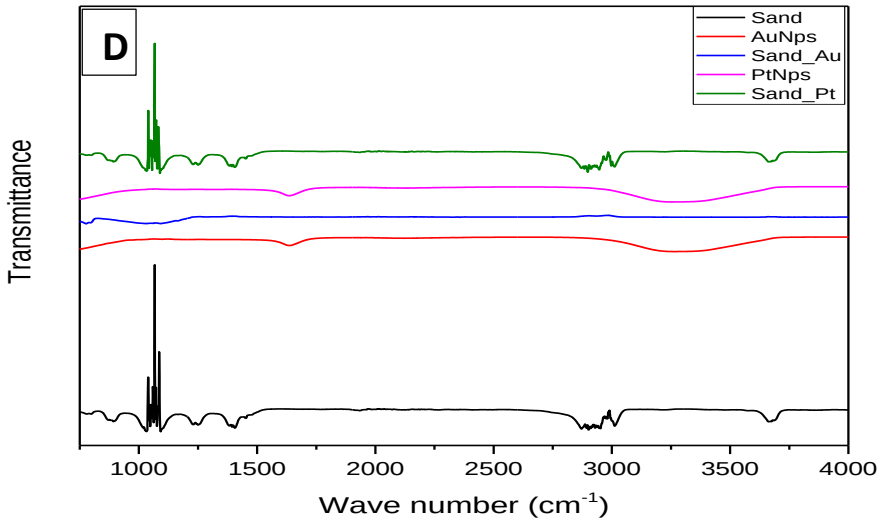
Wave number (cm-1)


D
Sand
AuNps
Sand_Au
PtNps
Sand_Pt
Transmittance
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Wave number (cm-1)

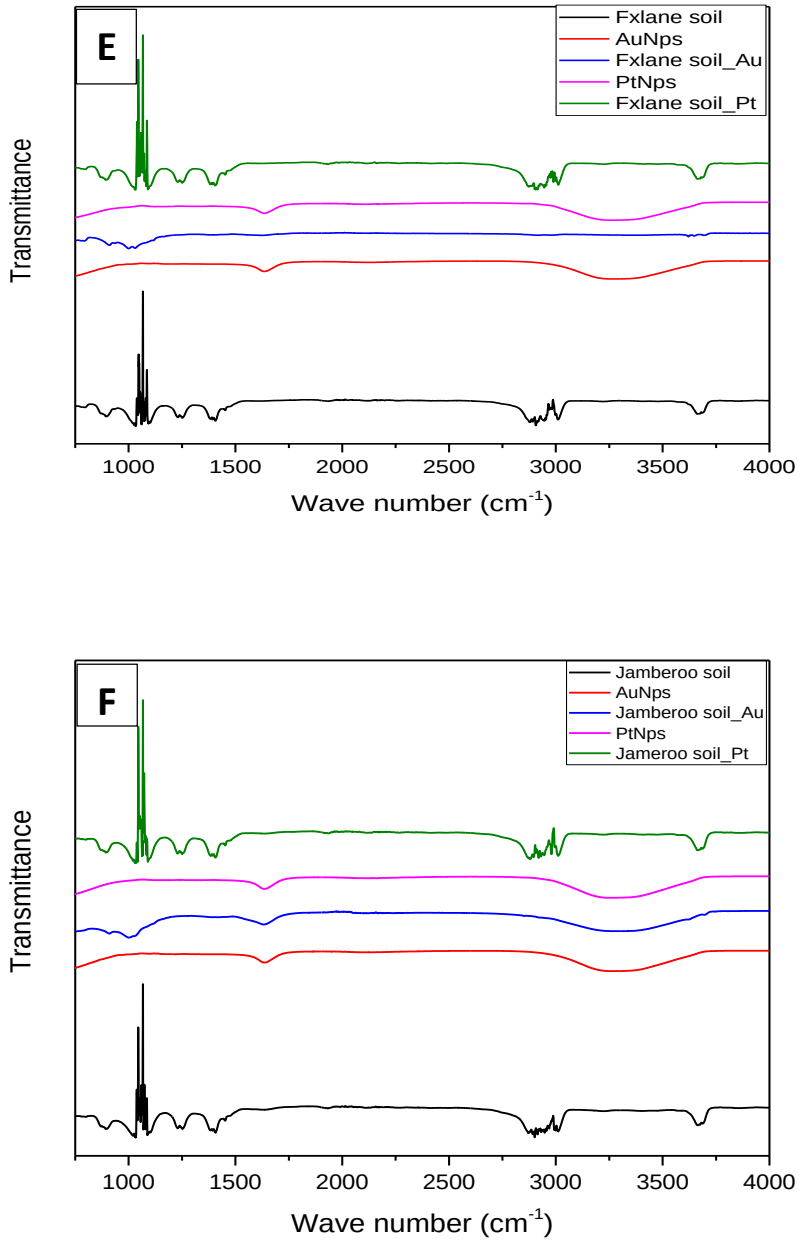



**Figure 3.5:** FTIR spectra of the samples from different column. sand (A), sand and clay (B), sand and humic acid (C), sand and iron oxide (D), JBR soil (E) and FLN soil (F).

**Note:** Figure 3.5A shows sand-Au, sand-Pt, sand (control; no NP), Au NP and Pt NP composite spectrum. Figure 3.5B is a composite spectrum of sand-clay-Au, sand-clay-Pt, sand (control; no NP), Au NP and Pt NP. Figure 3.5C is sand-FeO-Au, sand-FeO-Pt, sand (control; no NP), Au NP and Pt NP composite spectrum. Figure 3.5D is sand-humic acids-Au, sand-humic acids-Pt, sand (control; no NP), Au NP and Pt NP composite spectrum, Figure 3.5E is JBR-Au, JBR-Pt, sand (control; no NP), Au NP and Pt NP composite spectrum and 3.5F is FLN-Au, FLN-Pt, sand (control; no NP), Au NP and Pt NP composite spectrum

**Table 3.3:** Retention of PtNPs and AuNPs in different column (sand, sand and clay, sand and humic acid, sand and iron oxide, JBR soil and FLN soil.

| Column | PtNPs | AuNPs |
|---|---|---|
| Sand | 45% | 30% |
| Sand and clay | 75% | 70% |
| Sand and humic acid | 60% | 40% |
| Sand and iron oxide | 80% | 60% |
| JBR soil | 70% | 30% |
| FLN soil | 60% | 35% |

## 3.4 Discussion:

The pH of the eluted solution at the end of the experimental period was not significantly different from the pH observed at the start of the experiments. Therefore, no relationship between pH and NPs transportation was observed (data not shown). A similar observation has been reported by Wang (2016) which showed no pH effects on the transformation and availability of Ag NPs in experiments which had pH changes ranging from 5.4 to 7.1.

The highest percentages of NP elution from breakthrough curves points were different in each column due to different column components. Nanoparticle transportation is known to be affected by factors such as organic matter, clay and iron oxide as well as properties of the respective soils used as soil particles can form aggregates with the NPs. This appeared to be the case in this study as the size of the particles in the column at the highest breakthrough curve values was up to 3-fold greater than observed in samples at the start of the experiment (pre-NP spiking). TEM showed that Au and Pt NPs formed both homo- and hetero-aggregates in the columns used. However, the size of the aggregates appeared to vary in some instances (as observed in FLN soils).

In sand columns, analysis of the breakthrough curves showed that the highest elution of Pt NPs and Au NPs occurred relatively early (4 hours), although more Au NPs were mobilized than Pt NPs. This indicated that a rapid transportation of NPs had occurred. This could be because of the presence of chloride ions in the background solution (NaCl) as a previous study had indicated that increasing $Cl^-$ concentrations facilitated Ag NP transportation in agricultural soil (Wang et al., 2016). It could also be because sand was the column material as elevated sand concentrations have been reported to enhance Au and Pt NP mobility in soil (Reith and Cornelis, 2017).

In sand columns mixed with clay, higher Pt NPs and Au NPs concentrations were detected at different elution times (10 and 8 hours respectively). This late detection could have been due to the electrostatic binding of NPs to clay particle's which would have slowed down their movement in the column. In addition, saturation of the small pores in the sand-clay column may also have delayed NP transport through the column. The porosity and permeability of the soil is affected by the electrostatic surface charges of the clay. Clay particles would have attracted the ions in the solution forming a clay soil-ion complex or clay soil-ion aggregate, which would slow down the movement of nanoparticles (D'Williams, 2014). This has been observed in some studies where the large aggregates observed were confirmed to be NPs bound to clay (Sagee et al., 2012). In the presence of clay, the cumulative retention of Pt NPs and Au NPs in the column was 60 and 40% respectively. As observed in sand only columns, Au NPs were more mobile than Pt NPs. Clay is known to control (reduce) heavy metal mobility and it is therefore not unusual that an abundance of clay particles can hinder the mobility of Au NPs, Pt NPs and $TiO_2$ (Zhou et al., 2012, Petosa et al., 2010, Hotze et al., 2010, Reith and Cornelis, 2017).

In columns with sand and humic acids, 60% of Pt NPs and 40% of Au NPs (cumulative percentages) were retained because humic acids have a strong affinity for heavy metals and can bind NPs, hindering their transportation. The breakthrough curves also indicated a slower movement of NPs, with the highest breakthrough values observed at an elution time of 12 hours. Gold NPs were also more mobile than Pt NPs. The bioavailability of Cu, Cd, Zn and Pb in the soil has been reported to reduce in the presence of humic acid (Wu et al., 2017, Yates and Wandruszka, 1999). From TEM observations, Pt and Au NPs appeared to be bound to the humic acids, perhaps due to the interaction of humic acid (high in organic content) with these particular NPs. Dissolved organic matter plays a key role in the transport of Au NPs, Pt NPs and Ag NPs as it directly adsorbs nanoparticles, slowing down the deposition of coated hetero-

aggregates (Cornelis et al., 2013, Reith and Cornelis, 2017). Gold is also known to bind strongly to organic compounds (Boyles, 1979) (Gray et al., 1998). Organic acids such as humic acid can reduce gold ions and promote the formation of Au NPs which are then immobilized by these acids (as was the case for the NPs in columns used for this study) (Ling, 1969, Ong et al., 1970, Gray et al., 1998, Kenney et al., 2012, Etschmann et al., 2016).

In sand and iron oxide columns, the breakthrough curves showed that the best elution time for Pt NPs and Au NPs was 12 and 8 hours respectively. The cumulative retention percentage of Pt NPs and Au NPs in the column were 80 and 60% respectively. As reported by Carter (1990) and Cornelis (2013), the interaction with iron colloids accelerated NP transport due to size exclusion of Ag NP hetero-aggregates (Carter, 1990, Cornelis et al., 2013). Field and experimental studies have shown a substantial capacity of iron oxide for sorption of mobile gold complexes (Gray and Lintern, 1998, Reith and McPhail, 2006). High sorption and retention of Au NPs by Fe-oxides has also been shown in a recent study (Smith et al., 2015).

In JBR soil columns, breakthrough curves indicated that more Pt NPs and Au NPs were detected after 4 hours, with their cumulative retention percentage in the column 70 and 30% respectively. As observed in other columns, Au NP was more mobile than Pt NP in acidic JBR soils although TEM assays showed that both NPs formed aggregates with JBR soil particles. In FLN soil columns, breakthrough points for Pt NPs and Au NPs were detected after 6 hours and their retention in the column were 60 and 55% respectively. The TEM data derived from the FLN column was different from that of the JBR column; Pt NP aggregation were larger, with NPs bound to organic rich soil particles, whereas Au NPs showed small aggregations. This could have contributed to Au NP being more mobile in FLN soils than Pt NP.

Nanoparticle capping agents represent a factor that can affect the transportation of particles. A study showed that the breakthrough of citrate capped Ag NPs in soil was 10 times faster than in other media such as $H_2$−Ag NPs and PVP (El Badawy et al., 2013). Residual soil chloride and Ag NPs can react together to form silver chloride which forms precipitates with NPs, or coat particles changing the nanoparticle suspension stability and mobility (Li et al., 2010, Huynh and Chen, 2011, Prathna et al., 2011). Chemical interactions between soil residual chloride and Ag NP to form particles coated by a silver chloride layer can occur. If dissolved silver ions are released, the presence of citrate coating on the surface of Ag NPs can act as a barrier between the chlorides on soil minerals and the silver core of the Ag NPs, leading to higher NP transportation. However, it is not clear in this study whether the reaction between the silver and the chloride occurred with dissolved ions that were released from the NP, or whether the chloride ions reacted with silver molecules on the nanoparticle surface. If the latter were the case, the chloride ions would have replaced the citrate coating, in turn causing removal of the NP from suspension and eventual retardation.

Zeta potential is a measurement of the overall charge of the particles in media and it indicates the stability of the particles in the sense that the higher the zeta potential the more stable the particles are. Values that are larger than ±30 mV are considered stable (Ben-Moshe et al., 2010). In this study, the zeta potential of most of the particles tested at the start of the experiment (pre-spiking with NP) were around or above 30 mV (except FeO with -6 mV). This showed that the particles in the media were inherently stable. However, after spiking with NPs, the zeta potential fell below 30 mV (-8 to -26 mV). When soil-NP interactions reduce the magnitude of the zeta potential, the Au NPs may aggregate rather than remain in suspension as isolated particles. Aggregates typically are less reactive compared to individual nanoparticles and might pass through faster (as observed in some columns in this study) or may clog the pores depending on the ratio of the aggregate size to the pore size (D'Williams, 2014).

To elucidate the molecular interactions of various soils with the NPs, FT-IR analysis was performed on the soil samples obtained from the top of the column and non-spiked sand. FT-IR results showed that the sand (control) samples had peaks at ~1051 and 1161 $cm^{-1}$. Platinum NP-spiked samples still showed these peaks in sand, sand and clay, sand and humic acids, sand and iron oxide, JBR and FLN soils. This indicated that Pt NPs might not have substantially modified the surface properties of these soil particles. In contrast, Au NP spiking eliminated this peak in all samples (except small peaks with soils at ~2700 to ~2900, 3900 $cm^{-1}$ which are characteristic of soil humic compounds) suggesting that Au NPs had interacted with the particles and modified the functional groups of these particles. Gold NPs did not form any peak with either sand or soil columns, probably because Au was more reactive than Pt.

## 3.5 Conclusion

This study has shown that Au NP and Pt NP spiking of different column materials (sand, sand-clay, sand-humic acid, sand-FeO) and natural soils (JBR and FLN) caused a reduction in zeta potentials of their particles rendering them unstable and prone to aggregate formation. Analysis of particle sizes pre- and post- Au NP and Pt NP spiking showed up to 3-fold increase in particle size as a result of aggregates formation. Transmission Electron Microscopy confirmed that NPs formed both homo- and hetero-aggregates in the columns.

Breakthrough curve analysis showed that in all the tested columns, Au NP was more mobile and had a lower column retention percentage than Pt NP. Further analysis by FTIR showed that Au NP was more reactive than Pt NP. The highest breakthrough curve NP values for Au NP and PT NP were at 2 hours for both in sand columns, 4 hours for both in JBR columns, 6 hours for both in FLN columns, 8 (Au) and 10 (Pt) hours in sand-clay, 8 (Au) and 12 (Pt) hours sand-FeO columns and 12 hours for both in sand-humic acid columns.

# Chapter 4 Assessment of the impact of Au and Pt on the soil microbial community: soil respiration and enzyme activities.

## 4.1 Introduction

Heavy metal pollution is a global problem that poses serious risks to environmental and human health. The ecosystem's response to heavy metals pollution is complex and dependent on many factors such as metal concentrations, physicochemical factors, soil factors, microbial diversity and activity (Stone, 1997, Ehrlich et al., 2015, Lloyd, 2003). The presence of heavy metal pollution can have adverse effects on the soil microbial profile (size, structure and population), and microbial activities leading to lower microbial diversity, reduced soil organic matter decomposition, soil respiration and enzyme activity (Haferburg and Kothe, 2007, Ruhling and Tyler, 1973, Schmidt et al., 2005, Fashola et al., 2016, Smejkalova et al., 2003, Piotrowska-Seget et al., 2005). In humans, exposure to heavy metals may result in developmental abnormalities, cardiovascular diseases, neurological and neurobehavioral disorders, hearing loss, diabetes, hematologic and immunologic disorders and various types of cancer (Tchounwou et al., 2012, He and van Gestel, 2013, Havelkova et al., 2014).

Heavy metals can be divided into 2 groups with respect to their importance in biochemical reactions; essential and non-essential groups. Several heavy metal ions such as those belonging to cobalt (Co), copper (Cu), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn) are required in trace concentrations for biochemical reactions and belong to the essential group (Castaldi et al., 2004, Tchounwou et al., 2012). However, others such as silver (Ag), aluminium (Al), cadmium (Cd), gold (Au), lead (Pb) and mercury (Hg) which have no known biological role are classified as non-essential metals (Bontidean et al., 2004). Both essential and non-essential heavy metals can be toxic to living systems. These metals can exist in the environment in various forms; as native metals, ions, alloys, complexes

and nanoparticles. These forms determine their mobility, bioavailability and hence their toxicity to biotic components of the ecosystem. Gold Pt which are the focus of this study are toxic non-essential metals which can be found in the natural environment (Fashola et al., 2016) in these multiple forms.

In recent years, many studies have reported an elevation of Pt (in form of ions, complexes and nanoparticles) in the environment (Wiseman et al., 2016, Reith et al., 2014, Bocca et al., 2003, Schafer and Puchelt, 1998, Artelt et al., 1999, Pawlak et al., 2014). The high concentrations of Pt in environmental matrices is a consequence of human activities and exploitation of Pt ores (König et al., 1992); (Orecchio et al., 2012, Rubino et al., 2009. Gold (Au) exists naturally and gold mining may also contribute to an elevated concentration of gold and associated heavy metals from gold ore in the ecosystem {Durkin, 1994 #209, Edwards et al., 2000, Fashola et al., 2016).

Soil microorganisms are key components of the soil biotic community and as such are an effective indicator of not only the soil's health but also of "soil ill health" caused by pollution. Measurements of soil respiration can therefore be used to assess and predict the impact of contaminants such as pesticides and heavy metals in soil systems (Nielsen et al., 2002, Brookes, 1995, Friedlova, 2010). Heavy metals can reduce soil respiration rates when present at toxic levels. For example, the application of $TiO_2$ and ZnO NPs in soil environments has been reported to cause a significant reduction in soil biomass indicated by a reduction in substrate induced soil respiration (Ge et al., 2011). The addition of Cd, Zn and Cu to soils has also led to a reduction in soil respiration due to their toxicity leading to subsequent undesirable changes in the biochemistry of the soil matrix (Tyler, 1974); (Berard et al., 2014). However, the effect of metal NPs on soil respiration can be varied and is dependent on the type of NP, concentration and the dominant soil microbial groups (tolerant or

sensitive) as observed in a study of Ag NP at various soil concentrations (Samarajeewa et al., 2017).

Extracellular enzymes such as oxidoreductases (e.g. dehydrogenases and catalases) and hydrolases (e.g. acid phosphatase, alkaline phosphatase, urease, arylsulphatase and β-glucosidase) produced by the soil microbial community are involved in the biogeochemical cycling of elements in soils (Bell et al., 2013, Kucharski, 1997, Renella et al., 2006, Kumpiene et al., 2009, Wyszkowski and Wyszkowska, 2009, Dick et al., 2000). Extracellular enzymes activities can therefore be another indicator of the metabolic capacity of microbial communities in different types of soil and can be used to assess the impact of metals (such as Au, Pt, Zn, Ti, and Ag) in different soils (Gomez-Sagasti et al., 2012). Microbial enzyme activities can change metal speciation thereby affecting their bioavailability and ecological impact (Gianfreda et al., 1996, Drijber et al., 2000, Colombo et al., 2002, Nannipieri et al., 2002, Dick et al., 1997, Trasar-Cepeda et al., 2000, Bruins et al., 2000, Gianfreda et al., 2005). However, such effects can be metal -NP and -enzyme specific. Titanium oxide and ZnO NPs have been shown to inhibit soil protease, catalase and peroxidase activities while soil urease activity was unaffected (Du et al., 2011). The effects on enzyme can also be dependent on metal concentrations (Samarajeewa et al 2017).

The effect of Pt NPs and Au NPs on soil enzyme activities has yet to be fully investigated (to the best of the authors' knowledge). Given the fact that previous research had reported that Au was more toxic and mobile than Pt (Brugger et al., 2013, Reith et al., 2014, Etschmann et al., 2016, Reith et al., 2016) it is possible that Pt and Au may affect enzyme activities and soil respiration in different ways. Therefore, the aim of this study was to investigate the impact of Au and Pt on (i) soil microbial respiration and (ii) the activity of seven key soil enzymes, using five different Australian soils.

## 4.2 Methods

### 4.2.1 Soil sampling

Australian soils (0-10 cm) were collected from five different locations across Australia. The basis for selection of the soils were: (1) a high pH (alkaline) soil (Minnipa), (2) a low pH (acidic) soil (BrnGrounds), (3) an iron rich soil (Jamberoo), (4) an organic matter rich soil (FxLane), and (5) a high metal/silt soil (Pinpinio). The approximate location of each soil is shown in Fig. 4.1 while the soil properties are shown in Tables 4.1 and 4.2. Soils were air-dried, homogenized, sieved (<2 mm) and stored at room temperature until the desired experiments were performed**. Soil classified based on Australian classification (Isbell, 2015).**


**Figure 4.1:** Map showing five different locations across Australia (New South Wales (NSW), South Australia (SA) and Victoria (VIC)).

**Table 4.1:** Name, location and classification of soil used in this study

| Soil Name | Soil Code | Location | Classification | GPS |
|---|---|---|---|---|
| BrnGrounds | BGR | New South Wales (NSW) | Acidic soil | NA |
| FxLane, | FLN | South Australia (SA) | Organic soil | NA |
| Jamberoo | JBR | New South Wales (NSW) | Iron rich soil | 34°39′S 150°47′E |
| Minnipa | MNP | South Australia (SA) | Alkaline soil | 32°51′S 135°09′E |
| Pinpinio | PPN | Victoria (VIC) | High silt soil | 36°35′S 142°07′E |

**Table 4.2:** Physio-chemical properties of selected soils: FxLane (FLN), BrnGrounds (BGR), Jamberoo (JBR), Pinpinio (PPN), Minnipa (MNP).

| **Parameter** | **Unit** | **FLN** | **BGR** | **JBR** | **PPN** | **MNP** |
|---|---|---|---|---|---|---|
| WHC | % | 57.3 | 26.5 | 60.3 | 43.7 | 40.7 |
| Clay | % | 15.9 | 17.2 | 15.9 | 16.0 | 14.9 |
| Silt | % | 11.9 | 6.46 | 4.4 | 32.0 | 1.71 |
| Sand | % | 72.1 | 76.4 | 79.6 | 52.0 | 78.1 |
| pH | | 7.49 | 4.96 | 5.66 | 8.0 | 8.01 |
| EC | ds/m | 0.25 | 0.03 | 0.06 | 0.22 | 0.12 |
| N | % | 0.55 | 0.23 | 0.51 | 0.14 | 0.11 |
| TC | % | 6.16 | 5.40 | 6.90 | 1.30 | 1.02 |
| Na | % | 0.02 | 0.00 | 0.01 | 0.04 | Nd |
| Mg | % | 0.44 | 0.05 | 0.11 | 0.44 | Nd |
| Al | % | 2.05 | 0.73 | 4.62 | 2.81 | Nd |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ca | % | 0.70 | 0.03 | 0.05 | 0.42 | Nd |
| Fe | % | 1.33 | 0.25 | 5.02 | 2.27 | Nd |
| Cr | mg $kg^{-1}$ | 19.1 | 7.32 | 9.91 | 27.3 | Nd |
| Mn | mg $kg^{-1}$ | 91.6 | 7.21 | 265.4 | 254.1 | Nd |
| Co | mg $kg^{-1}$ | 1.5 | 0.22 | 6.07 | 7.82 | Nd |
| Ni | mg $kg^{-1}$ | 4.78 | 0.80 | 8.5 | 12.2 | Nd |
| Cu | mg $kg^{-1}$ | 4.76 | 7.61 | 139.3 | 9.60 | Nd |
| Zn | mg $kg^{-1}$ | 17.9 | 9.34 | 46.2 | 22.6 | Nd |
| As | mg $kg^{-1}$ | 2.56 | 1.41 | 5.98 | 3.40 | Nd |
| Mo | mg $kg^{-1}$ | 0.08 | 0.12 | 0.64 | 0.10 | Nd |
| Ag | mg $kg^{-1}$ | 0.05 | 0.07 | 0.08 | 0.07 | Nd |
| Cd | mg $kg^{-1}$ | 0.04 | 0.02 | 0.03 | 0.02 | Nd |
| Pb | mg $kg^{-1}$ | 6.37 | 12.1 | 15.9 | 11.4 | Nd |
| Pt | mg $kg^{-1}$ | ND | ND | ND | ND | ND |
| Au | mg $kg^{-1}$ | ND | ND | ND | ND | ND |

**ND-not detected**

### 4.2.2 Soil incubation experiment

An aliquot (135 g) of each soil (n = 3) was weighed and spiked with 10 mL of either $NaAuCl_4$ or $PtCl_4$ solutions at appropriate concentrations; 0, 1, 25, 100, 500 and 2,000 mg $kg^{-1}$. The $NaAuCl_4$ and $PtCl_4$ solutions were applied in three doses of 10 mL (measured by weight) and the soils were mixed between dose additions. The soils were left overnight to equilibrate. Excess chloride ($Cl^-$) was removed from the soil using artificial rainwater as described previously (Oorts et al., 2007). After chloride removal, samples were removed from the cups and air-dried at room temperature or at a maximum temperature of 37˚C.

After drying, clumps of soil were broken into smaller fragments and then each sample (135 g) was divided in three replicates of approximately 40 g (per tested concentration per soil type) and placed in Ziploc plastic bags. Equivalent controls for each soil type were set up in triplicate with unamended soil (no metal spiking). The samples were incubated with the bags opened at 25 ˚C at constant humidity in the dark throughout the experimental period. During the incubation, relative humidity was maintained at ~30%. After 180 days of incubation, soil respiration and soil enzyme activities were measured following standard procedures described previously (Campbell et al., 2003) and (Bell et al., 2013).

### 4.2.3 Soil respiration assay.

Micro-respiration or the MicroResp$^{TM}$ method provides 'whole soil' information on the role of microbial communities and measures the production of carbon dioxide released by microorganisms in a short period of time after incubation (Campbell et al., 2003, Wakelin et al., 2013). The MicroResp$^{TM}$ technique directly correlates microbial soil respiration with substrate consumption (activity) following the addition of a specific carbon (C) substrate and helps to detect changes in soil function (Banning et al., 2012).

Microresp$^{TM}$ kits were used to measure the soil respiration in five different Australian soils using the protocol previously developed (Berard et al., 2014). Soil samples (n=165) were placed in the wells of a 96-deep-well microplate (1.2 mL volume) and an aqueous carbon substrate added (substrate-induced respiration (SIR)) (Campbell et al., 2003, Wakelin et al., 2013). The plates were sealed with a colorimetric $CO_2$-trap microplate, which is a detection plate, using a gasket and incubated in the dark at room temperature (~25°C) for 6 h. The detection plate contains a gel-based bicarbonate buffer with an indicator dye that responds to pH change within the gel resulting from carbon dioxide evolution from the soil. The colour change was read after 6 h of incubation on a standard laboratory microplate reader “FLUOstar OPTIMA Microplate reader (BMG LABTECH, Ortenberg, Germany (Campbell et al., 2003).

The carbon substrate used for the SIR toxicological bioassays was D-glucose (25 µL solution, supplied at a concentration of 30 mg C $mL^{-1}$ soil water). The incubation with glucose (6 h, 25ºC) was then performed as described by Campbell et al. (2003) with the total water holding capacity maintained at 60%. The absorbance of the detection microplate was measured at 570 nm.

### 4.2.4 Soil enzyme assays.

The activity of key enzymes involved in the biogeochemical cycling of C, N, P and S in soil was assessed with seven different enzymes; N-acetyl glucosaminidase, phosphatase, β-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, α-D-glucosidase and arylsulphatase. These enzymes were selected based on their key roles (which are summarized in Table 4.3) in the environment. The effects Au and Pt-soil incubation (180 days) on enzyme levels in these soil samples were determined and compared with un-spiked soils (control soil without the addition of Pt and Au) (Fig 4.2 and 4.3).

Aliquots (2.75 g) of each soil in triplicate were weighed and blended with 91 mL of 50 mM Tris base buffer. Aliquots of soil slurries (800 µL) were transferred to 96 well microtitre plates and mixed with C, N, P and S substrates as appropriate (200 µL, 200 µM); eight replicates were prepared for each substrate. Plates were sealed, mixed and incubated at 25 °C for 6 h. After 6 h incubation, plates were centrifuged at 2900 x g for 3 min. Following this, an aliquot of the supernatant (250 µL) was transferred to 96 black flat-bottomed microtitre plates. Plates were read using a fluorometric plate-reader at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Standards of 4-methylumbelliferone (MUB) were routinely included and read at the same wavelength (Bell et al., 2013). Tris buffer (50 mM) was prepared using 9.085 g of Tris base added to 1.5 L of water). Standards were prepared using 4-methylumbelliferone (MUB) by adding 1.7 mg to 100 mL of $H_2O$, creating a 0.1 mM MUB stock standard. Dilutions of this stock (2, 4, 10, 20 and 40-fold dilutions) were prepared. Each

enzyme was prepared in a substrate solution of concentration 0.2 mM in a total volume of 50 mL (Table 4.4).

**Table 4.3:** The function of the seven representative soil enzymes selected for use in this study (Koyama et al., 2013, Dong et al., 2015)

| Enzyme | Abbreviation | Function |
|---|---|---|
| N-acetyl glucosaminidase | NAG | N-enzyme is the catalyst that breaks down the N-rich residues to nitrogen and degrades chitin. |
| Phosphatase | PHOS | Releases phosphate ions from phosphate group. |
| β-D-glucosidase | BG | Releases glucose from cellulose. |
| β-D-cellobiohydrolase | CB | Releases disaccharides from cellulose. |
| β-xylosidase | XYL | Degrades hemicellulose. |
| α-D-glucosidase | AG | Releases glucose from soluble saccharides. |
| Arylsulphatase | ARYL | Degrades organic compounds containing sulphur |

**Table 4.4:** Preparation of enzymes substrates

| Enzyme name | Abbreviation | Concentration mM | Substrate (mg) | $H_2O$ mL |
|---|---|---|---|---|
| 4-methylumbelliferyl N-acetyl-β-D-glucosaminidase | NAG | 0.2 | 2.5 | 50 |
| 4-methylumbelliferyl phosphate | PHOS | 0.2 | 2.6 | 50 |
| 4-methylumbelliferyl β-D-glucosidase | BG | 0.2 | 3.4 | 50 |
| 4-methylumbelliferyl β-D-cellobioside | CB | 0.2 | 5 | 50 |
| 4-ethylumbelliferyl α-D-glucopyranoside | AG | 0.2 | 3.4 | 50 |
| 4-methylumbelliferyl β-D-xylosidase | XYL | 0.2 | 3 | 50 |
| 4-methylumbelliferyl-sulphate | SUL | 0.2 | 3 | 50 |

### 4.2.5 Measurement of pH.

For each soil aliquot (5 g) ultra-pure water (25 mL) and $CaCl_2$ (0.5 M, 0.5 mL) were added and mixed for 1 h using a shaker. The soil samples were then left to settle for 30 min. Electrical conductivity (EC) and pH were then recorded for each soil. The pH and EC were measured using a Hanna Instruments (H11134 pH-electrode equipped with a CyberScan pH 310 meter) following a standard protocol (Gee and Bauder, 1986, Rayment and Higginson, 1992).

### 4.2.6 Statistical analysis

Data was analysed using Graphpad prism 7.02 and SPSS version 23. Multiple T-tests were used to test the significance among controls and treatments representing the different concentrations of Pt or Au. Differences at $P< 0.05$ level (using the corresponding two-tailed P value) were considered to be significant.

## 4.3 Results

### 4.3.1 Effect of Au and Pt on microbial respiration.

The effects of Au and Pt at different concentrations on soil respiration in soil samples from five different locations (Table 4.1) with different characteristics (Table 4.2) were assessed and compared to the soil respiration rate in control (non-amended) soils. All five soils were found to have an initial concentration of Au and Pt that were below detection limits prior to soil spiking with these heavy metals (Table 4.2). The initial soil respiration rates differed substantially among the five soils, with the BrnGrounds soil having the highest $CO_2$ production followed by Jamberoo, FxLane, Minnipa and Pinpinio (Table 4.5).

**Table 4.5:** Initial soil respiration rates(n=16), water holding capacity (%) and pH of the five different soil samples (mmol/g/h) (n = 3)

| Soil Name | Initial microbial respiration (mmol/g/h) | Water Holding Capacity (WHC) % | pH |
|---|---|---|---|
| BrnGrounds | 0.12 ±0.01 | 26.5 | 4.96 |
| FxLane | 0.04 ±0.01 | 57.3 | 7.49 |
| Pinpinio | 0.02 ±0.03 | 43.7 | 7.95 |
| Jamberoo | 0.08 ±0.03 | 60.3 | 5.6 |
| Minnipa | 0.04 ±0.01 | 40.7 | 8.01 |

In BrnGrounds soils (an acidic soil), significant reductions in soil respiratory rates were observed with the addition of 1, 25 and 100 mg $kg^{-1}$ (but not 500 mg $kg^{-1}$) of Au. However, at the highest concentration (2,000 mg $kg^{-1}$), a significant increase in respiration was observed (Fig 4.2.A) ($P<0.05$). The addition of Pt at 1, 25, 100 and 2,000 mg $kg^{-1}$ also caused a statistically significant decrease in soil respiration ($P<0.05$). The reduction in soil respiration

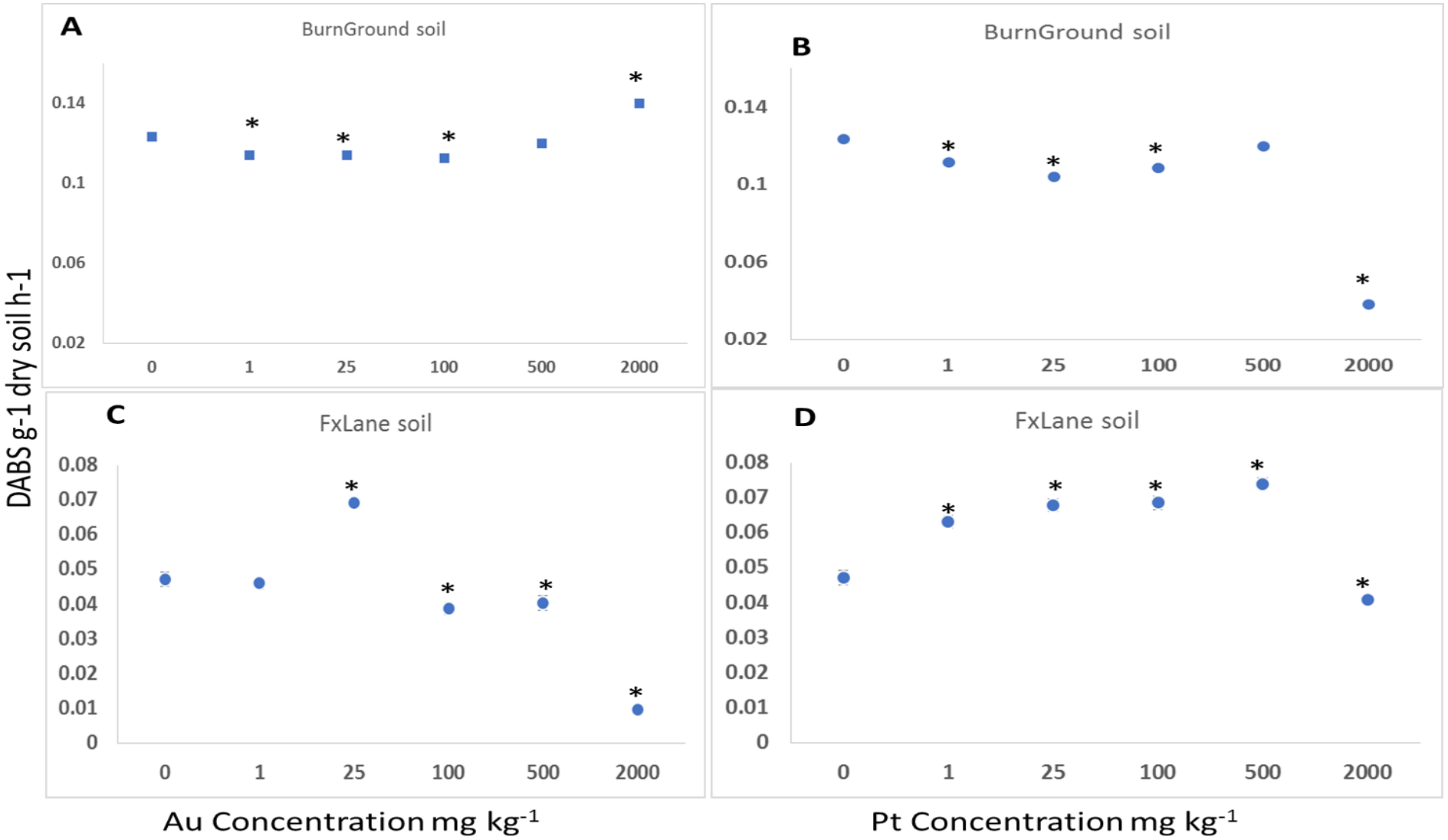
observed at 500 mg $kg^{-1}$ was however not significant. For the BrnGrounds soil samples, the addition of Pt and Au resulted in reduction in respiratory activity except at 2,000 mg $kg^{-1}$ of Au where a significant increase in soil respiration was recorded.

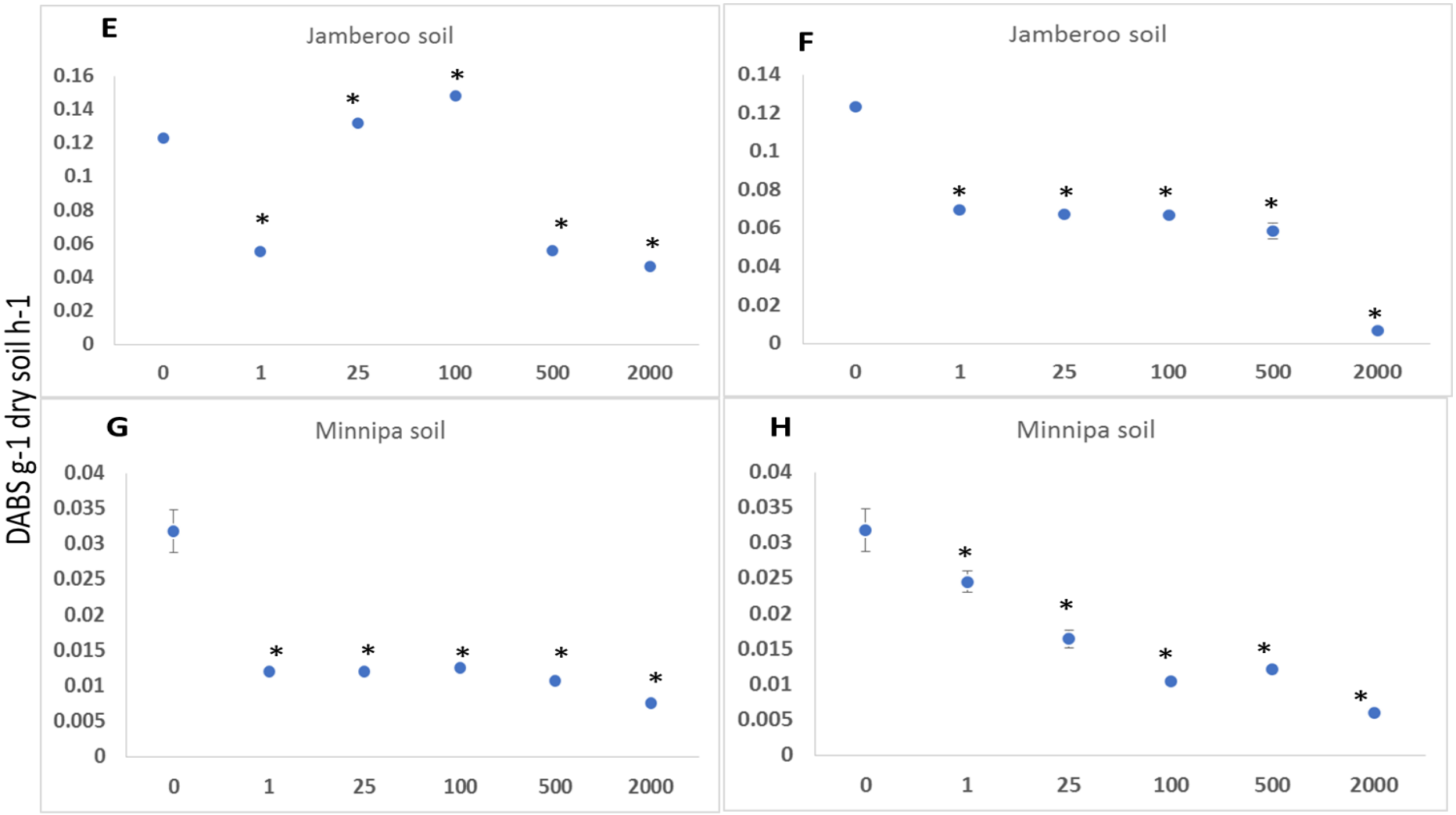
In FxLane soil samples, the addition of Au at a concentration of 25 mg $kg^{-1}$ led to a substantial increase in respiratory activity. However, with all other concentrations (100, 500 and 2,000 mg $kg^{-1}$ of Au) a reduction was observed in soil respiration which were statistically significant (Fig 4.2.C). With regards to Pt, its addition at the four concentrations tested resulted in increased respiratory activities (Fig 4.2.D).

The addition of Au to Jamberoo soil samples caused a significant reduction in soil respiratory activity at Au concentrations of 1, 500 and 2.000 mg $kg^{-1}$ ($P<0.05$) while respiration rates increased at 25 and 100 mg $kg^{-1}$ (Fig. 4.2.E). Significant decreases in soil respiratory activity were observed with the addition of each Pt concentrations, with a reduction reaching up to 80% when 2,000 mg $kg^{-1}$ of Pt was added (Fig.4.2.F).

In Minnipa soils (highest pH), there was a clear trend of reduction in soil respiration in all samples spiked Au and Pt at all concentrations (Fig. 4.2.G, H). The addition of Au at any concentration resulted in significant reductions in respiratory activity ($P<0.05$). The addition of Pt also showed significant reductions in respiration rates in all tested concentrations; greater reductions were observed at higher concentrations of Pt.

In Pinpinio soil samples, the initial soil respiration rate was lower than the respiration rated observed in the other four soil types. The addition of different concentrations of Au caused a significant decrease in soil respiration in most of the tested concentrations with a similar trend being observed in soils amended with Pt with significant reductions observed only at 2,000 mg $kg^{-1}$ of Pt (Fig. 2.1.I, J).


A
BurnGround soil
B
BurnGround soil
C
FxLane soil
D
FxLane soil
DABS g-1 dry soil h-1
0.14
0.1
0.06
0.02
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
1
25
100
500
2000
Au Concentration mg kg-1
Pt Concentration mg kg-1


E
Jamberoo soil
F
Jamberoo soil
G
Minnipa soil
H
Minnipa soil
DABS g-1 dry soil h-1
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
1
25
100
500
2000
Au Concentration mg kg-1
Pt Concentration mg kg-1

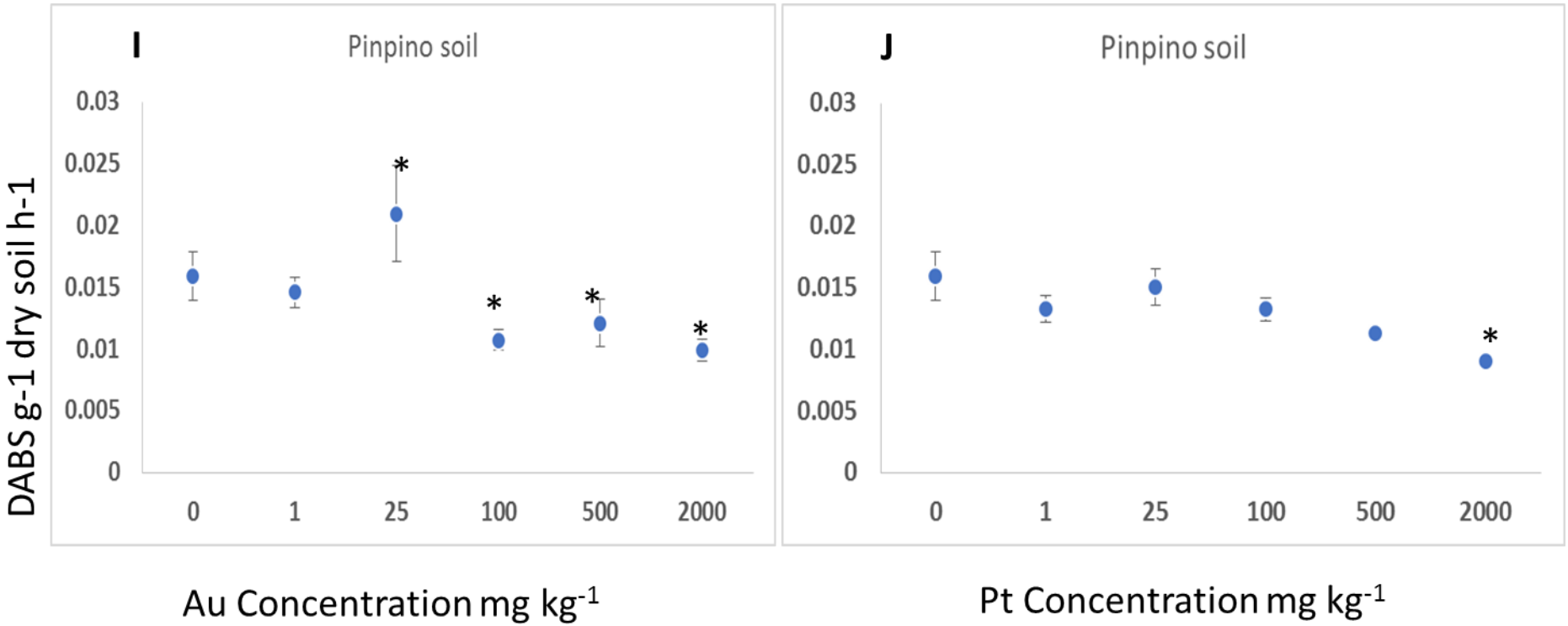



**Figure 4.2 :** Assessment of respiratory activity in soils treated with different concentrations of Au (left) and Pt (right). A, B are BrnGround soil; C, D are FlxLane soil; E, F are Pinpinio soil; G, H are Jamberoo soil; I, J are Minnipa soil. Vertical bars indicate the standard errors of mean of replicates (16 replicates each) at 95 % confidence level. * means significant change at the 0.05 probability compared to values at 0 mg $kg^{-1}$.

**pH of soil**

Over the 180 days of the experiment, the pH increased in all soils treated with Au and Pt; a reduction was observed only in the BGR soil at high concentration of Pt 2,000 mg $kg^{-1}$ with a pH of 4.4 (Table 4.6).

**Table 4-6: pH values of soils spiked with Pt and Au, presented as averages and standard deviation of three replicates.**

| Soil | pH | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 mg $kg^{-1}$ amendment | | 25 mg $kg^{-1}$ amendment | | 100 mg $kg^{-1}$ amendment | | 500 mg $kg^{-1}$ amendment | | 2000 mg $kg^{-1}$ amendment | |
| | | Pt | Au | Pt | Au | Pt | Au | Pt | Au | Pt | Au |
| BGR | 4.5 ± 0.2 | 5 ± 0.2 | 5.3 ± 0.2 | 5.4 ±0.3 | 5.2 ± 0.2 | 5.2 ± 0.3 | 5.3 ± 0.1 | 4.9 ± 0.3 | 5.5 ± 0.1 | 4.4 ± 0.3 | 5 ± 0.2 |
| FLN | 7.8 ± 0.1 | 7.7 ± 0.1 | 7.6 ± 0.2 | 7.6 ±0.1 | 7.9 ± 0.1 | 8 ± 0.1 | 8.3 ± 0.1 | 8 ± 0.2 | 8.4 ± 0.2 | 8 ± 0.3 | 8.3 ± 0.2 |
| PPN | 7.9 ± 0.1 | 7.8 ± 0.3 | 8.3 ± 0.2 | 8.2 ±0.2 | 8.1 ± 0.3 | 7.9 ± 0.3 | 8.1 ± 0.1 | 8.1 ± 0.2 | 8.2 ± 0.2 | 8.1 ± 0.3 | 8.2 ± 0.2 |
| JBR | 5.5 ± 0.2 | 5.4 ± 0.3 | 5.2 ± 0.1 | 5.5 ± 0.3 | 6.5 ± 0.2 | 6 ± 0.3 | 6.2 ± 0.3 | 5.5 ± 0.2 | 6.3 ± 0.1 | 5.7 ± 0.1 | 5.9 ± 0.3 |
| MNP | 7.5 ± 0.1 | 7.8 ± 0.3 | 7.4 ± 0.2 | 7.9 ± 0.1 | 7.5 ± 0.3 | 7.5 ± 0.3 | 7.6 ±0.1 | 7.6 ± 0.2 | 7.7 ± 0.2 | 7.9 ± 0.3 | 7.8 ± 0.2 |

Note: BrnGrounds (BGR), FxLane (FLN), Pinpinio (PPN), Jamberoo (JBR) and Minnipa (MNP).

### 4.3.2 The effect of Au and Pt on enzyme activities in five different Australian soils.

Examination of the five unamended soils prior to soil spiking and incubation confirmed that all soils exhibited enzymatic activities from the all seven chosen enzymes, with the exception of PPN and JBR soils in which no ARYL and no AG activity were detected. The level of enzyme activity was different in each soil (Table 4.7). In general, BGR had the lowest detectable enzyme activities for PHOS, BG, CB, AG, XYL and ARYL and the third lowest activity for NAG. PPN soils had the lowest NAG activities. In contrast, FLN soil samples had the highest detectable enzyme activities for PHOS, BG, CB, AG, XYL and ARYL while JBR soil samples had the highest enzyme activity for NAG (Table 4.7). The following sections present the resulting effects on the activity of seven different soil enzymes. The impact the additions of Au and Pt are presented as a percentage of the initial activities shown in Table 4.7.

**Table 4-7:** Enzyme activities in the 5 soils (nmol/g/h) for seven selected enzymes prior to metal spiking and incubation.

| Soil | NAG nmol/g/h | PHOS nmol/g/h | BG nmol/g/h | CB nmol/g/h | AG nmol/g/h | XYL nmol/g/h | ARYL nmol/g/h |
|---|---|---|---|---|---|---|---|
| BGR | 8.0 ±0.03 | 42.0 ±30 | 3.0 ±0.70 | 2.0 ±0.10 | 1.0 ±0.30 | 1.0±0.05 | 1.0 ±0.10 |
| FLN | 19.0 ±0.80 | 454.0±24 | 97.0±16.5 | 43.0 ±2.5 | 32.0±4.3 | 78.0±0.2 | 2.0 ±6.10 |
| JBR | 120.0±15.7 | 45.0 ±5.90 | 10.0 ±3.4 | 2.0 ±1.03 | 0.0 ±0.0 | 3.0 ±0.1 | 2.0 ±0.90 |
| MNP | 7.0 ±0.90 | 162.0±6.10 | 85.0 ±1.7 | 20.0 ±1.1 | 22.0 ±0.8 | 52.0 ±0.1 | 1.0 ±1.20 |
| PPN | 1.0 ±6.50 | 202.0±6.70 | 85.0 ±6.7 | 12.0±1.04 | 12.0 ±0.4 | 29.0 ±0.2 | 0.0 ±0.00 |

Note: NAG- N-acetyl glucosaminidase, PHOS- Phosphatase, BG- β-D-glucosidase, CB- β-D-cellobiohydrolase, AG- α-D-glucosidase, XYL- β-xylosidase, ARYL- Arylsulphatase).

Three different responses in terms of enzyme activities were observed with the addition of Au and Pt in the five different soils: firstly, inhibition or inactivation of the enzyme activities, secondly, enhancement of the enzyme activities, and thirdly no change in activity (Fig. 4.7). In BrnGrounds soil, Au inhibited all the activities of the seven enzymes evaluated at all the concentrations tested (Fig. 4.7 A). Conversely, Pt increased only NAG enzymatic activity by up to 40% at lower concentrations (1, 25 and 100 mg $kg^{-1}$) and decreased the activities by 80% at higher concentration of 2,000 mg $kg^{-1}$. For the other enzymes (PHOS, BG, CB, AG, XYL and ARYL) inhibition of their activities were observed with the addition of Pt (by 10 to 35 %) (Fig. 4.7 B). Therefore, Au was more toxic to soil enzymatic activities than Pt in alkali BrnGrounds soil.

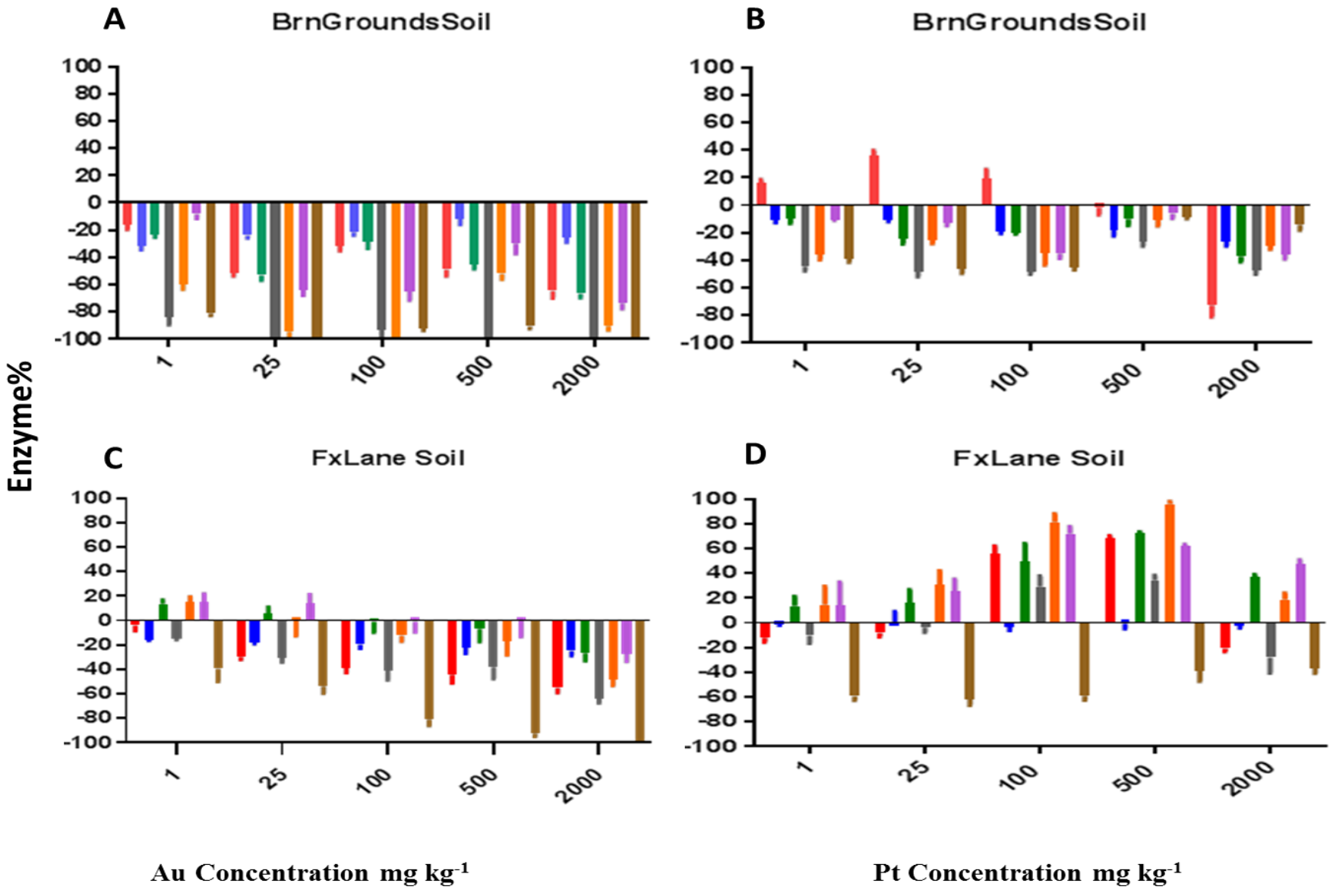
In FxLane soil, the addition of Au resulted in increased and decreased enzyme activities depending on the concentrations of Au added. Low concentrations of Au of 1 and 25 mg $kg^{-1}$ increased the activities of NAG, BG and XYL enzymes by 20%. The addition of higher concentrations of Au (500 and 2,000 mg $kg^{-1}$) resulted in the inhibition of enzyme activities, inhibiting NAG activity by 50%, PHOS by 20% and BG by 20%. Other enzymes activities (CB, AG and ARYL) were also inhibited with their activity reduced by 20, 60 and 100 % respectively (Fig. 4.7 C). However, Pt largely enhanced more enzyme activities at different concentrations than Au. The activities of BG, AG and XYL were enhanced by 20-100% at all the tested Pt concentrations. NAG activities were enhanced only at 100 and 500 mg $kg^{-1}$ and reduced at other concentrations. Even at 2,000 mg $kg^{-1}$ Pt concentration, BG, AG and XYL activities increased (Fig. 4.7 D).

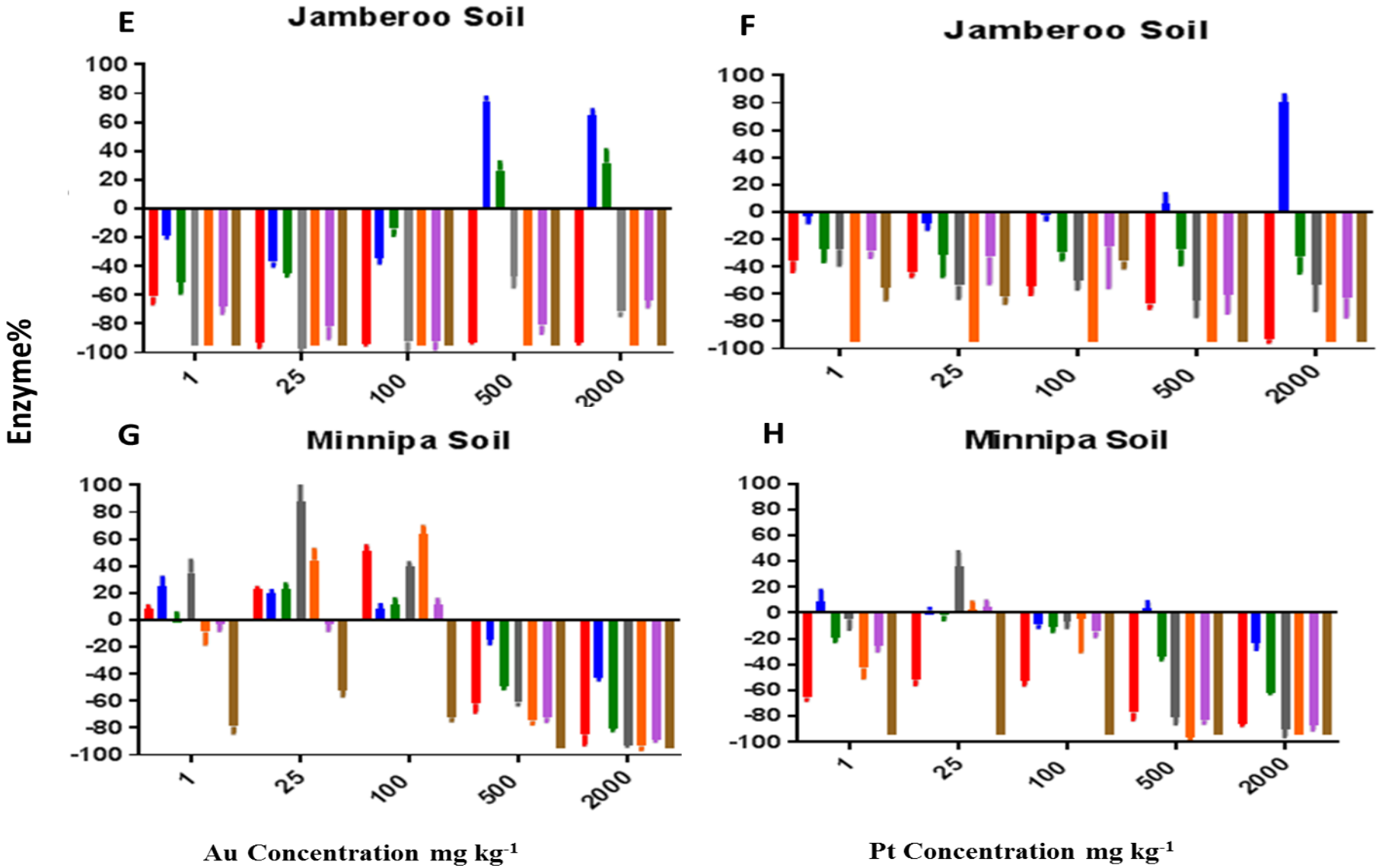
In PPN soil Au increased the activity of XYL and BG by 20% at low concentrations of 1, 25 and 100 mg $kg^{-1}$. At high concentrations of Au (500 and 2,000 mg $kg^{-1}$) these enzymes activities were inhibited by 100 and 80% respectively except for ARYL activity enhancement at 2,000 mg $kg^{-1}$ by up to 20%. On addition of Pt, NAG, PHOS and XYL activities increased

at most tested concentrations while inhibiting the activities of other enzymes. A high Pt concentration (2,000 mg $kg^{-1}$) increased ARYL activity by 20%. For other enzymes (BG, CB, AG, XYL), the addition of Pt inhibited their activity by up to 90% (Fig.4.7 E,F).

In JBR soil, high concentrations of Au (500 and 2,000 mg $kg^{-1}$) increased the activity of PHOS (80%) and BG (50%) but inhibited the other enzymes (NAG, CB, AG, XYL and ARYL) by almost 100 %. All enzyme activities were inhibited at the other tested concentrations. High concentrations of Pt (2.000 mg $kg^{-1}$) increased PHOS activity by 90% but inhibited the other enzymes (NAG, BG, CB, AG, XYL and ARYL) by 100%, 30%, 50%, 100%, 70% and 100%, respectively (Fig.4.7 G, H). Enzyme inhibition was observed with all other Pt concentrations evaluated.

In MNP soil, Au increased the activity of NAG, PHOS, BG, CB, and AG at low concentrations (1, 25 and 100 mg $kg^{-1}$) by up to 50%, 20%, 25%, 85% and 65%. At higher Au concentrations of 500 and 2000 mg $kg^{-1}$ these enzymes were inhibited by almost 100 %. A low Pt concentration of 25 mg $kg^{-1}$ increased the activity of CB by 40%, whereas the other enzyme activities (NAG, PHOS, BG, CB, AG, XYL and ARYL) were inhibited by 80%, 40%, 95%, 100%, 95% and 100%, respectively (Fig.4.7 I ,J).


A
BrnGroundsSoil
B
BrnGroundsSoil
C
FxLane Soil
D
FxLane Soil
Enzyme%
100
80
60
40
20
0
-20
-40
-60
-80
-100
1
25
100
500
2000
Au Concentration mg kg-1
Pt Concentration mg kg-1


E
Jamberoo Soil
F
Jamberoo Soil
G
Minnipa Soil
H
Minnipa Soil
Enzyme%
100
80
60
40
20
0
-20
-40
-60
-80
-100
1
25
100
500
2000
Au Concentration mg kg-1
Pt Concentration mg kg-1

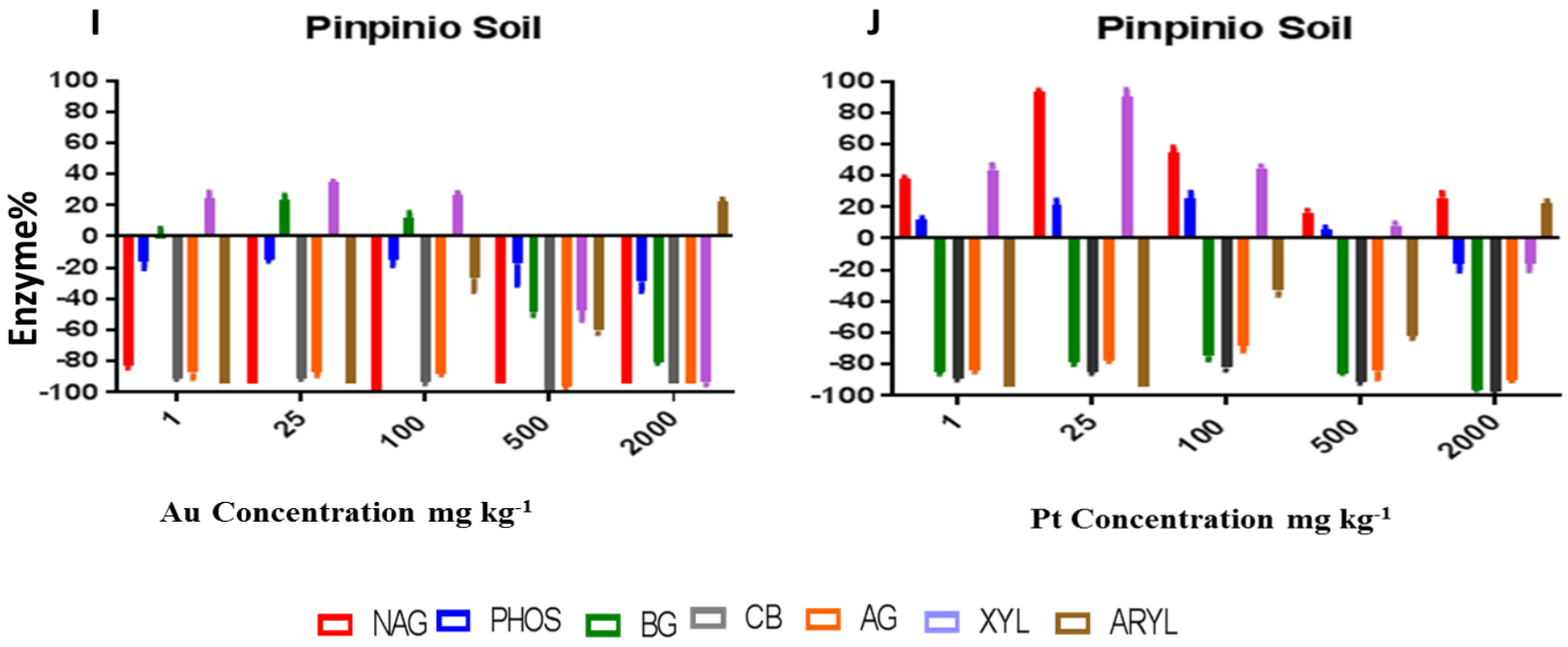



**Figure 4.3:** Soil enzymes activities in soils exposed to (0-2000 mg $kg^{-1}$) Au or Pt concentration. Results are expressed as a percentage of initial activities of each enzymes in untreated soils compared with the addition of Au and Pt (n=8).

Note: NAG- N-acetyl glucosaminidase, PHOS- phosphatase, BG- β-D-glucosidase, CB- β-D-cellobiohydrolase, AG- α-D-glucosidase, XYL- β-xylosidase, ARYL- arylsulphatase).

## 4.4 Discussion:

### 4.4.1 Effects of Au and Pt on soil microbial respiration.

In this study, the addition of Au and Pt showed varied effects on soil microbial respiratory activity, increasing or decreasing soil respiration in different soils and at different metal concentrations. The soil respiration rates in the BGR (acidic), PPN (metal/silt) and MNP (basic) soils largely decreased following the addition of Au and Pt. Soil respiration rate, an index of soil microbial activity can significantly decrease in polluted soils as a result of the toxicity associated with the pollutants (Lighthart et al., 1983, Laskowski et al., 1994, Cotrufo et al., 1995, Dumat et al., 2006, Verma et al., 2010). Therefore, the decrease observed in soil respiration rates in this study was likely due to Au and Pt toxicity to soil microbial groups. While there are limited reports of Au and Pt effects on soil respiration, such reports when available showed that increasing the concentration of Pt in forest soils decreased soil respiration (Giller et al., 1998, Kalbitz et al., 2008). Significant reductions in soil respiration have been reported in most soils contaminated with heavy metals such as Cu, Zn and Cd (Kaplan et al., 2014, Nordgren et al., 1988, Deng et al., 2015), and so the trend observed in this study is not unusual.

Metal induced ecotoxicity reduce or impair the efficiency of energy use by microbial metabolic processes, with adverse effects on microbial biomass (Valsecchi and Gigliotti, 1995). Heavy metals such as Au and Pt can also cause a reduction in microbial diversity (population) as soil microbial groups unable to tolerate these heavy metals are suppressed or eliminated (leading to a net reduction in soil respiration) (Fliessbach et al., 1994). Whether the soil was acidic, basic or metal/silt rich appeared to have had minimal effects in ameliorating Au and Pt toxicity in this study.

While soil respiration rate decreased in BGR, PPN, MNP soils, it increased in FLN soil with Pt amendments at most concentrations tested. Soil respiration rates also increased in JBR soils following amendments with Au but only at 500 and 1,000 mg $kg^{-1}$ concentration. The reasons for this are not yet clear but it could be related to the soil type, metal concentration and the microbial groups (heavy metal resistant/tolerant) present in the soil. FLN soils were classified as organic matter rich soils and large amounts of organic matter by itself can increase soil respiration (Sassi et al., 2012, McDowell and Sharpley, 2003). Organic matter can also adsorb some heavy metals, forming stable complexes and rendering such metals not bioavailable to the soil microbiota (Violante et al., 2010, Park et al., 2011). Therefore, the organic matter in FLN soil was probably beneficial to soil respiration in that it adsorbed most of the introduced Au while making available ample resources for microbial activities (respiration).

Organic matter rich soils are also rich in fungal population. One study has shown that increases in soil respiration with heavy metal contamination were due to increased fungal contribution to soil respiration probably as result of metal effects on other soil microflora (Fliessbach et al., 1994). Increased soil respiration in the presence of heavy metals such as Pt and Au could also occur as a result of a stress response, caused by increased diversion of energy from growth to maintenance-energy requirements (Anderson and Domsch, 1993, Sawada et al., 2009, Dilly et al., 2008, Masto et al., 2011). For example, the application of sewage sludge with high metal (Pb, Zn, Cr, Cu, Ni, and Hg) content increased respiration and $qCO_2$ in an arable and woodland soil (Fliessbach et al., 1994). In soil samples from pine forestry, Killham (1985) found that high metal load increased $qCO_2$, and Chander and Brookes (1991b), measured higher respiration in soil contaminated with sewage sludge. Therefore, the observed increase in soil respiration in these soils could have been due to a combination of these and other unknown factors. In summary, the hypothesis that the presence of Au and Pt would affect

soil respiration differently was generally observed. However, the response was varied, with both soil type and concentration highlighting the complexity of the interaction of heavy metals with soils (Killham, 1985, Chander and Brookes, 1991).

### 4.4.2 The effects of Au and Pt on soil enzyme activity.

It is well known that soil enzyme activities are greatly influenced by soil properties (Maboeta et al., 2006, Sannino and Gianfreda, 2001); indeed enzymes have been suggested as sensitive indicators of heavy metal toxicity in soil (Xian et al., 2015). Due to the different chemical affinities of the enzymes in soil systems, different metals influence enzyme activities in different ways (Sethi and Gupta, 2015). The amount of inhibition of soil microbial activities is related to the tolerance and adaptation of the microbial community to heavy metals (Moreno et al., 2003).

In acidic BGR soil, all the tested enzymes were inhibited by the addition of Au and Pt (except NAG at 1, 25, 100 mg $kg^{-1}$ Pt concentration), suggesting that under acidic conditions Au and Pt were toxic to most enzymatic activities. Previous studies have shown similar findings; in acidic conditions Cu inhibited enzyme function (Tyler and Mcbride, 1982, Gupta and Aten, 1993, Romić et al., 2014). Also, acidic environments can lead to decreased growth in some microorganisms as more metabolic energy is used for maintenance instead of respiration and enzyme function (Sherameti and Varma, 2010).

In the organic matter rich FLN soil, the presence of Au led to greater inhibition of enzymes activities when compared to Pt application. While many studies have shown that heavy metals inhibit enzyme activities, the type of heavy metals and soil factors such as organic matter may modulate this effect (Karaca et al., 2010, Effron et al., 2004, Doelman and Haanstra, 1989, Giller et al., 1998). In this study, Pt application at 1-2,000 mg $kg^{-1}$ stimulated more enzymatic activities (except in ARYL) compared to Au containing soils. This was

perhaps the result of Pt binding to the organic matter, providing protection from microbial degradation, resulting in the heavy metals not being bioavailable (Hattori, 1996, Post and Beeby, 1996). However, this benefit was heavy metal-specific as the presence of organic matter was of little benefit in Au containing FLN soil. Therefore, organic matter appears to reduce the toxicity of Pt not Au indicating that the latter may be unable to form organic matter-metal complexes, critical to protecting soil activities.

In PPN metal/silt rich soil Au inhibited most of the enzymes compared to Pt enriched soils. As observed in FLN soil, Au was more toxic to soil enzyme activities than Pt in PPN soil samples. In JBR Fe-rich soil both Au and Pt inhibited most of the enzymes confirming the toxicity of these heavy metals to most of the enzymes evaluated in this study. In the alkaline MNP soil, the presence of Au was beneficial to some enzymes as their activities increased whereas Pt inhibited most of the enzymes. While the reasons for these differences were not investigated, it appears that the effect on enzymes was heavy metal-specific suggesting that these metals (Au and Pt) may have different behaviours in different soil types and against specific enzymes. It is possible that this could be related to the solubility of these metals in soils of different pH, heavy metal bioavailability and the indigenous microbial community.

Heavy metals such as Au and Pt are toxic to some enzymes primarily due to their protein-binding capacity (Dick et al., 1997). They can reduce enzymatic activity by interacting with the enzyme–substrate complex, denaturing the enzyme by binding amino acids in enzymes and inactivating them or the microorganisms that produces the active enzymes (Doelman and Haanstra, 1984, Kuperman and Carreiro, 1997, Bandick and Dick, 1999, Kunito et al., 2001). Gold complexes have previously been found to be toxic to the soil microbial community, resulting in the inhibition of enzyme activities as well as causing oxidative stress at the cellular level (Oren, 1999, Reith et al., 2009). An indirect effect is also possible because changes in the community structure can modify enzyme activities (Nannipieri, 1994, Gao et

al., 2010). If Au and Pt were to selectively remove some soil microbial groups, the enzymatic activities related to such groups are likely to be limited or reduced in the absence of soil functional redundancy.

There are many reports of heavy metals inhibiting soil enzyme activity. BG activity was inhibited by heavy metals such as Cu (Haanstra and Doelman, 1991, Deng and Tabatabai, 1995, Wenzel et al., 1995) and PHOS activities was decreased by metals such as Zn, Cu, V, Ni and Cd (Wang et al., 2007, Kandeler et al., 1996, Kandeler et al., 2000); Moreno et al. (2003). N-acetyl glucosaminidase can be inhibited by increasing concentrations of metals (Cd, Cu, and Pb) (Wang et al., 2009) or may be unaffected by the presence of Zn, Cu, and Cd (Sarosiek et al., 2009).

## 4.5 Conclusion

The application of Pt or Au at most of the concentrations evaluated in this study largely caused reductions in soil respiration rates in BGR, JBR, MNP and PPN soils, while in FLN soil, there was some reduction in soil respiration because of Pt application; Au application

caused increases in FLN soil respiration at all tested Au concentrations (except 2,000 mg $kg^{-1}$). In addition, the results obtained in this study showed three different enzymatic interactions with the addition of Au and Pt in five different soils at different concentrations: inhibition or inactivation of the enzyme activities, enhancement of enzyme activities and no change in the activity. Platinum application inhibited all or most of the enzymes tested in BRG, FLN, JBR and PPN soils while largely enhancing enzyme activities in MNP soils (1-100 mg $kg^{-1}$). Gold application on other hand largely inhibited most of the enzymes tested in BGR, JBR and MNP while enhancing their activities in FLN and PPN soils. The explanation for these differential results may be a combination of specific enzyme sensitivity, metal concentration and soil type. A striking feature of the results is the differential response of the microbial community in the presence of Pt and Au at different concentrations in each soil. Generally, heavy metal pollution caused an inhibition of microbial respiration and soil enzyme activities, but different doses of heavy metal and incubation time showed different effects on soil respiration and enzymatic activity. However at low metal concentrations (1 mg $kg^{-1}$), other undetermined factors alongside the applied metals could have caused the observed decrease in activities.

# Chapter 5 The impact of short term exposure of gold and platinum on microbial communities of different Australian soils.

## 5.1 Introduction:

Microorganisms are crucial for the biogeochemical cycling of elements, essential to the normal functioning of the ecosystem (Whitman et al., 1998). Soil microorganisms, in particular, play an important role in the decomposition of organic materials (Wardle and Ghani, 1995) converting complex compounds into simpler and elemental forms making them available for subsequent steps in the cycle. Microorganisms interact with heavy metals in the soil environment. Some heavy metals are required in trace quantities for microbial growth and biological reactions. However, the release of heavy metals in the environment at elevated concentrations can cause a disruption in microbial structure and function in the soil (Chander et al., 2001, Gao et al., 2010).

Microbial responses can be varied and these can include modulation of the metal form which may render it less or more toxic, through alterations in the adsorption and intake of metals via microbial influx systems. Extensive reviews of microbial interactions have been carried out (Gadd, 2010) with adverse outcomes observed in interactions between some heavy metals, proteins (enzymes) and metabolic processes (Gasic and Korban, 2006, Wang et al., 2010). Heavy metal pollution can lead to the loss of sensitive microbial groups with negative effects on ecosystem functioning when those groups are important to nutrient cycling and in the absence of functional redundancy (Piotrowska-Seget et al., 2005). Reductions or shifts in microbial population, diversity and enzymatic activities are associated with short and long-term exposure to heavy metals (Varrica et al., 2003) (Doelman and Haanstra, 1979); (Pereira et al., 2014).

The release of Au and Pt in the environment in elevated concentrations results mainly from human activities (Tchounwou et al., 2012). Gold mines are major sources of environmental pollution, contributing largely to the emissions of metals and metalloids in the environment as well as the release of harmful elements from the tailings and other mine wastes

(Fashola et al., 2016). Similarly, the emission of Pt is elevated in industrialised areas (Ek et al., 2004) (Kalbitz et al., 2008). These metals are distributed in an uncontrolled manner into surrounding environments through water or by wind. For instance, the Pt complex cis-diaminedichloroplatinum(II), commonly known as cisplatin, has been found to inhibit the binary fission in the bacterium *Escherichia coli* by cross linking DNA (Rosenberg et al., 1967). Other Pt complexes, such as Pt-thiosulfate and -chloride, complexes with Low- and high-molecular weight organic acids (LMWOAs) and Pt NP, may also affect biota which in turn influences the solubilisation, transport and precipitation of Pt in surface environment.

Traditional molecular approaches such as Polymerase-chain reaction (PCR), Denaturing gradient gel electrophoresis (DGGE) and Terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing have been used to study the effect of heavy metal on soil microorganisms (Qing et al., 2007, Martinez-Inigo et al., 2009, Kozdroj and van Elsas, 2001, Chodak et al., 2013). However, Next Generation Sequencing (NGS) approaches are available which generate more accurate, higher resolution, detailed information on microbial community's interactions with environmental components and pollutants such as heavy metals when compared with traditional approaches. These NGS approaches can be amplicon based which provides detailed taxonomic information on the microorganisms in the environment being studied or whole genome based (information on microbial taxonomy and function provided). Extensive reviews of NGS tools, platforms, applications and cost are available (Liu et al., 2012b) (Shokralla et al., 2012).

The NGS- based metagenomic approach is now available for examining microbial diversity and its adaptation and evolution (Riesenfeld et al., 2004, Handelsman et al., 2007, He et al., 2010, Hemme et al., 2010). This provides the opportunity to assess ecosystem responses to environmental change. Despite the availability of technologies for the high-throughput

sequencing of microbial communities, data interpretation is still challenging because of the complexity and magnitude of the microbial communities. Low-complexity microbial communities are more suitable for high-resolution, in-depth metagenomic studies (Allen and Banfield, 2005). These low complexity microbial communities are generally found in extreme environments, such as acidic geothermal hot springs and heavily contaminated sites (including heavy metal contaminated sites). There is an increasing number of studies applying NGS to the study of heavy metal-contaminated environments (Keshri et al., 2015, Gołębiewski et al., 2014, Kuppusamy et al., 2016).

Considering the negative impact of the increasing Au and Pt introduction into the environment, it is essential to fully understand their effect on microbial community using high resolution NGS based approaches. Additional research is required to develop these biological exploration techniques into standard tools for use in environmental remediation and biotechnology. To do this and develop a comprehensive bioindicator approach, there is a need to study the metagenomes of metal-rich and background soils, use bioinformatics and multivariate statistics to generate baseline data, identify key microorganisms and functional genes (Reith 2013) involved in metal resistance and transformation for potential biotechnological and remediation applications.

In this study, we have chosen to target 16S rRNA genes, as a means of providing detailed information on microbial community changes associated with Pt and Au introduction into a soil system. Therefore, the aim of this chapter is to assess the change in soil microbial community in response to short-term exposure to Au and Pt using an amplicon based NGS approach on an Illumina platform (MiSeq).

## 5.2 Materials and methods:

### 5.2.1 Soil samples

Four different soil types were selected for use. These were acidic BGR soil, organic matter rich FLN soil, metal/silt rich PPN soil and alkali MNP soil. There were soil supply issues with JBR during the experimental period and the use of this soil type was therefore discontinued. The soil samples used for the experiments described in this section had been amended with either Pt or Au at different concentrations (1, 25 and 100 mg $kg^{-1}$) and incubated for 180 days as described in earlier chapters (chapter 4, section 4.2.2, P 91). Soils with higher

Pt and Au amendments (500 and 2,000 mg $kg^{-1}$) were not used in this study as they were deemed to reflect rare, extreme pollution levels only.

### 5.2.2 DNA extraction

Replicate soil samples (triplicates) were used for DNA extraction which was carried out using PowerLyzer DNA isolation Kits (Qiagen, Australia). Briefly, 0.25 g of soil sample was added to the PowerLyzer glass bead tubes containing glass beads (0.1 mm). Bead solution (750 µL) was added to this mixture, vortexed gently for a few seconds and solution C1 added (60 µL). The mixture was inverted at least 10 times to mix properly and then subject to bead beating for 10 minutes on a bead beater fitted with a Mo Bio vortex adapter. The rest of the protocol as described by the manufacturer was followed. A final DNA elution led to the generation of 100 µL of purified DNA and 5 µL of this was subject to agarose gel electrophoresis to confirm DNA extraction, Thereafter, the DNA was stored at -20$^{o}$C for other downstream applications.

### 5.2.3 Next Generation Sequencing (NGS)

#### 5.2.3.1 Primers, PCR, library preparations and sequencing

Purified DNA samples were sent to AGRF (Australia) for Next Generation Sequencing in Illumina's MiSeq platform. Primer, PCR and library preparations were prepared according to AGRF guidelines. Briefly, the primers used were **ILM_27F_Uv3 (forward)** AATGATACGGCGACCACCGAGATCTACACTATGGCGAGTGAAGAGTTTGATCMT GGCTCAG and **ILM_519R_NNNN (reverse)*** CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG GGGWATTACCGCGGCKGCTG. These primers already have appropriate Illumina adapter sequences attached to them. The reverse primer includes a 12 base Golay barcode (Caporaso et al., 2010). Each sample was amplified with a PCR reaction mix containing 14.55 µL MO BIO PCR water, 0.5 µL dNTPs, 1.25 µL of 50mM

MgCl2, 2.5 µL each of the forward and reverse primers (5 µM final concentration), 1.0 µL genomic DNA, 2.5 µL of ImmoBuffer, and 0.2 µL DNA polymerase (5U/ µL). Reactions were held at 95°C for 10 min to denature the DNA, with amplification proceeding for 35 cycles at 94 °C for 30 s, 55 °C for 10 s, and 72 °C for 45 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. Amplicons were cleaned and quantified using Picogreen dsDNA. Sequencing was carried out using sequencing primers (Read 1 Primer; ACACTATGGCGAGTGAAGAGTTTGATCMTGGCTCAG and Read 2 Primer AGTCAGTCAGGGGWATTACCGCGGCKGCTG) and index primer (CAGCMGCCGCGGTAATWCCCCTGACTGACT. Sequencing was completed on the MiSeq V3 600 cycle chemistry.

### 5.2.4 Metagenomic analysis

Image analysis was performed in real time by the MiSeq Control Software (MCS) v2.5.0.5 and Real Time Analysis (RTA) v1.18.54, running on the instrument computer. RTA performs real-time base calling on the MiSeq instrument computer. The Illumina bcl2fastq 2.17.1.14 pipeline was used to generate the sequence data. The data generated here meet the AGRF quality standards. Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5). Assembled sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8)4 USEARCH2.3 (version 8.0.1623) and UPARSE software. Using search tools sequences were quality filtered; full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using “rdp_gold” database as reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Using Qiime, taxonomy was assigned using Greengenes database5 (Version 13_8, Aug 2013).

### 5.2.5 Data analyses

Next generation sequencing data were subject to analysis using Primer 6 software and Microsoft Excel. Dominant bacterial groups in each soil sample were determined using Class OTU data by dividing the abundance of each OTU with the total abundance of all OTUs in the sample in order to express the selected OTUs as fractions. The values expressed as fractions were converted into charts using Microsoft Excel and as a percentage in the texts of the results.

Assessment of soil bacterial community diversity was carried out using Primer 6 software as described in the software manual (Clarke and Warwick, 2001). Operational Taxonomic Units (OUT) family table was imported in Primer 6 software; the data was standardized and transformed using square root. Shannon Weaver diversity index was calculated using the formula H'= -SUM(Pi*Log(Pi)) and expressed as bar charts. The standardized and transformed data were subject to resemblance analysis using S17 Bray Curtis similarity resemblance measure and cluster analysis applied to generate plot dendrograms using the group average cluster mode. Principal component analysis was carried out on standardized data with appropriate labelled factors as described by (Clarke and Warwick, 2001). SIMPER (similarity percentages) analysis was carried out on standardized and transformed (using the fourth root) OTU family data. Replicate samples were grouped together and compared with other grouped replicates from the same and other soil types. Dissimilarity percentages of samples of interest were extracted from the SIMPER results and used to construct desired tables of dissimilarity percentages between samples with different Pt or Au concentrations.

### 5.2.6 Statistical analyses

Shannon Weaver diversity values were subject to statistical analysis using IBM SPSS statistics version 23. One-way ANOVA analysis was carried out on data that had passed

Shapiro-Wilk normality test with Tukey HSD post hoc tests. P values ≤0.05 were taken as being significant.

Statistically significant differences in family abundances between any selected two samples (Pt and Au at 100 mg $kg^{-1}$) for each of the four soil types (BGR, FLN, PPN and MNP) were determined with the Statistical Analysis of Metagenomic Profiles (STAMP) software package (Parks and Beiko, 2010). Normalized OTU tables at Family level were used. The average of replicate OTU values at desired Pt or Au concentrations for each soil type were imported into STAMP software. Two-sided Fisher's exact test (Fisher, 1958) was used for statistical analysis with p-values (q-values) set at 0.05. Confidence intervals for differences between proportions were calculated using the Asymptotic method(Newcombe, 1998). False discovery rates were corrected with Storey's FDR method (Storey and Tibshirani, 2003) and the results presented as profile plots with extended error bars.

## 5.3 Result:

### 5.3.1 The effect of Pt and Au amendments on dominant groups in the soil bacterial community

The effects of Au and Pt amendments were assessed on the bacterial community in the four different soil types; BGR-acidic soil, FLN-organic matter rich soil, PPN- high metal/silt soil and MNP-alkali soil (Chapter four). For ease of presentation, the data at Class level is presented.

#### 5.3.1.1 The effects of Pt and Au amendments on dominant soil bacterial groups in BGR soil

In the BGR control soil, the relative abundance of *Proteobacteria* was 32 % (most dominant group), followed by *Acidobacteria* at 19 %, *Actinobacteria* at 19% and *Chloroflexi* at 7%. The addition of Pt and Au (1, 25, 100 mg $kg^{-1}$) caused a decrease in the abundance of *Proteobacteria* to 12-16%, which represents a 50% decrease compared to the control. A shift from *Proteobacteria* dominance to dominance by bacterial groups belonging to Kazan-3B-28 (28-41%) was also observed. This was the most dominant community in all the evaluated concentrations of Pt and Au (but was not detected in the original soil (control)). The second most dominant group was WPS-2 (20-40%) in most tested metal concentrations. Therefore, the addition of Au and Pt at different concentrations to BGR soils caused a shift

from the Proteobacterial groups to Kazan-3B-28 and WPS-2 groups (Au) and Kazan-3B-28 and WPS-2/ *Verrumicrobia* groups (Pt) (Fig. 5.1A).

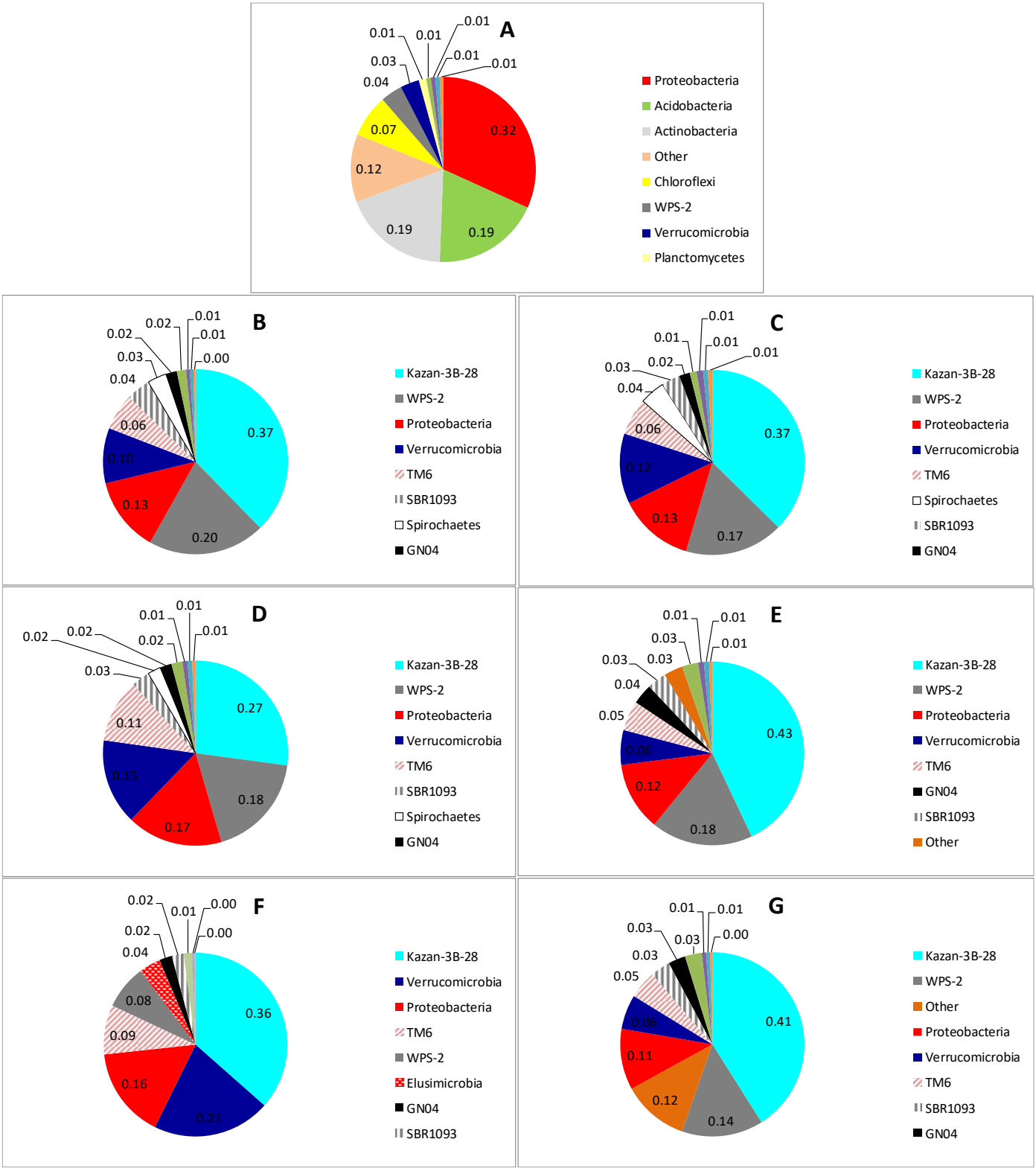



**Figure 5.1:** The class abundance of bacterial communities in BurnGround (BGR) soil spiked with different concentrations (mg kg-1) of $PtCl_4$ and $NaAuCl_4$.

NOTE: A -no Pt or Au added, B- 1 mg $kg^{-1}$ of Pt added, C- 1 mg $kg^{-1}$ of Au added, D- 25 mg $kg^{-1}$ of Pt added, E- 25 mg $kg^{-1}$ of Au added, F- 100 mg $kg^{-1}$ of Pt added and G- 100 mg $kg^{-1}$ of Au added.

#### 5.3.1.2 The effects of Pt and Au amendments on dominant soil bacterial groups in FLN soil

Unlike BRG soils, amendment of FLN soils caused no shift in the dominant bacterial groups. In control and FLN soils amended with Pt and Au at 1, 25 and 100 mg $kg^{-1}$, the dominant bacterial species belonged to *Proteobacteria* and *Actinobacteria* irrespective of heavy metal concentrations (Fig. 5.2). The same top 10 bacterial classes were found in control and Au and Pt amended FLN soil (Fig. 5.2) with a slight increase in the dominance percentages of the top 2 classes.

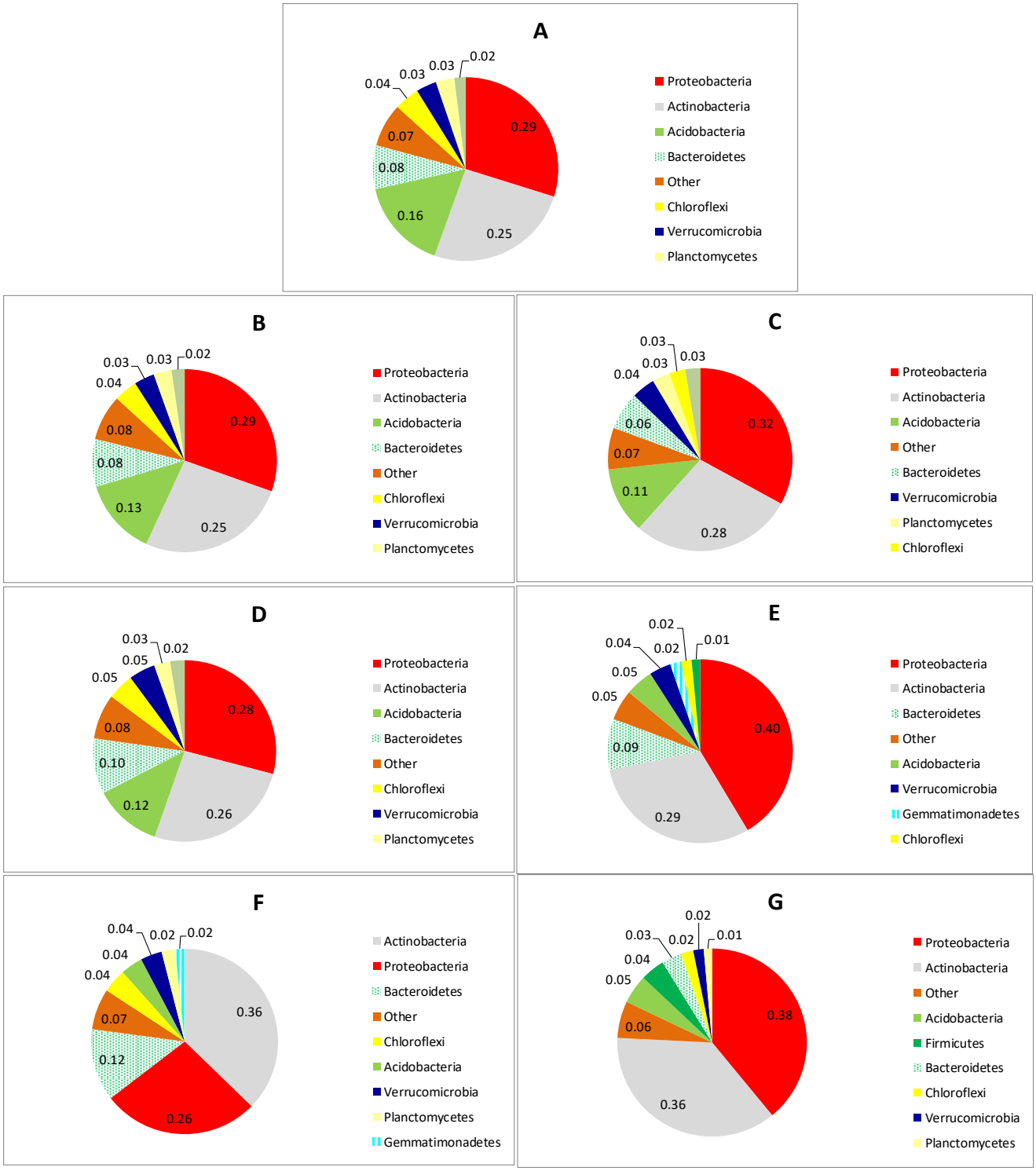



**Figure 5.2:** The class abundance of bacterial communities in FxLane (FLN) soil spiked with different concentrations (mg kg-1) of $PtCl_4$ and $NaAuCl_4$.

NOTE: A -no Pt or Au added, B- 1 mg $kg^{-1}$ of Pt added, C- 1 mg $kg^{-1}$ of Au added, D- 25 mg $kg^{-1}$ of Pt added, E- 25 mg $kg^{-1}$ of Au added, F- 100 mg $kg^{-1}$ of Pt added and G- 100 mg $kg^{-1}$ of Au added.

#### 5.3.1.3 The effects of Pt and Au amendments on dominant soil bacterial groups in PPN soil

In PPN soil, the percentage abundances the dominant top 3 bacterial groups, Actinobacteria, *Proteobacteria* and *Acidobacteria* were 43, 28 and 7 % respectively in the control soils Adding Pt and Au shifted the top 2 dominant groups to *Firmicutes* and *Caldithrix* at all tested concentrations with the *Acidobacterial* groups eliminated from the top 9 dominant classes (Fig. 5.3). In Pt amended soils, the percentage dominance of *Firmicutes* was positively correlated with increasing the Pt concentrations.

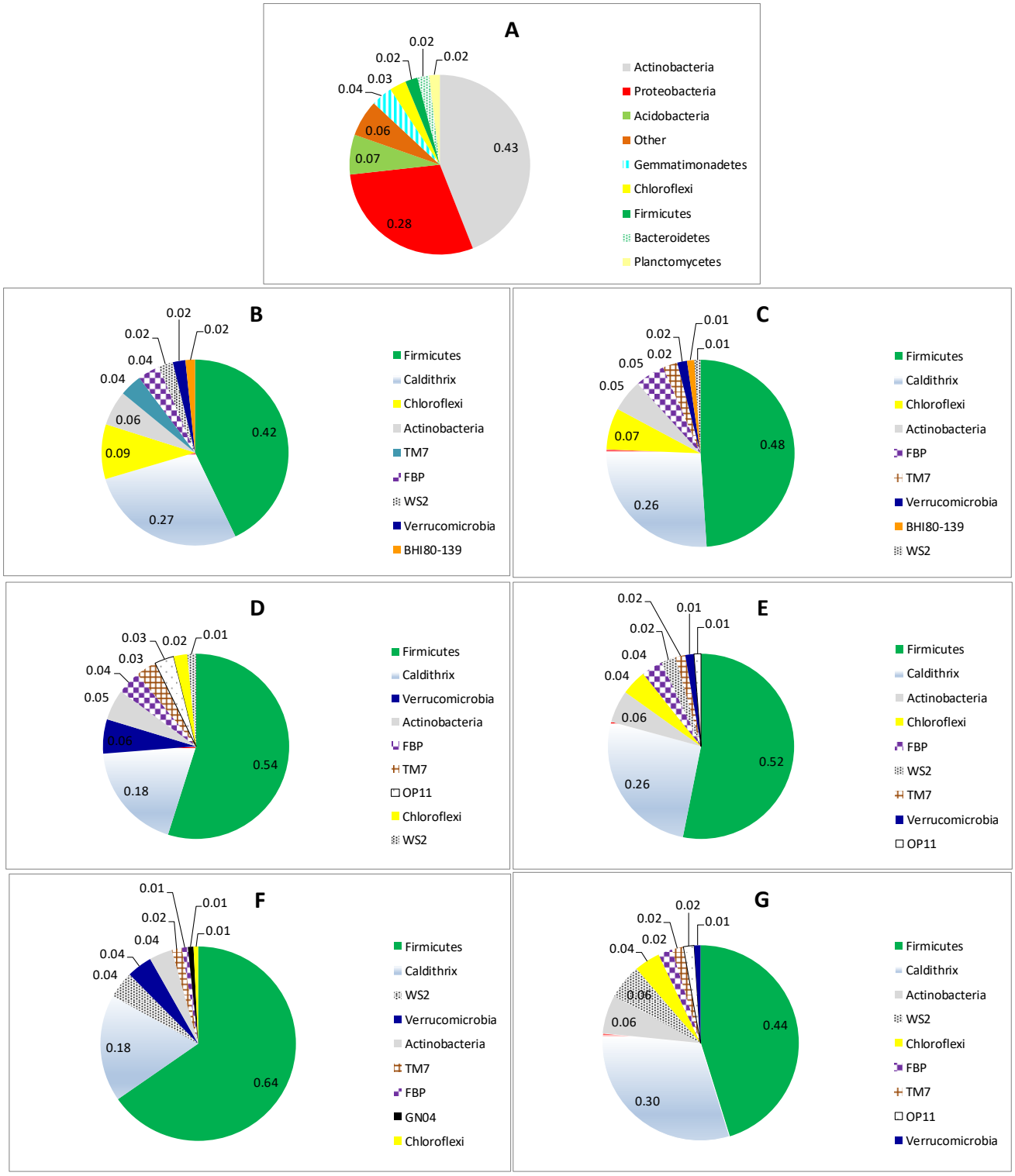



**Figure 5.3:** The class abundance of bacterial communities in Pinpinio (PPN) soil spiked with different concentrations (mg kg-1) of $PtCl_4$ and $NaAuCl_4$.

NOTE: A -no Pt or Au added, B- 1 mg $kg^{-1}$ of Pt added, C- 1 mg $kg^{-1}$ of Au added, D- 25 mg $kg^{-1}$ of Pt added, E- 25 mg $kg^{-1}$ of Au added, F- 100 mg $kg^{-1}$ of Pt added and G- 100 mg $kg^{-1}$ of Au added.

#### 5.3.1.4 The effects of Pt and Au amendments on dominant soil bacterial groups in MNP soil

In MNP soils, the dominant bacterial groups belonged to the class *Proteobacteria* (33%) and *Actinobacteria* (27%) in the control soil (Fig. 5.4). The addition of Au did not cause a shift in the dominance of these top 2 bacterial groups in soils with 1 to 100 mg $kg^{-1}$ Au amendments but the percentage of largely dominant bacterial group, *Proteobacteria* increased from 33% to 41%. In Pt amended soils, there was a shift from *Proteobacteria* dominance to *Actinobacteria* dominance at 25 and 100 mg $kg^{-1}$.

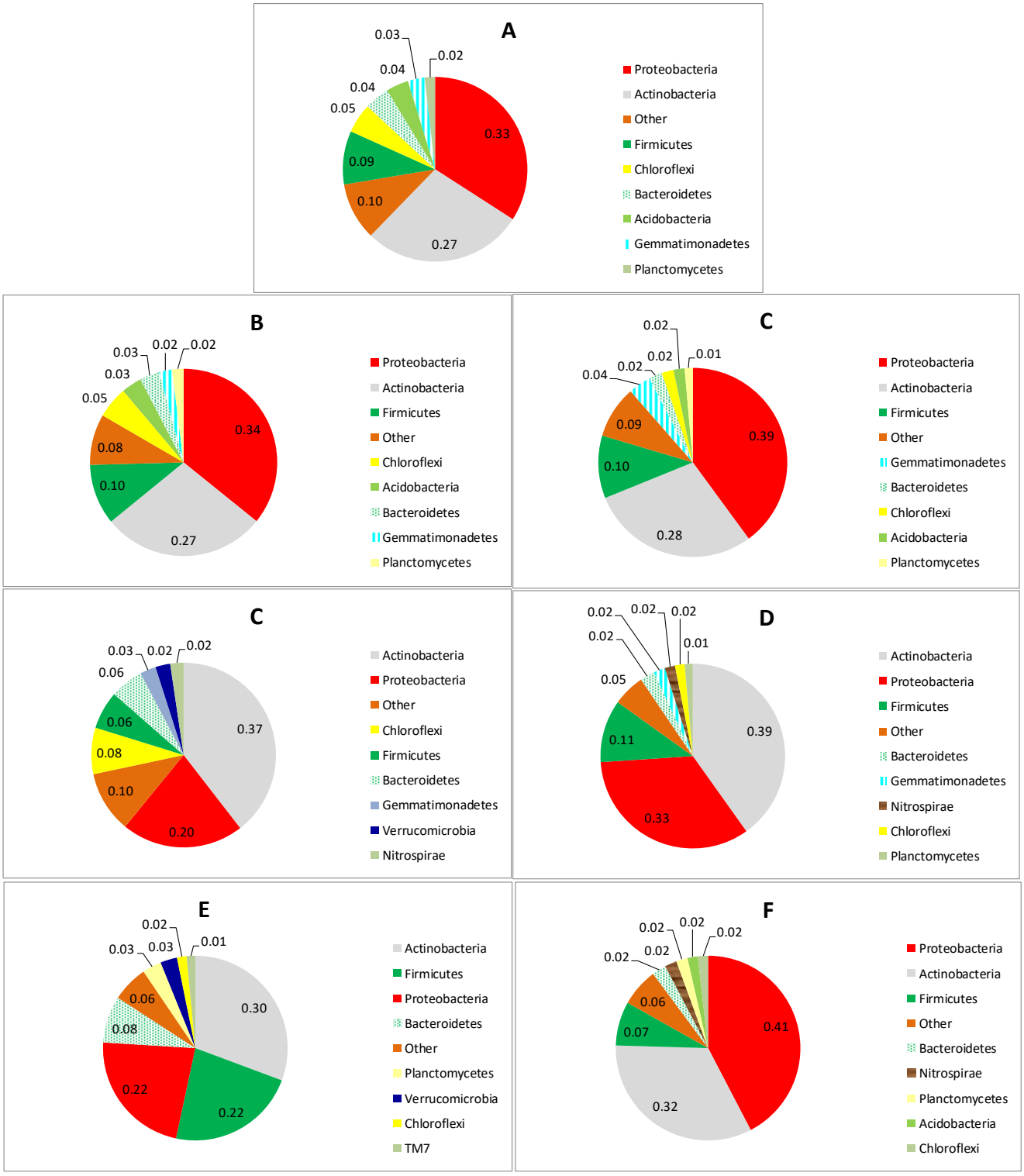



**Figure 5.4:** The class abundance of bacterial communities in Minnipa (MNP) soil spiked with different concentrations (mg kg-1) of $PtCl_4$ and $NaAuCl_4$.

NOTE: A -no Pt or Au added, B- 1 mg $kg^{-1}$ of Pt added, C- 1 mg $kg^{-1}$ of Au added, D- 25 mg $kg^{-1}$ of Pt added, E- 25 mg $kg^{-1}$ of Au added, F- 100 mg $kg^{-1}$ of Pt added and G- 100 mg $kg^{-1}$ of Au added.

### 5.3.2 The effects on Pt and Au amendments on bacterial community diversity.

Having observed substantial shifts in bacterial group dominance at class level only in BRG and PPN soil, the impact of Pt and Au amendments at 1, 25 and 100 mg $kg^{-1}$ on soil microbial community diversity were evaluated using Shannon Weaver diversity index. The metal effects were assessed at the Family level in this instance.

#### 5.3.2.1 The effects on Pt and Au amendments on bacterial community diversity in BGR soil

In acidic BGR soil, Pt amendment had little or no effect on bacterial diversity until 100 mg $kg^{-1}$ when a significant reduction in diversity was observed in the bacterial community. However, increasing Au concentration in BGR soil had no significant effect on bacterial community diversity (Fig. 5.5).

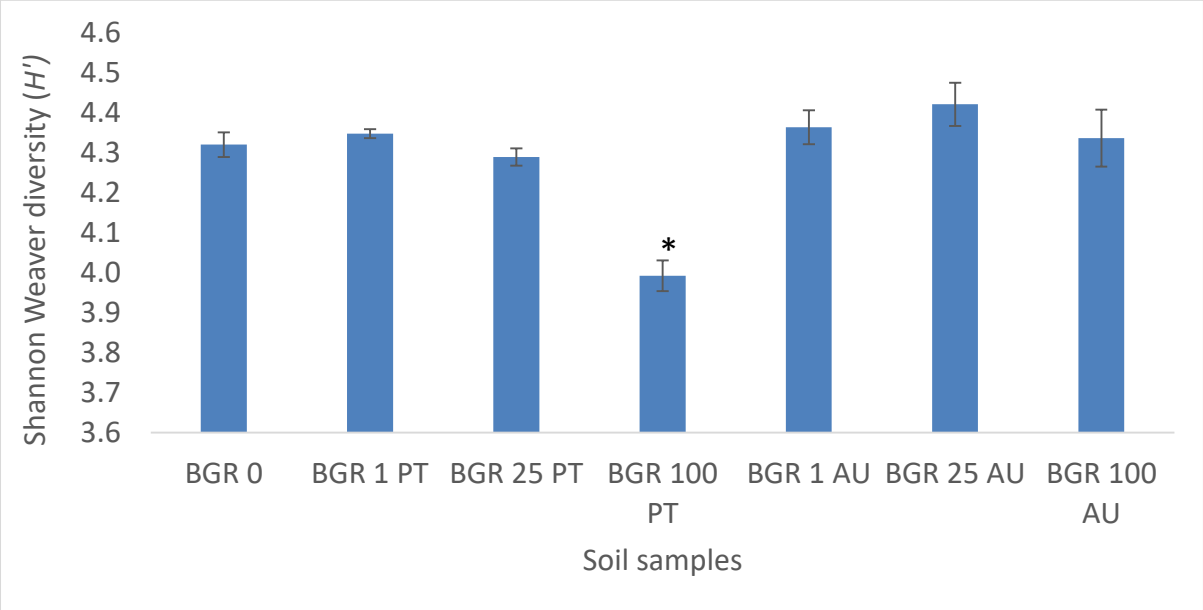



**Figure 5.5:** Shannon Weaver diversity of bacterial community in amended and non-amended BGR (BurnGrounds) soil after 180 days of incubation. Soils were amended with Au (gold) or Pt (platinum). n=3. Diversity determined at family level. BGR is an acidic soil. * indicates significant difference from control BGR 0.

Note: BGR 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$, 100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU - Au amendment at 100 mg $kg^{-1}$.

### 5.3.2.2 The effects on Pt and Au amendments on bacterial community diversity in FLN soil

In organic matter rich FLN, Pt amendment at 1 mg $kg^{-1}$ caused an initial increase in bacterial diversity but the diversity decreased as Pt concentrations increased. However, in Au-amended soil, bacterial community diversity fluctuated over the range of concentrations tested. A significant decrease in bacterial community diversity was observed at Au concentration of 25 mg $kg^{-1}$ at P≤ 0.05 (Fig. 5.6).

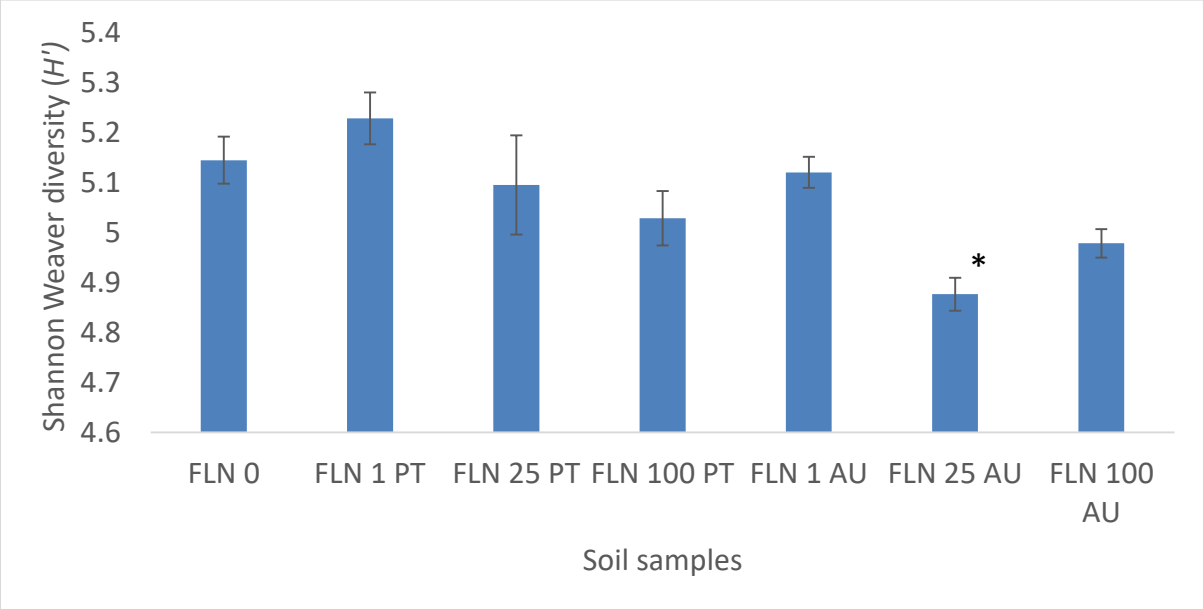



**Figure 5.5:** Shannon Weaver diversity of bacterial community in amended and non-amended FLN (FxLane) soil after 180 days of incubation. Soils were amended with Au (gold) or Pt (platinum). n=3. Diversity determined at family level. FLN is an organic matter rich soil. * indicates significant difference from control FLN 0.

Note: FLN 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$, 100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU - Au amendment at 100 mg $kg^{-1}$.

### 5.3.2.3 The effects on Pt and Au amendments on bacterial community diversity in PPN soil

In metal/silt rich PPN, Pt amendment largely caused a reduction in bacterial diversity over the range of Pt concentrations tested. Significant diversity reduction was observed at 100 mg $kg^{-1}$, $P< 0.05$. Bacterial community diversity fluctuated in Au amended soil (Fig.5.7).

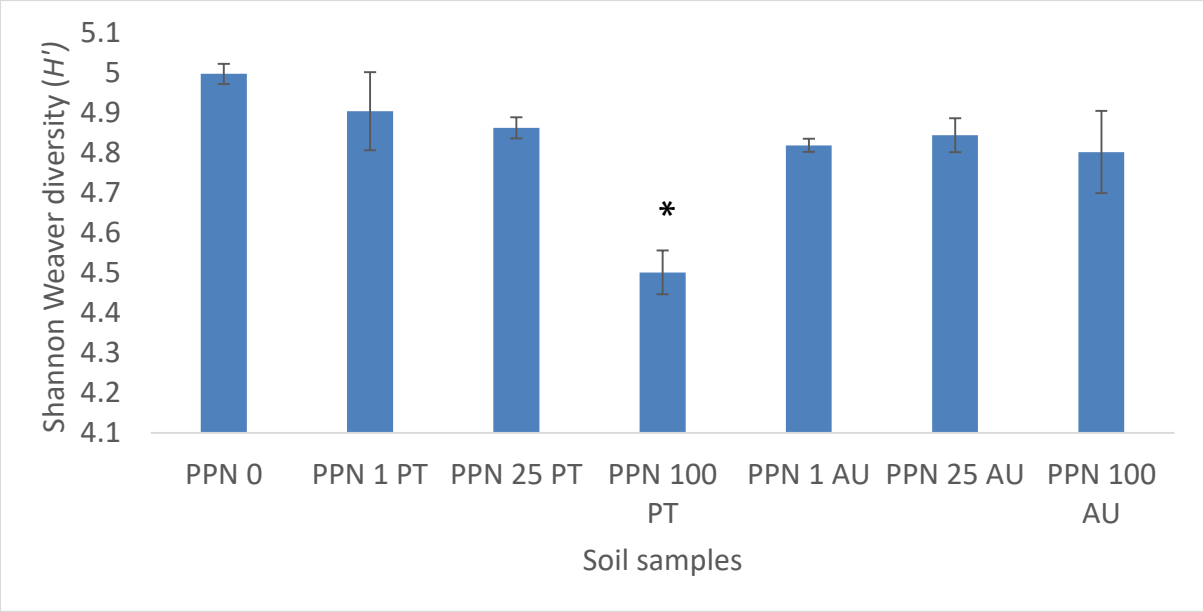



**Figure 5.6:** Shannon Weaver diversity of bacterial community in amended and non-amended PPN (Pinpinio) soil after 180 days of incubation. Soils were amended with Au (gold) or Pt (platinum). n=3. Diversity determined at family level. PPN is a metal/ silt rich soil.* indicates significant difference from control PPN 0.

Note: PPN 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$, 100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU - Au amendment at 100 mg $kg^{-1}$.

### 5.3.2.4 The effects on Pt and Au amendments on microbial community diversity in MNP soil

In alkaline MNP soil, Pt amendment had little or no effect on bacterial diversity until 100 mg $kg^{-1}$ when a significant reduction in diversity was observed. However, in Au-amended soil, bacterial community diversity reduced compared to the control after which the diversity fluctuated over the range of concentrations tested (Fig. 5.8).

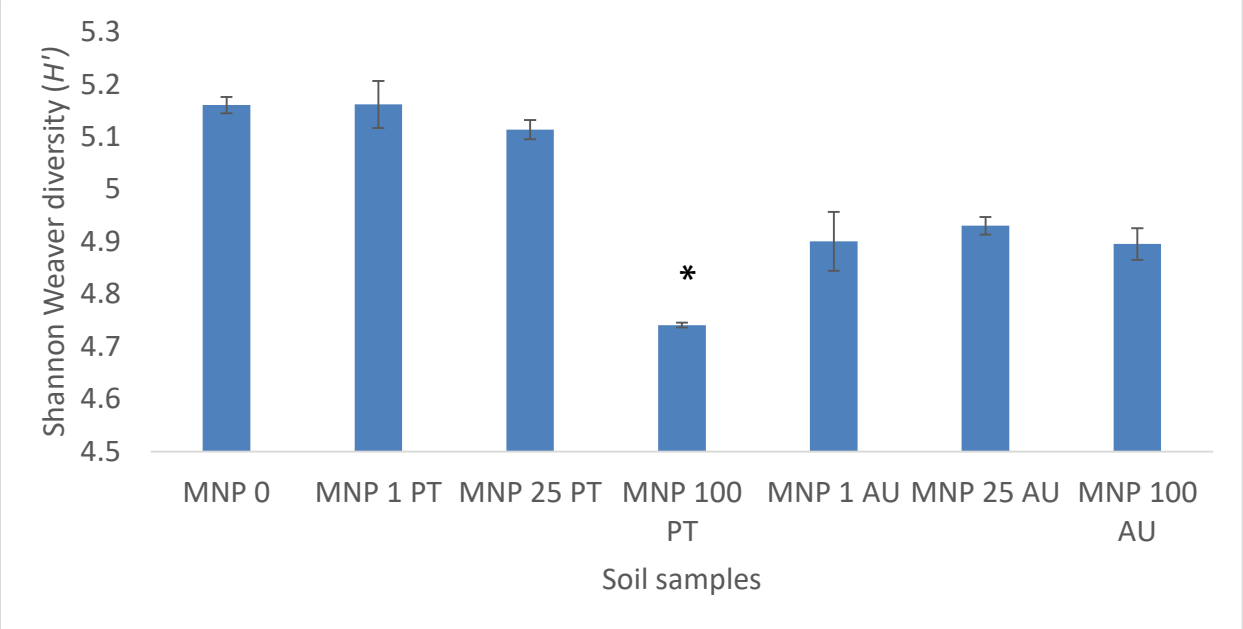



**Figure 5.7:** Shannon Weaver diversity of bacterial community in amended and non-amended MNP (Minnipa) soil after 180 days of incubation. Soils were amended with Au (gold) or Pt (platinum). n=3. Diversity determined at family level. MNP is an alkali soil. * indicate significant difference from control MNP 0.

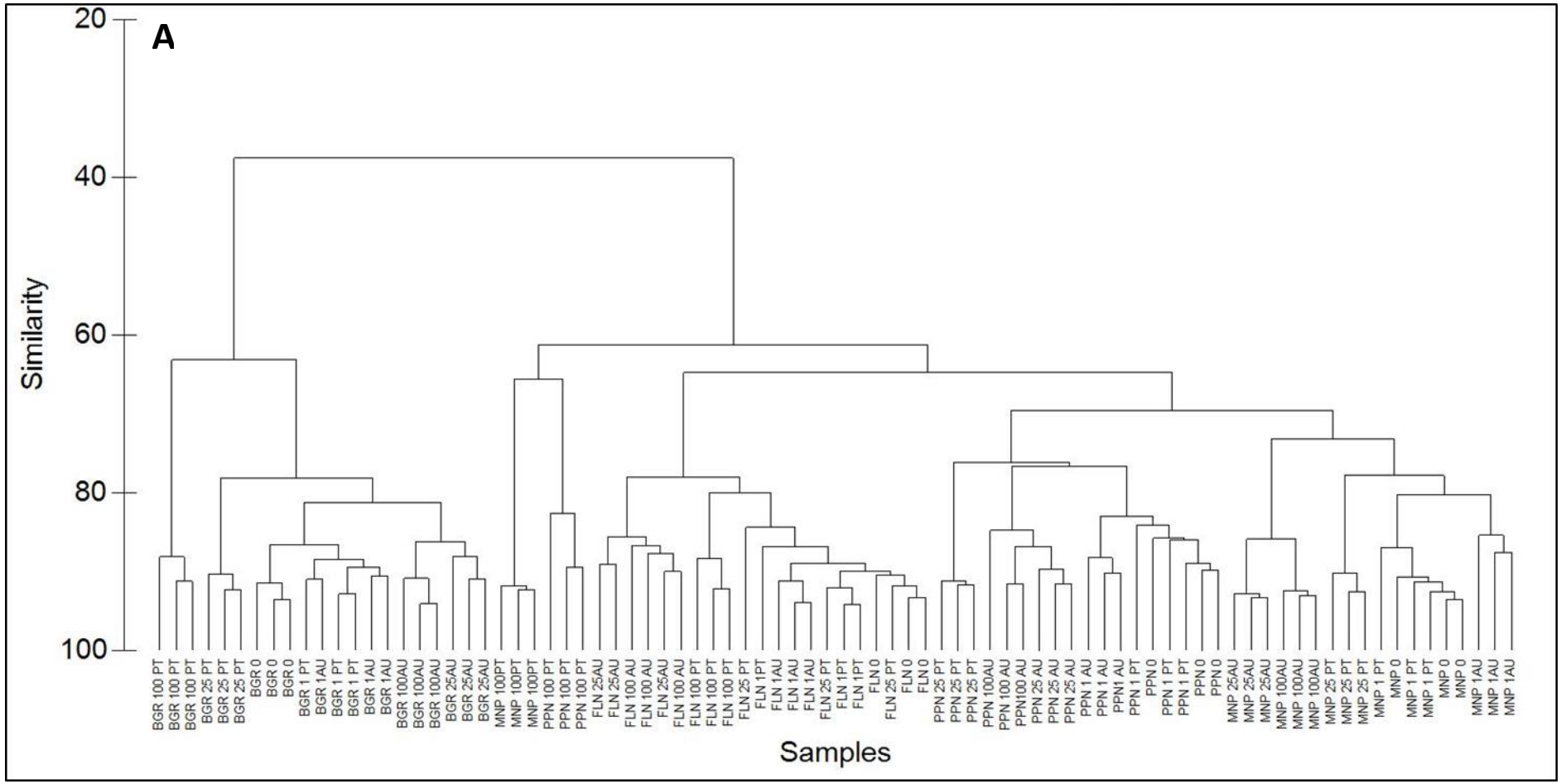
Note: MNP 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$, 100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU - Au amendment at 100 mg $kg^{-1}$.

### 5.3.3 The effects on of Pt and Au amendment on soil bacterial community structure

Having observed changes in bacterial group dominance and significant reduction in bacterial community diversity at specific heavy metal concentrations in Pt and Au amended soils, it was important to evaluate the effects of these reductions in bacterial community on bacterial community structure. The results of dendrograms, PCA and SIMPER based evaluations at the Family level are presented for the different soil types. However, an initial analysis was carried out on all the 4 soil types to determine how important Au and Pt contamination and soil type were to the microbial community structures in these soil samples.

#### 5.3.3.1 Composite bacterial community structure in BRG, FLN, PPN and MNP soil

The dendrogram in Figure 5.9A showed two major clusters formed when all the 4 soil types were analysed at family level. Cluster 1 consisted of bacterial community in BGR soils with or without Au and Pt. Cluster 2 contained communities in all other soils (FLN, PPN and MNP) with or without Au or Pt amendment. Overall, cluster 1 showed approximately 62 % similarity to cluster 2. This suggested that the BRG soil bacterial community was substantially different from those in other soils. The data was then subjected to a more rigorous assay of principal component analysis. Figure 5.9B shows that there were three distinct clusters formed. Cluster 1 (BRG bacterial community), cluster 2 (PPN and MNP bacterial communities) and cluster 3 (FLN bacterial communities). In each cluster, samples with and without Au or Pt clustered together. However, communities in PPN (high metal/silt) (cluster 2) soil were very similar to those in MNP (alkaline) soils (cluster 2). Distinct communities were observed in acidic BRG soil (cluster 1) and organic matter rich FLN soils (cluster 3). Overall, bacterial community clustering pattern appeared to be driven by soil type rather by Au and Pt amendments (Fig. 5.9A and B).


A
Similarity
20
40
60
80
100
Samples
BGR 100 PT
BGR 100 PT
BGR 100 PT
BGR 25 PT
BGR 25 PT
BGR 25 PT
BGR 0
BGR 0
BGR 0
BGR 1 PT
BGR 1AU
BGR 1 PT
BGR 1 PT
BGR 1AU
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BGR 100AU
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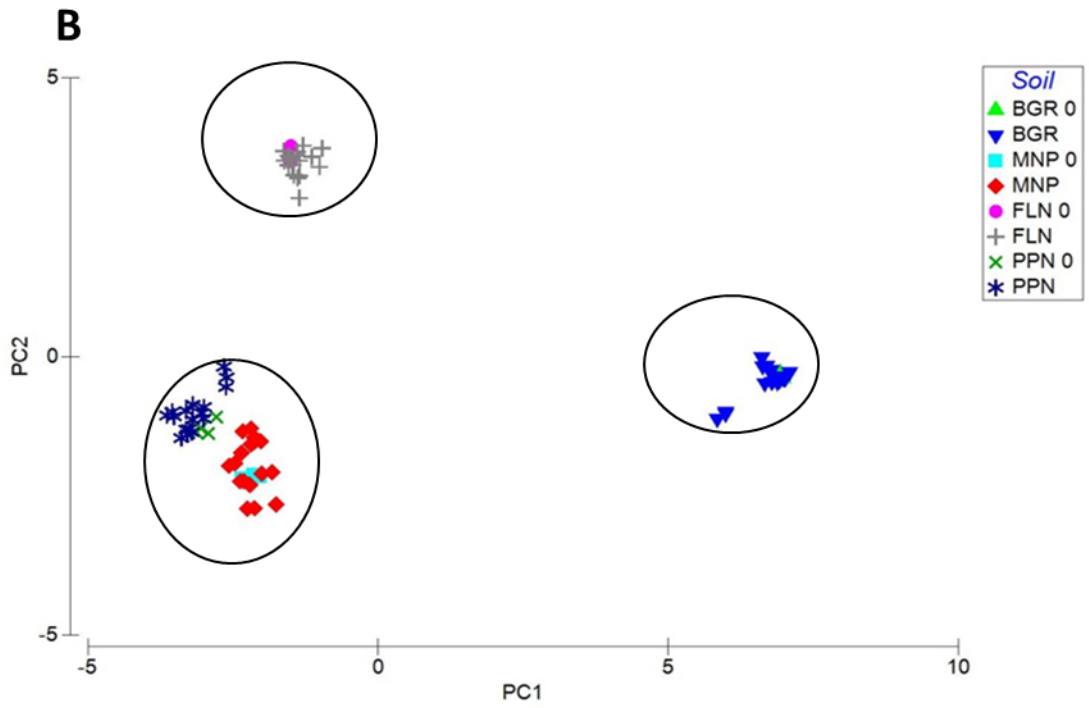


**Figure 5.8:** Dendrogram (A) and principal component analysis (B) of bacterial communities in BGR (BrnGrounds), FLN (FxLane), MNP (Minnipa) and PPN (Pinpinio) soils amended with Gold (Au) and Platinum (Pt) at different concentrations and incubated for 180 days. BGR is an acidic soil, FxLane is an organic matter rich soil, MNP is an alkaline soil and PPN is a high metal/silt containing soil. Dendrogram and PCA generated with community at the Family level.

Note: 5.9A 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$,100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU- Au amendment at 100 mg $kg^{-1}$.

5.9B -BGR 0, MNP 0, FLN 0 and PPN 0 - refer to no Au or Pt amendment in soil, BGR, MNP, FLN and PPN – Soil samples with Pt or Au amendments at 1-100 mg $kg^{-1}$.

#### 5.3.3.2 The effects of Pt and Au amendment on bacterial community structure in BRG soil

At low concentrations of 1 mg $kg^{-1}$, there were no difference between the bacterial community with Au or Pt. Increasing concentrations of Au and Pt (25, 100 mg $kg^{-1}$) caused shifts in bacterial communities. Therefore, separation of Au and Pt bacterial communities was observed at 25 and 100 mg $kg^{-1}$ concentrations (Fig. 5.10A).

PCA showed 4 clusters in BGR soil. At 0 and 1 mg $kg^{-1}$ of Au and 0, 1 and 25 mg $kg^{-1}$ Pt, the bacterial communities were very similar (formed a single cluster). The bacterial community in soils with 100 mg $kg^{-1}$ Pt also formed a highly distinct cluster (2). Increasing concentrations of Au (25 and 100 mg $kg^{-1}$) caused detectable shifts in bacterial communities (clusters 3 and 4). However, while increasing Au and Pt concentrations caused shifts in the bacterial community, this occurred earlier in Au (Fig. 5.10B).

As the shift in bacterial community occurred earlier in Au at 25 mg $kg^{-1}$, the bacterial community at this concentration was slightly more dissimilar to BGR 0 (18%) than when Pt was added (17%) to BGR 0 at the same concentration (Table 5.1). The bacterial communities at Au and Pt at 100 mg $kg^{-1}$ were 34% dissimilar to each other. Compared to control BGR soils, Au application at 100 mg $kg^{-1}$ caused 19% dissimilarity while Pt application resulted in a greater difference (28%). Increasing metal amendments correlated with increased community dissimilarity (Table 5.1). Overall, shifts in bacterial community were caused by heavy metal type and concentration in BGR soils.

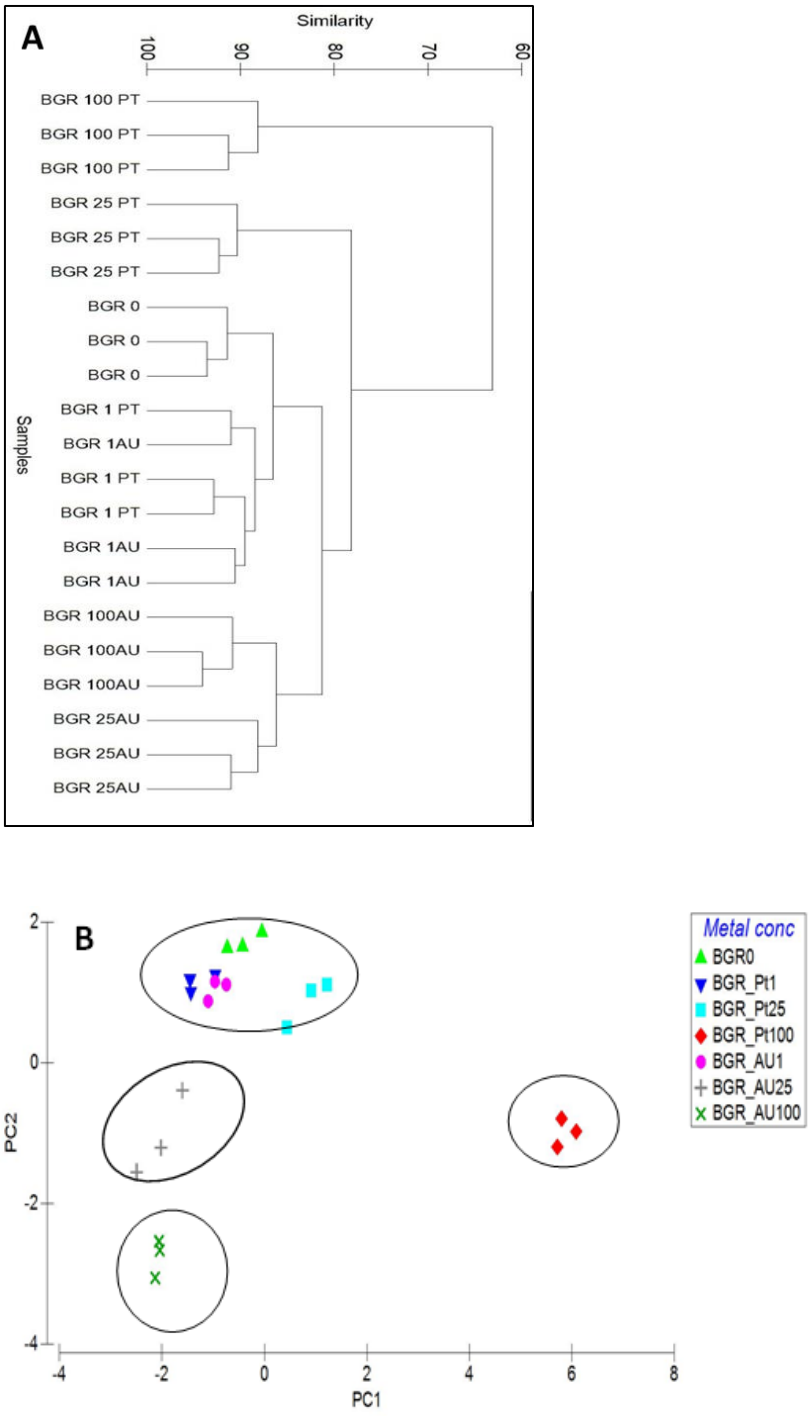


**Figure 5.9:** Dendrogram (A) and principal component analysis (B) of bacterial communities in BGR (BrnGrounds) soils amended with Gold (Au) and Platinum (Pt) at different concentrations and incubated for 180 days. BGR is an acidic soil. Dendrogram and PCA generated with community at Family level. Note: 5.10A 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$,100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU- Au amendment at 100 mg $kg^{-1}$.

Note:5.10B 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1AU- Au amendment at 1 mg $kg^{-1}$, 25PT- Pt amendment at 25 mg $kg^{-1}$, 25AU-Au amendment at 25 mg $kg^{-1}$,100PT- Pt amendment at 100 mg $kg^{-1}$, and 100AU- Au amendment at 100 mg $kg^{-1}$.

**Table 5.1:** The dissimilarity percentages of microbial communities between Au and Pt amended soil samples and non-amended controls in BRG soils after 180 days of incubation.

| | Dissimilarity percentages | | | |
|---|---|---|---|---|
| | BRG 0 | BRG Au1 | BRG Au25 | BRG Au100 |
| BRG 0 | 0 | 13.02 | 18.42 | **18.96** |
| BRG Pt1 | 13.83 | 11.93 | 14.97 | 16.51 |
| BRG Pt25 | 16.95 | 17.41 | 20.80 | 20.92 |
| BRG Pt100 | **28.40** | 30.72 | 34.69 | **34.13** |

Note: BGR 0 refers to no Au or Pt amendment, Pt1 – Pt amendment at 1 mg $kg^{-1}$, Au1- Au amendment at 1 mg $kg^{-1}$, Pt25- Pt amendment at 25 mg $kg^{-1}$, Au25-Au amendment at 25 mg $kg^{-1}$, Pt100- Pt amendment at 100 mg $kg^{-1}$, and Au100- Au amendment at 100 mg $kg^{-1}$. n=3. Dissimilarity percentages derived from SIMPER analysis of community at family level using primer 6 software.

#### 5.3.3.3 The effects of Pt and Au amendment on bacterial community structure in FLN soil

In FLN soil, two major but diffuse clusters were observed (Figure 5.11A). At 0, 1 and 25 mg $kg^{-1}$ of Pt, 1 mg $kg^{-1}$ of Au, the soil bacterial community were largely similar resulting in a diffuse cluster. In addition, the bacterial community in soils with Au (25 & 100 mg $kg^{-1}$) and Pt (100 mg $kg^{-1}$) formed another diffuse cluster. However, separation of Au and Pt bacterial communities at 100 mg $kg^{-1}$ was not as distinct as seen in BGR soil (Fig 5.11A).

As observed in BGR soils, PCA showed greater clarification of heavy metal induced shifts (Fig. 5.11B). In FLN soil, three clusters were identified; (i) at 0 and 1 mg $kg^{-1}$ of Au and 0, 1 and 25 mg $kg^{-1}$ of Pt, (ii) at 100 mg $kg^{-1}$ of Pt and (iii) at Au 25 and 100 mg $kg^{-1}$. Increasing Au and Pt concentration caused shifts in bacterial community (at earlier concentrations in Au than in Pt amended soils). Bacterial community in Au and Pt at 100 mg $kg^{-1}$ were different. Increasing metal amendments was correlated with increases in community dissimilarity only in Pt amended soils. The shift in bacterial community occurred earlier in Au at 25 mg $kg^{-1}$ and the bacterial community at this concentration was more dissimilar to FLN 0 (20%) than in Pt amended soils (13%) was to FLN 0 at the same concentration (Table 5.2). At 100 mg $kg^{-1}$, of Au the community was 18% dissimilar to the community in control soils while at 100 mg $kg^{-1}$ of Pt, the community was 17% dissimilar to the control soils**.** The bacteria community in soils with Au amendment of 100 mg $kg^{-1}$ was 17% dissimilar to the community in soils with Pt amendment of 100 mg $kg^{-1}$ (Table 5.2).

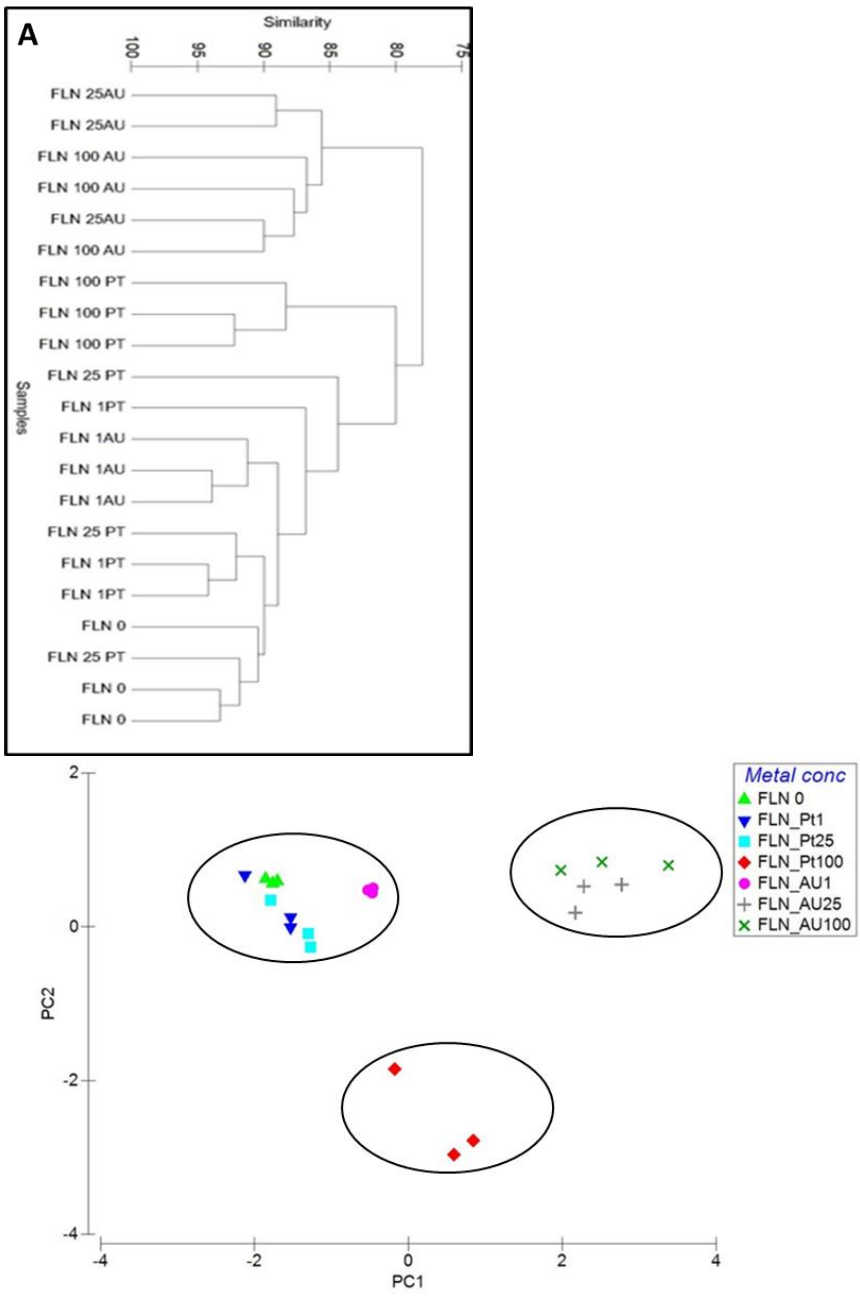


**Figure 5.10:** Dendrogram (A) and principal component analysis (B) of bacterial communities in FLN (FxLane) soils amended with Gold (Au) and Platinum (Pt) at different concentrations and incubated for 180 days. FLN is an organic matter rich soil. Dendrogram and PCA generated with community at the Family level.

**Note:** 5.11A 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$,100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU- Au amendment at 100 mg $kg^{-1}$.
5.11B Note: 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1AU- Au amendment at 1 mg $kg^{-1}$, 25PT- Pt amendment at 25 mg $kg^{-1}$, 25AU-Au amendment at 25 mg $kg^{-1}$,100PT- Pt amendment at 100 mg $kg^{-1}$, and 100AU- Au amendment at 100 mg $kg^{-1}$.

**Table 5.2:** The dissimilarity percentages of microbial communities between Au and Pt amended soil samples non-amended controls in FLN soils after 180 days of incubation.

| | Dissimilarity percentages | | | |
|---|---|---|---|---|
| | FLN 0 | FLN Au1 | FLN Au25 | FLN Au100 |
| FLN 0 | 0 | 11.28 | 19.80 | **18.35** |
| FLN Pt1 | 10.48 | 11.17 | 20.96 | 18.16 |
| FLN Pt25 | 12.67 | 13.29 | 20.52 | 19.50 |
| FLN Pt100 | **16.74** | 15.46 | 18.27 | **16.76** |

Note: FLN 0 refers to no Au or Pt amendment, Pt1 – Pt amendment at 1 mg $kg^{-1}$, Au1- Au amendment at 1 mg $kg^{-1}$, Pt25- Pt amendment at 25 mg $kg^{-1}$, Au25-Au amendment at 25 mg $kg^{-1}$, Pt100- Pt amendment at 100 mg $kg^{-1}$, and Au100- Au amendment at 100 mg $kg^{-1}$. n=3. Dissimilarity percentages derived from SIMPER analysis of community at family level using primer 6 software.

#### 5.3.3.4 The effects of Pt and Au amendment on bacterial community structure in PPN soil

In PPN soil (Fig. 5.12A),the bacterial community at 0 and 1 mg $kg^{-1}$ of Pt were largely similar, forming one cluster, whereas, at 1 mg $kg^{-1}$ of Au, the bacterial community has started shifting from control soils. Similarity percentage of bacterial community in soils with Au (25, 100 mg $kg^{-1}$) was 86%, and with Pt (25, 100 mg $kg^{-1}$) 78% to the bacterial community in the

controls soils. A clear separation of Au and Pt bacterial communities was observed at 100 mg $kg^{-1}$ in MNP soils (Fig. 5.12A).

In PPN soil (Fig.5.12B), four PCA clusters were identified; (i) bacterial community at 0 and 1 mg $kg^{-1}$ of Au and Pt, (ii) bacterial community at 25 mg $kg^{-1}$ of Pt, (iii) bacterial community at 100 mg $kg^{-1}$ of Pt and (iv) bacterial community at 25 & 100 mg $kg^{-1}$ of Au. Increasing Au and Pt concentrations caused shifts in the bacterial community with the bacterial community in Au and Pt at 100 mg $kg^{-1}$ being different (25.82% dissimilarity) (Table 5.3). At this concentration, the community in Au amended PPN soils were 23% dissimilar (32% in Pt amended soils) to the original community (Table 5.3).

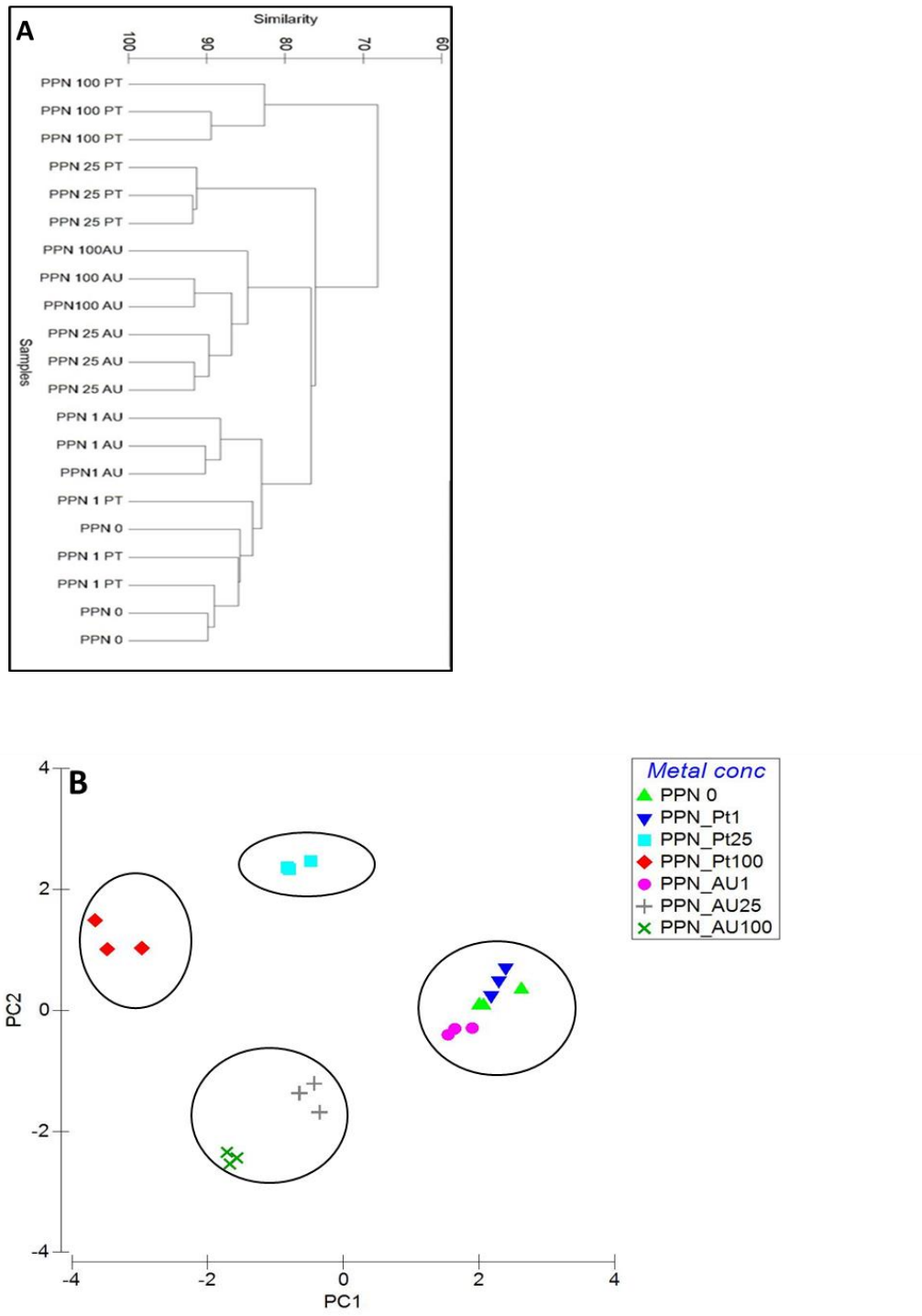


**Figure 5.11:** Dendrogram (A) and principal component analysis (B) of bacterial communities in PPN (Pinpinio) soils amended with Gold (Au) and Platinum (Pt) at different concentrations and incubated for 180 days. PPN is a metal/silt rich soil. Dendrogram and PCA generated with community at the Family level.

**Note:** 5.12A 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$,100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU- Au amendment at 100 mg $kg^{-1}$.

5.12D -PPN Note: 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1AU- Au amendment at 1 mg $kg^{-1}$, 25PT- Pt amendment at 25 mg $kg^{-1}$, 25AU-Au amendment at 25 mg kg-1,100PT- Pt amendment at 100 mg kg-1, and 100AU- Au amendment at 100 mg kg-1.

**Table 5-3:** The dissimilarity percentages of microbial communities between Au and Pt amended soil samples and non-amended controls in PPN soils after 180 days of incubation.

| | **Dissimilarity percentages** | | | |
|---|---|---|---|---|
| | PPN 0 | PPN Au1 | PPN Au25 | PPN Au100 |
| PPN 0 | 0 | 17.49 | 19.65 | **23.19** |
| PPN Pt1 | 15.77 | 18.81 | 21.83 | 25.12 |
| PPN Pt25 | 19.59 | 19.78 | 19.53 | 22.11 |
| PPN Pt100 | **32.16** | 28.99 | 25.62 | **25.82** |

Note: PPN 0 refers to no Au or Pt amendment, Pt1 – Pt amendment at 1 mg $kg^{-1}$, Au1- Au amendment at 1 mg $kg^{-1}$, Pt25- Pt amendment at 25 mg $kg^{-1}$, Au25-Au amendment at 25 mg $kg^{-1}$, Pt100- Pt amendment at 100 mg $kg^{-1}$, and Au100- Au amendment at 100 mg $kg^{-1}$. n=3. Dissimilarity percentages derived from SIMPER analysis of community at family level using primer 6 software.

#### 5.3.3.5 The effects of Pt and Au amendment on bacterial community structure in MNP soil

In MNP soil (Fig.5.13A), the bacterial community at 0 and 1 mg $kg^{-1}$ of Pt were largely similar, forming one cluster, whereas at 1 mg $kg^{-1}$ of Au, the soil bacterial community structure

started shifting from control soils. The community was 87% similar to that in Au (25 and 100 mg $kg^{-1}$) and substantially different from control samples. At 25 mg $kg^{-1}$ of Pt, the soil bacterial community was 82% similar to controls. The bacterial community in soils with Pt (100 mg $kg^{-1}$) was 75% similar to controls. Therefore, there was a clear separation of Au and Pt bacterial communities at 100 mg $kg^{-1}$ in MNP soils (Fig. 5.13A).

PCA analysis of the bacterial community in MNP soil (5.13B) resulted in the formation of three clusters. Cluster 1, at 0 and 1 mg $kg^{-1}$ Au and 0, 1 and 25 mg $kg^{-1}$ Pt bacterial communities, Cluster 2, bacterial community in soils with 100 mg $kg^{-1}$ Pt and Cluster 3 bacterial communities in Au soils at 25 and 100 mg $kg^{-1}$. Increasing Au and Pt concentrations caused shifts in bacterial community (earlier in Au than in Pt) with the bacterial community

At a soil Au concentration of 25 mg $kg^{-1}$, the community was 21% dissimilar to MNP 0 compared to the Pt community at the same concentration which was only 16% similar to MNP 0. Gold and Pt at 100 mg $kg^{-1}$ concentration were different (25% dissimilarity) (Table 5.4). At this concentration, the community in Au amended PPN soils were 30% dissimilar (30% also in Pt amended soils) to the original community (Table 5.4).

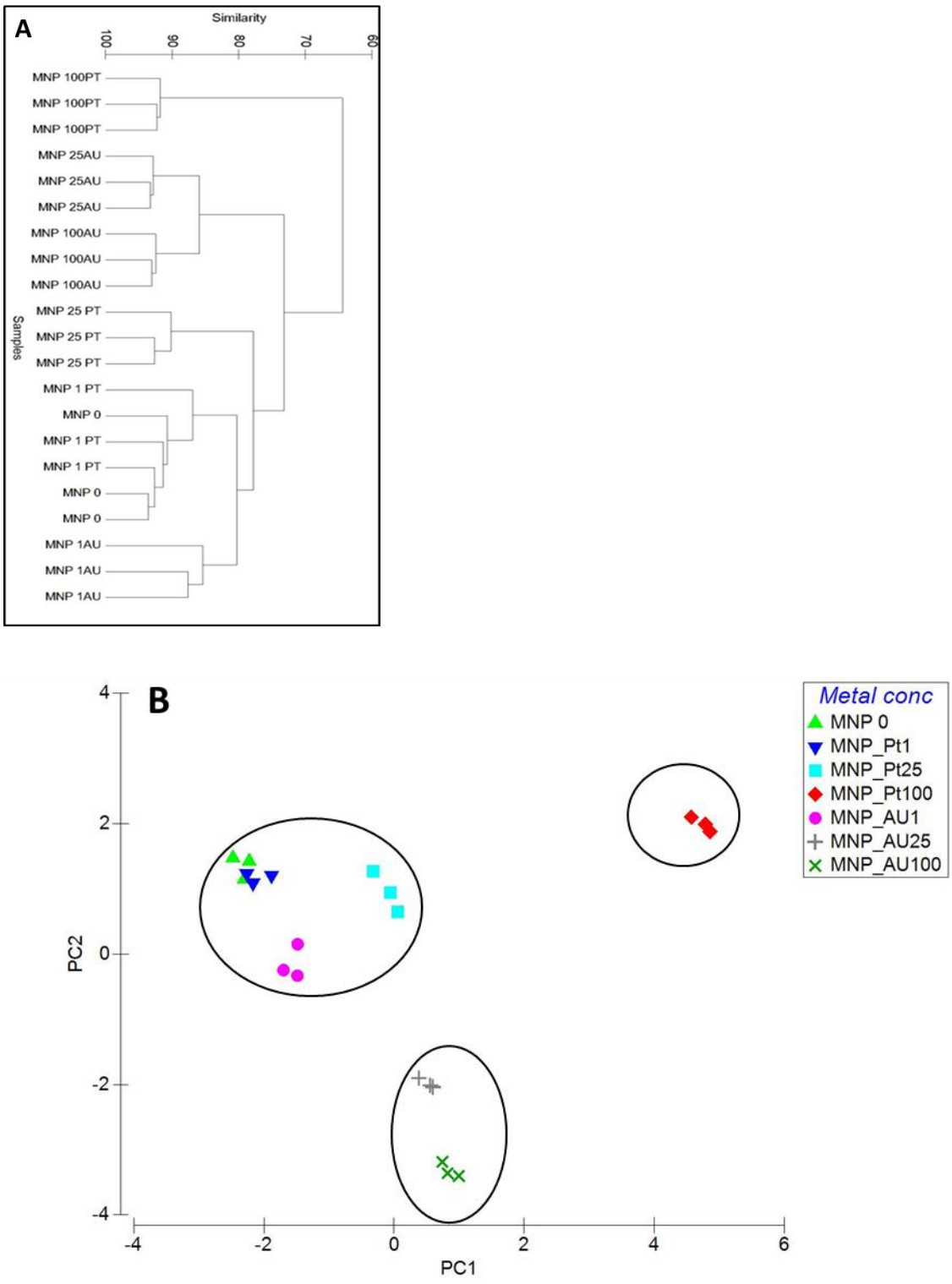


**Figure 5.12:** Dendrogram (A) and principal component analysis (B) of bacterial communities in MNP (Minnipa) soils amended with Gold (Au) and Platinum (Pt) at different concentrations and incubated for 180 days. MNP is an alkali soil. Dendrogram and PCA generated with community at the Family level.

Note: 5.13A 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1 AU- Au amendment at 1 mg $kg^{-1}$, 25 PT- Pt amendment at 25 mg $kg^{-1}$, 25 AU- Au amendment at 25 mg $kg^{-1}$,100 PT- Pt amendment at 100 mg $kg^{-1}$, and 100 AU- Au amendment at 100 mg $kg^{-1}$.

5.13B -MNP Note: 0 refers to no Au or Pt amendment, 1 PT – Pt amendment at 1 mg $kg^{-1}$, 1AU- Au amendment at 1 mg $kg^{-1}$, 25PT- Pt amendment at 25 mg $kg^{-1}$, 25AU-Au amendment at 25 mg $kg^{-1}$,100PT- Pt amendment at 100 mg $kg^{-1}$, and 100AU- Au amendment at100mg $kg^{-1}$**.**

**Table 5.4**: The dissimilarity percentages of microbial communities between Au and Pt amended soil samples and non-amended controls in MNP soils after 180 days of incubation.

| | **Dissimilarity percentages** | | | |
|---|---|---|---|---|
| | MNP 0 | MNP Au1 | MNP Au25 | MNP Au100 |
| MNP 0 | 0 | 18.36 | 21.33 | **22.79** |
| MNP Pt1 | 9.75 | 18.72 | 21.30 | 22.80 |
| MNP Pt25 | 15.81 | 20.52 | 19.50 | 20.64 |
| MNP Pt100 | **29.82** | 27.82 | 23.12 | **24.25** |

Note: MNP 0 refers to no Au or Pt amendment, Pt1 – Pt amendment at 1 mg $kg^{-1}$, Au1- Au amendment at 1 mg $kg^{-1}$, Pt25- Pt amendment at 25 mg $kg^{-1}$, Au25-Au amendment at 25 mg $kg^{-1}$, Pt100- Pt amendment at 100 mg $kg^{-1}$, and Au100- Au amendment at 100 mg $kg^{-1}$. n=3. Dissimilarity percentages derived from SIMPER analysis of community at family level using primer 6 software.

### 5.3.4 STAMP Analysis

#### 5.3.4.1 BRG soil

STAMP analysis showed that different families in BGR soil were over represented (enriched) by either Pt or Au application at 100 mg $kg^{-1}$ in BGR soils. After statistical analyses, groups belonging to families such as *Burkholderiaceae*, *Acidobacteriales*, *Actinomycetales*, *Actinospicaceae*, *Alicyclobacillaceae* and *Ktedonobacteraceae* were over-represented in Pt amended soils (Figure 5.14) (please see Figure 5.14 for other families enriched by Pt amendments). However, groups enriched by Au amendments belonged to different families which included *Sphingobacteriaceae*, *Koribacteraceae*, *Rhodospirillaceae*, *Xanthomonadaceae*, *Hyphomicrobiaceae* and others shown in Fig. 5.14.

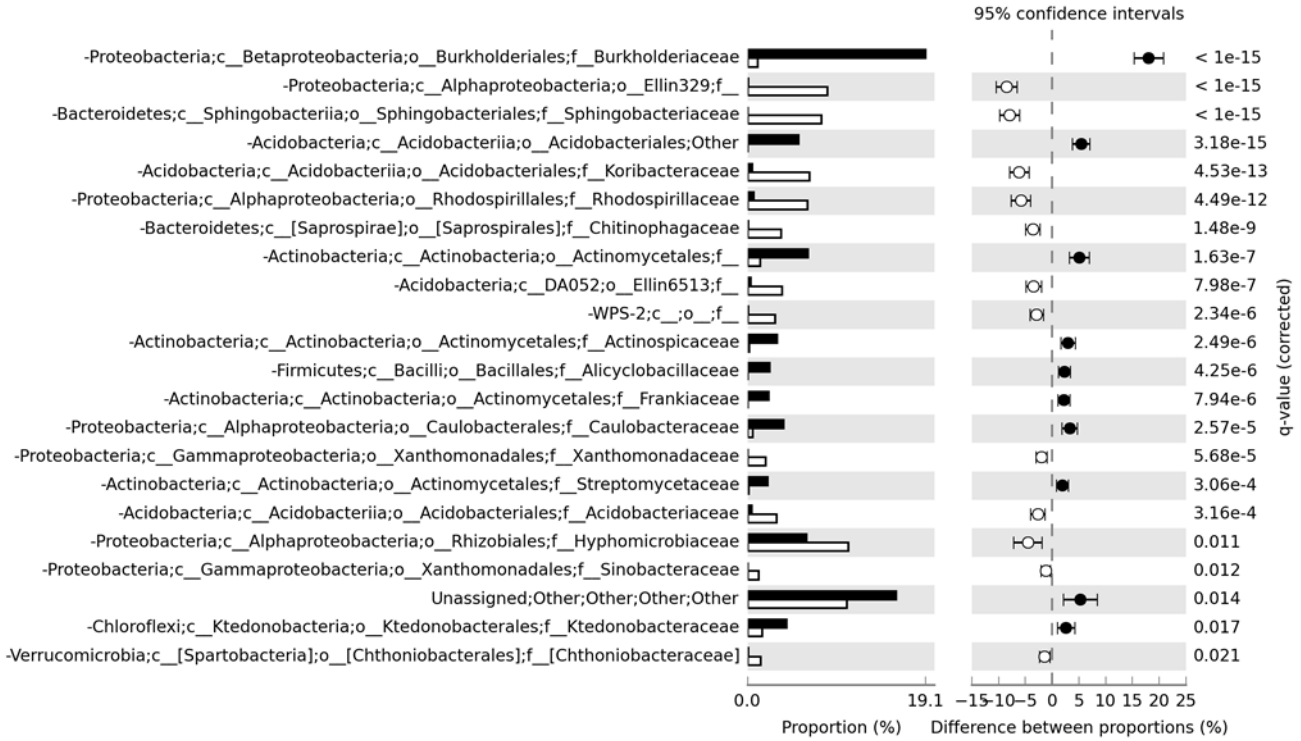


**Figure 5.13:** STAMP analysis of bacterial groups enriched or depleted in BRG soils. Enriched groups in Pt amended soils at 100 mg $kg^{-1}$ (black; positive differences between proportions) compared to enriched groups in Au amended soils at 100 mg $kg^{-1}$ (white; negative d ifferences between proportions). False discovery rate corrected with Storey's FDR approach. BRG is an acidic soil.

### 5.3.4.2 FLN soil

Initially, there were eighteen families enriched by either Pt or Au amendment at 100 mg $kg^{-1}$ in FLN soil (data not shown) in the original uncorrected profiles. This decreased to four families once Storey's FDR was applied to correct for false discovery rates. Two families, *Chitinophagaceae* and *Cytophagaceae* were significantly enriched in Pt amended soils compared to microbial groups belonging to the families such as *Erythrobacteraceae* and *Sphingomonadaceae* which were significantly enriched in Au amended soil samples (Fig. 5.15).

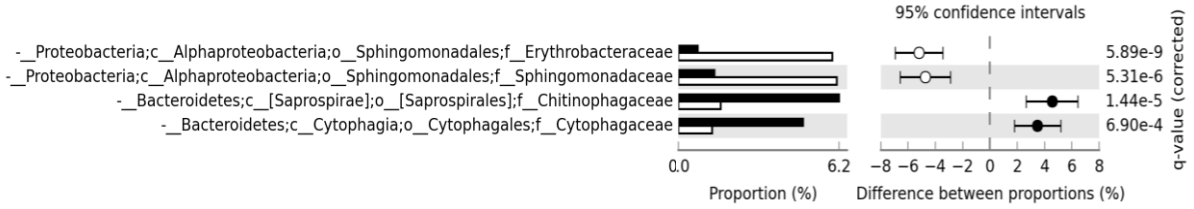


**Figure 5-14:** STAMP analysis of bacterial groups enriched or depleted in FLN soils. Enriched groups in Pt amended soils at 100 mg $kg^{-1}$ (black; positive differences between proportions) compared to enriched groups in Au amended soils at 100 mg $kg^{-1}$ (white; negative difference between proportions). False discovery rate corrected with Storey's FDR approach. FLN is an organic matter rich soil.

### 5.3.4.3 PPN soil

A similar trend was observed in PPN soil where there were sixteen families enriched by either Pt or Au amendment at 100 mg $kg^{-1}$ in FLN soil (data not shown) in the original uncorrected profiles. This decreased to four families once Storey's FDR was applied to correct for false discovery rates. The families significantly enriched in Pt amended soils included bacterial groups belonging to the families *Rubrobacteraceae* and *Oxalobacteraceae* while *Sphingomonadaceae* and families in the order *Acidobacteriales* were enriched in Au amended soils (Figure 5.16).

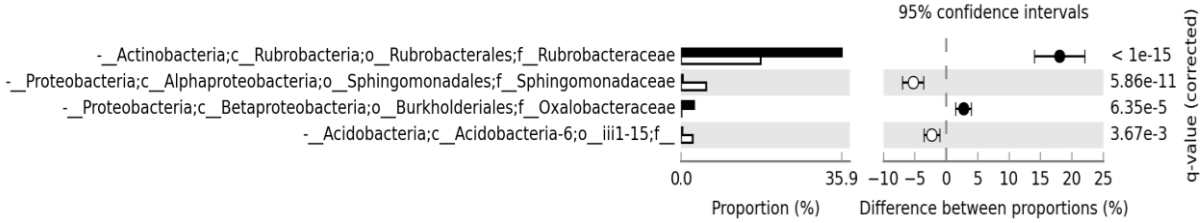


**Figure 5-15:** STAMP analysis of bacterial groups enriched or depleted in PPN soils. Enriched groups in Pt amended soils at 100 mg kg-1 (black; positive differences between proportions) compared to enriched groups in Au amended soils at 100 mg kg-1 (white; negative differences between proportions). False discovery rate corrected with Storey's FDR approach. PPN is a metal/silt rich soil.

#### 5.3.4.4 MNP soil

STAMP analysis showed that more families were over represented (enriched) by either Pt or Au application at 100 mg $kg^{-1}$ in MNP soils than in FLN and PPN soils. After Storey's FDR was applied to correct for false discovery rates, microbial families/orders such as *Alicyclobacillaceae*, *Paenibacillaceae*, *Oxalobacteraceae*, *Cytophagaceae*, *Clostridiaceae*, *Chitinophagaceae*, *Burkholderiales* (order) and *Rubrobacteraceae* were over-represented or enriched in Pt amended soils. Please see Figure 5.17 for other families enriched in Pt amended soils. In contrast, microbial groups belonging to the families such as *Sphingomonadaceae*, *Caulobacteraceae*, *Rhodospirillaceae*, *Bradyrhizobiaceae* and *Geodermatophilaceae* were enriched in Au amended soils (Figure 5.17).

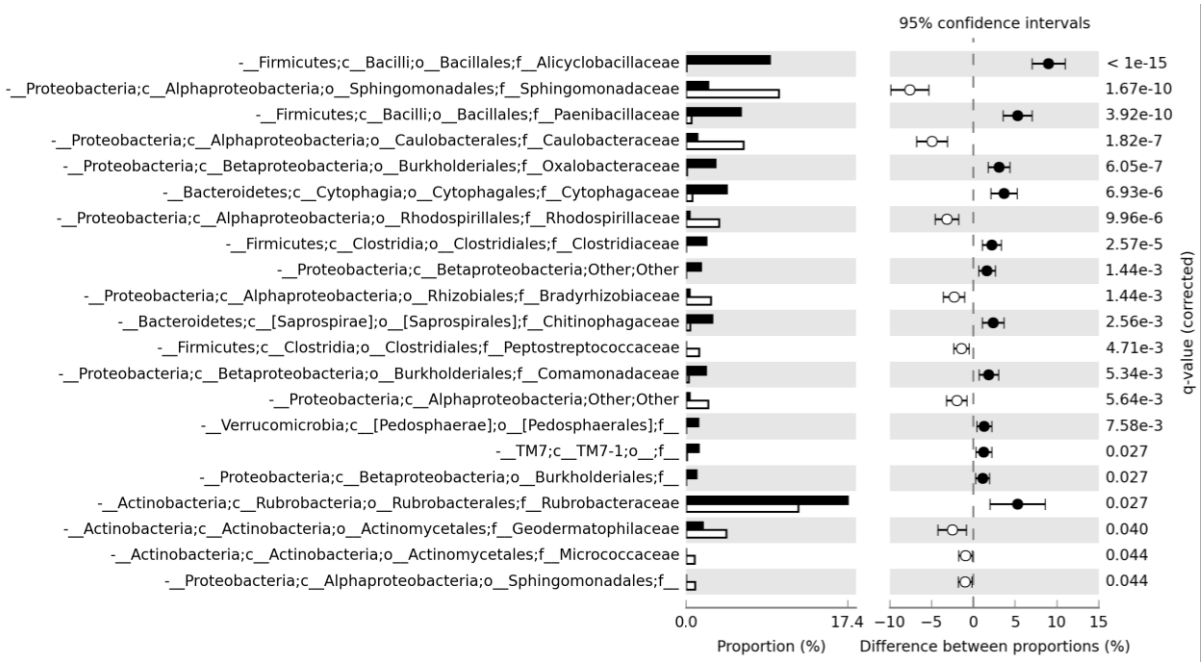


**Figure 5-16:** STAMP analysis of bacterial groups enriched or depleted in MNP soils. Enriched groups in Pt amended soils at 100 mg kg-1 (black; positive differences between proportions) compared to enriched groups in Au amended soils at 100 mg kg-1 (white; negative differences between proportions) compared to enriched groups in Au amended soils at 100 mg $kg^{-1}$ (white; negative differences between proportions). False discovery rate corrected with Storey's FDR approach. MNP is an alkali soil.

### 5.3.4.5 Identification of bacterial groups of interest in Pt or Au amendments

Analysis of the bacterial STAMP profiles also allowed for the detection of groups that were over-presented only either in Pt or Au amendments irrespective of the soil type. Soil amendment with Pt significantly enriched only bacterial groups belonging to the order/family *Burkholderiales/ Burkholderiaceae* (BRG, PPN and MNP soils), *Alicyclobacillaceae* (BRG and MNP soils), *Rubrobacteraceae* (BRG and MNP soils), *Cytophagaceae* (MNP and FLN soils), *Oxalobacteraceae* (PPN and MNP soils) (irrespective of soil types). The families significantly enriched only in Au amendments were *Sphingomonadaceae* (BRG, FLN, PPN and MNP soils) and *Rhodospirillaceae* (BRG and MNP soils). The family *Chitinophagaceae* was enriched in Pt amended FLN and MNP soils and Au amended BRG soils.

## 5.4 Discussion:

This study investigated the effects of a range of Pt and Au amendments on soil microbial communities in four different soil types ranging from acidic BGR, organic matter rich FLN, metal/silt rich PPN to alkali MNP soils. Given the varied nature of the soil, the microbial communities would be expected to be different. What was not clear was whether the application of Pt or Au at 1, 25 and 100 mg $kg^{-1}$ could lead to changes in the bacterial group dominance. The results obtained from this study showed two trends.

Firstly, a shift in the top dominant classes in BGR and PPN soils was observed. *Proteobacteria* and *Acidobacteria* (BGR), *Actinobacteria* and *Proteobacteria* (PPN) which were the dominant top two classes in non-amended soil samples were replaced by *Kazan-3B-28* and *Verrumicrobia* groups (BGR) and *Firmicutes* and *Caldithrix* groups (PPN) in Pt at 100 mg $kg^{-1}$ soil amendment. The same trend was observed in the Au microbial community at this concentration with the *Verrumicrobia* group replaced by *WS2* group in BGR soils. In these soil types, both Pt and Au bioavailability probably led to the elimination or suppression of sensitive *Proteobacterial, Acidobacterial* and *Actinobacterial* groups and replacement by other groups. Some of the dominant bacterial groups belonging to *Kazan 3B-28* and *Caldithrix* have been detected in wastes, soils and heavy metal polluted environments (Zhang et al., 2017, Gupta et al., 2017, Neyestani, 2016)and so their presence in soils used for this study is not unusual.

Secondly, Pt and Au amendments generally caused no substantial change in the dominant bacterial classes in FLN and MNP soils. This could mean that there were bacterial groups that could tolerate or were resistant to the metals in these soils. Many bacterial species belonging to the class *Proteobacteria* and *Actinobacteria* have been observed to be heavy metal resistant (Sun et al., 2010) (Mergeay and Van Houdt, 2015) (Karelova et al., 2011). A possible explanation of this lack of change in bacterial groups could also be the replacement of heavy

metal sensitive *Proteobacterial* and *Actinobacterial* species (eliminated by heavy metal addition) with metal resistance species in the same classes.

Given that some shifts in the dominant bacterial community were observed it was important to assess the impact of these heavy metal-induced shifts on bacterial community diversity at the Family level. Pt amendment, especially at 100 mg $kg^{-1}$ had greater adverse effects on the bacterial community diversity than those observed in Au treated soils. Significant reductions in the Shannon diversity at 100 mg $kg^{-1}$ Pt concentration were observed in three (acidic BGR metal/silt rich PPN and alkali MNP) out of the four soils tested. This indicated that the shifts in bacterial group dominance observed in some of these soils may be associated with a reduction in overall bacterial diversity (species richness and abundance). However, in FLN soil (organic matter rich) the presence of Au caused greater reduction in community diversity than Pt (significant reduction observed at 25 mg $kg^{-1}$ of Au). This suggests that soil type could be playing a role in determining the effects of heavy metal eon soil bacterial diversity.

Reductions in microbial diversity have been linked to heavy metal toxicity (Chen et al., 2014);(Kandeler et al., 1996); (Chodak et al., 2013). This toxicity is a reflection of the bioavailability of the heavy metal of interest (Pt and Au in this study) for biological interactions. However, other factors such as soil type (sand and clay component) and components (organic matter, humic acids and metal oxides) can affect Pt and Au transportation rate in soils (Reith and Cornelis, 2017), and affect metal toxicity. Au amendment largely had no effect on bacterial diversity in most of the soil types except FLN soils. The reason for this remains unclear but it could be due to a combination of factors related to microbial soil groups and accessibility to Au. Overall, Pt at 100 mg kg adversely altered bacterial diversity in acidic, basic and silt rich soils in this study.

The effects of this reduction of bacterial diversity was further evaluated using principal component analysis (PCA) of the bacterial community. PCA provided greater clarity with respect to Pt and Au effects on bacterial community structure. Two questions were addressed in this section. The first involved determining the primary driver for the microbial community response in different types of heavy metal polluted soils while the second question involved evaluating heavy metal induced effects in the bacterial community in each soil type.

Initial analyses were carried out on all the 4 soil types to determine how important Au and Pt contamination and soil type were to the microbial community structures. The results showed that soil type was the primary clustering determinant. Soil samples from the same soil type clustered together irrespective of heavy metal type or concentrations and were largely distinct from other soil types (Fig 5.9). This has been reported in other soils (Girvan et al., 2003) and shows the importance of soil type in heavy metal pollution studies. Different soil types will have different microbial groups and soil components and these will influence the effect of heavy metal contamination. This distinction is important especially with Au and Pt experimental investigations as their transportation and toxicity is strongly affected by soil type. Therefore, results obtained from this study may differ from those of other studies on different soil types.

Focusing on each individual soil type, it was observed that Pt and Au amendment caused a detectable shift in community structure that was in most samples, positively correlated with increasing metal concentrations. Gold is believed to be more soluble and therefore more mobile and toxic than Pt in some environment (Brugger et al., 2013) and was expected to cause greater alterations in the microbial community structure. However, it appears that heavy metal type and concentrations are also important.

Based on the cluster formed, the dissimilarity of the metal amended community to and distance from the original unamended soil community, Au amendment caused a greater shift in bacterial groups than Pt at 25 mg $kg^{-1}$ metal concentration. In three of the soils (BRG, FLN and MNP); changes in the community occurred earlier in Au-amended soils resulting in the formation of distinct clusters from controls. This was in contrast to Pt based communities which largely clustered with the controls. The SIMPER generated dissimilarity values showed that the community in these samples were different from the controls (Tables 5.1-5.4). This indicated that Au was more mobile and probably toxic to some microbial groups at this concentration than Pt. Gold complexes can react with microbial cells blocking key protein functions, generating free radicals which create oxidative stress that results in cell death (Reith et al., 2009) (Nies, 1999).

However, at 100 mg $kg^{-1}$, Pt appeared to cause greater alterations as validated by the clusters formed (Figures 5.10-5.13) and higher dissimilarity percentages (to control communities) compared to Au at the same concentration (Tables 5.1-4). This showed that this was the effective concentration of Pt. It was possible that at 1 and 25 mg $kg^{-1}$, much of the Pt applied was adsorbed to soil components such as soil particles, organic matter and humic acids rendering them unavailable for microbial interactions. A four-fold increase in Pt concentrations (100 mg $kg^{-1}$) would then have made it more available resulting in the shifts observed at this elevated concentration. These shifts resulted from the selection of Pt tolerant bacterial community and the elimination or suppression of Pt sensitive microbial groups. Therefore, Au was probably more toxic to soil bacterial communities than Pt at 25 mg $kg^{-1}$ probably due to its high mobility but Pt was more toxic at 100 mg $kg^{-1}$.

STAMP analysis was carried out on both Pt and Au amended samples at the highest concentration of 100 mg $kg^{-1}$ to determine the microbial groups that were enriched by either Pt

or Au amendment in each soil types. Prior to the application of the multiple comparison factor (correction factor, FDR Storey), some of the soils (FLN and PPN) had a higher number of enriched but different families (data not shown) than after the application of the correction (reduction to four in both soil types). This suggested that under the strict parameters employed by the correction factor and at a q value of 0.05, only these four families were statistically significantly enriched compared to the other families in community. The other eliminated families were enriched by either Au or Pt but not at the level of significance used in this study. The different families that were selected by either Pt or Au were identified in each soil type. This allowed for the determination of qualitative changes associated with either Pt or Au amendment in the different soils (Figures 5.14-5.17). It was therefore possible that the shifts in bacterial community structure by Pt and Au communities at this elevated concentration were due to these different bacterial families over-represented in each soil type.

One interesting finding of this study is the identification of some families that were only selectively enriched by either Pt or Au. These families were found in significant numbers only in Pt or Au amended soils largely irrespective of soil type. This meant that their population increased under the selective pressure of the heavy metals. Soil amendment with Pt significantly enriched only bacterial groups belonging to the order/family *Burkholderiales/ Burkholderiaceae*, *Alicyclobacillaceae*, *Rubrobacteraceae*, *Cytophagaceae*, *Oxalobacteraceae* (irrespective of soil types). This meant that the bacterial species in these families tolerated and thrived at Pt levels that were toxic to other soil microorganisms.

The number of common families significantly enriched only in Au amendments irrespective of soil type was comparatively lower (2); *Sphingomonadaceae* and *Rhodospirillaceae*. The reason for this lower number of families significantly enriched only in Au amendment compared to Pt's is unclear. However, it might be related to the fact that Au

was more mobile and toxic than Pt in soil which could have meant fewer families were able to tolerate Au related metal stress (Brugger et al., 2013).

Most of the bacterial families whose population significantly increased compared to other families in either Pt or Au amended soils are well known heavy metal resistance families. For example, some members of the family Burkholderiaceae are resistant to Pt, Au and other heavy metals. *Cupriavidus metallidurans* (Burkholderiaceae) can tolerate Pt and Au toxicity, successfully mediating heavy metal biomineralization processes without any substantial loss of cell viability (Gadd, 2010, Monsieurs et al., 2011, Reith et al., 2009, Fairbrother et al., 2013). Other Burkholderia sp, have been isolated from heavy metals polluted environments or shown to be resistance to heavy metals such as Pb, Cd,Cr,Cu and Zn (Jiang et al., 2008, Acosta-Navarrete et al., 2014, Guo et al., 2015). Members of Oxalobacteraceae, Xanthomonadaceae, Sphingomonadaceae and Rubrobacteraceae have also been detected or isolated in heavy metals contaminated environments (Rastogi et al., 2010, Bamborough and Cummings, 2009) .

The mechanisms of survival that these identified families in Pt or Au amended soils used in this study have not investigated. However, it could have been by any of the mechanisms already described in the literature. Bacterial groups can tolerate, resist or circumvent heavy metal-related stress and reduce metal toxicity by different mechanisms some of which are plasmid-borne (Schmidt and Schlegel, 1989). This include activating efflux systems that can pump metal ions outside the cell, adsorption of metals by cell surface ligands or intracellular polymers, biomethylation and metal transformation to less toxic forms (Ma et al., 2016, Rajkumar et al., 2013). No single microorganism possesses all these abilities and different mechanisms are utilized by different bacterial species. For example, some *Burkholderia* sp. and members of Oxalobacteraceae have active protection against heavy metals via efficient

efflux systems/pumps or use of transmembrane proteins (the chromate ion transporter family in *Burkholderia*) (Acosta-Navarrete et al., 2014, Karelova et al., 2011).

## 5.5 Conclusion:

This study has shown that soil type was the primary driver of the bacterial community response to Pt and Au amendments. Different responses were observed in bacterial community response to Pt and Au amendments at 1, 25 and 100 mg $kg^{-1}$ with new dominant groups

observed only in BGR and PPN soils. Au amendments only significantly affected bacterial community in the organic matter rich FLN soil while Pt amendments substantially reduced microbial diversity in the three other soil types. In each soil type, Pt and Au amendment caused a detectable shift in community structure that was in most samples, positively correlated with increasing metal concentrations. However, this effect was concentration related. The microbial groups enriched by metal amendment in each soil type were identified through STAMP analysis. This analysis also allowed for the detection a few microbial groups that were only observed in Pt or Au amended soils irrespective of soil type. Generally, Au was observed to cause greater alterations (shifts) in soil bacterial community structure than Pt at 25 mg $kg^{-1}$ while the effects of Pt amendments on community structure (including adverse effects on diversity) was greater at 100 mg $kg^{-1}$.

# Chapter 6 General Discussion

Platinum and gold are precious heavy metals that have widespread application in the modern world in the jewellery, medical and automobile industries (Freyschlag and Madix, 2011). They are non-essential metals and are not needed for any biochemical processes. As a

consequence of their domestic and industrial applications, these heavy metals can be introduced into the environments at elevated concentrations. Multiple studies have shown that the presence of Pt and Au in the environment has a significant impact on both the biotic and abiotic ecosystem components especially in soils where they are preferentially deposited in their ionic forms, Au and Pt complexes (Akpor and Muchie, 2010, Rene et al., 2017).

Platinum and Au complexes have some similarities in terms of their chemical composition and behavior in the environment (although the environmental mobility of Au is higher than that of Pt) (Brugger et al., 2013). Improved transportation/ mobility of Pt and Au in any environmental medium renders them bioavailable for interactions with biotic and abiotic components of the ecosystems. However, several environmental and edaphic factors such as thermodynamics, solubility, microbial structure and activities can influence the mobility of the complex forms of Au and Pt in the environment (Reith et al., 2009, Reith et al., 2014, Brugger et al., 2013). Gold is generally believed to be more mobile and reactive than Pt in the same state, especially after microbial interactions (Reith et al., 2014, Brugger et al., 2013). Gold and Pt can react with biological systems (including microorganisms), adversely affecting their structure and function at elevated concentrations (Reith et al., 2016)

The study of Pt and Au is important for many reasons. Understanding their interactions with microorganisms in different environmental media provides detailed information on how microorganisms, (i) affect their speciation, (ii) affect their bio-availability and (iii) respond to metal related toxicity (sensitive or resistant). This knowledge can be potentially exploited for biotechnological purposes. These could include exploiting microbial Pt and Au mobilizing capacities for the recovery of these precious metals from low grade ores or for bioremediation purposes using heavy metal resistant microorganisms. Apart from this, understanding the mechanisms of Pt and Au mediated microbial death could be useful in

developing medications or metal based drug delivery systems for use against specific human pathogens. The knowledge of Pt and Au behaviour in the environment is crucial to developing environmental management approaches designed to reduce human exposure to toxic metallic pollutants or in the restoration of metal polluted environments.

To date, there is limited information available specifically on the behaviour of Pt and Au in different aquatic systems and soil types. Therefore, this study was focused on experiments designed to assess the bioavailability of these heavy metals (in various forms) in different environmental media to generate baseline data for future studies. The effects of Pt and Au on important soil (microbial) processes were also evaluated, a crucial process in developing biological markers which could potentially be used to risk assess Pt and Au polluted environments. Finally, the latest molecular microbiological tools were applied for semi-quantitative and qualitative determination of metal effects on soil micro-organisms in different soil types.

The first question for this study was related to the mobility of Pt and Au in the soil environment. Platinum and Au can exist in their native states, as part of a complex compound, in ionic forms and as nanoparticles. Earlier studies by members of this researcher's group had generated useful data on Au mobility in the soil environment using sand-filled columns. Using Au complexes such as Au (I) thiosulfate, they demonstrated the biomineralization of Au by a bacterium, *C. metallidurans* via the uptake of Au (I/III)- complexes. When sand column was amended with Au, *C. metallidurans* retained 99% of Au via passive sorption mechanisms leading to the formation of Au nanoparticles (Fairbrother, 2013). Cell toxicity of Au was dependent on the cell up take of this heavy metal (i.e. detoxification of Au(I)-complexes by active reductive precipitation to Au (0)). Some bacterial groups can interact with Au more than Pt; Pt is thought to be less mobile, less toxic and less susceptible to biogeochemical cycling

mediated by microorganisms than Au (Etschmann 2016). Therefore, the first result chapter of this thesis was focused on replicating this experimental approach on Pt, with the biomineralization of Pt by *C. metallidurans* and *E. coli* in sand columns**.** This was done to determine whether the trends observed in previous Au biomineralization studies were applicable to Pt biomineralization,

This study found that both *C. metallidurans* CH34 and *E. coli* interacted with $PtCl_4$ and transformed it into other forms. Both bacteria were involved in Pt uptake with *C. metallidurans* CH34 showing greater resistance to Pt toxicity than *E. coli*. In comparison to similar work on Au, Pt retention by *C. metallidurans* CH34 was not as high as the 99% reported for Au (Fairbrother (2013)). At 87% retention, Pt retention by *C. metallidurans* CH34 was higher than the 75% observed in *E. coli*, demonstrating that this bacterium can biomineralize Pt. Both bacteria transformed Pt into its nanoparticulate form with large particles found within *E. coli*; in contrast, the Pt nanoparticles in *C. metallidurans* CH34 were found in its exopolymeric biofilm. Therefore, this study has shown that *C. metallidurans* can biomineralize Pt in sand columns almost as well as it biomineralized Au.

However, gaps remain in our knowledge of Pt and Au biomineralization in different soil types. While sand is an ideal medium for the study of the biomineralization of Pt and Au under controlled conditions, it is not representative of the variety of soil types found in the natural environments in which Pt and Au may occur naturally or as contaminants from anthropogenic sources. It is therefore important to plan future experiments with other soil types in order to properly characterize Pt and Au mineralization in the natural environment. Therefore, different soil types and media were used to assess Pt and Au mobility in this study in subsequent studies.

Microorganisms (bacterial species) can play a role in transforming Pt into Pt nanoparticles (Pt NP). A review of literature showed that there was very limited information available on Pt and Au nanoparticles mobility and interactions with microorganisms. Therefore, this second study investigated the transportation of both Pt and Au nanoparticles in soil columns. In order to properly understand the mobilization of these metals, multiple media were used in the column experiments. This included sand only columns, sand-clay columns, sand-humic acids columns and sand-FeO columns. Two natural soil based columns (an organic matter rich FLN soil and iron rich JBR soil) were used to create a realistic environmental scenario. As expected, Pt and Au were rapidly and substantially eluted in sand columns (~ 2 hours). More interestingly, most of the mobile Pt and Au were eluted earlier (~ 4 hours in JBR and 6 hours in FLN soil) than in sand-clay, sand-FeO and sand-humic acid columns (8-12 hours). This demonstrated that Pt and Au nanoparticle transportation can occur at a relatively high rate in natural soils, information which is currently not available in the literature for the soil types used in this study. In addition, this study showed that Au NP was possibly more reactive than Pt NP as the FTIR-based analysis showed that Au NP modified soil particle surfaces compared to Pt NP. Evidence of heavy metal-particle aggregate (homo- and hetero-aggregate) formation was obtained from zeta potential measurements with TEM analysis shedding more light on the transportation of these particles in natural soil.

Nanoparticles are reported to travel faster in sand and natural soil columns in comparison to the columns containing clay, humic acids and iron oxide. Previous studies (Sagee, 2012) showed that Ag NPs travelled faster in soil and sand medium. A possible explanation for the faster breakthrough point of Ag NPs could be related to the effect of soil heterogeneity and the negative charge of the particles ($\zeta$ potential = −39 mV). While soil aggregates contain solution in the inner aggregate pores, they are impenetrable to Ag NPs. In this case, the volume in which Ag NPs travel would be smaller than the total pore volume

(Sagee et al., 2012). This highlighted the limitation of our study of Au NP and Pt NP. While the mechanisms underpinning other heavy metal NPs such as Ag NPs in soil are known, that of the two metals used in this study are not yet properly investigated and should be part of planned future studies.

The results of the current study on other soil combinations (apart from FLN and JBR soils) showed that Au- and Pt nanoparticles displayed similar mobility patterns suggesting that organic matter, clays and Fe-oxides play important roles in nanoparticle sorption in Australian soils. It might be that the elevated Au- and Pt concentrations detected in anomalous soils may be the result of sorbed nanoparticles rather than complexes. Gold and Pt nanoparticles are highly stable with regards to dissolution; some remain dispersed while others are remobilized, and therefore they may play a leading role in the formation of soil anomalies. Indeed, a number of recent field studies provide evidence that nanoparticle mobility, sorption, deposition and enrichment play an important role for the (trans)formation of Au and Pt grains as well as surface anomalies (Reith et al., 2012, Reith et al., 2016, Shuster et al., 2017, Anand et al., 2016). The understanding that organic matter and clays are strong sorbents for these metals in complexes and nanoparticulate form, as shown in this study, suggested that measuring Au and Pt contents of clay and OM fractions (Reith, 2017) is important for understanding metal transportation in soil.

Apart from adsorbing to different soil components, Pt and Au in their various forms can react with soil micro-organisms affecting microbial structure and function. Since the mobility/transportation of Pt and Au in complexes and as nanoparticles had been demonstrated, it was important to examine their effects on soil microbial activities. In this study, the effects of Pt and Au on soil respiration and enzyme activities (key enzymes involved in the biogeochemical cycling of C, N, P and S in soil) were evaluated using multiple test concentrations; 0, 1, 25, 100, 500 and 2,000 mg $kg^{-1}$. The enzymes selected were N-acetyl

glucosaminidase, phosphatase, β-D-glucosidase, β-D-cellobiohydrolase, β-D-xylosidase, α-D-glucosidase and arylsulphatase. Soil respiration and enzymes based assays were carried out on 5 different soil types ((1) a high pH (alkaline) soil (Minnipa), (2) a low pH (acidic) soil (BrnGrounds), (3) an iron rich soil (Jamberoo), (4) an organic matter rich soil (FxLane), and (5) a high metal/silt soil (Pinpinio).

Findings from this study showed that metal effects were related to soil types. The soil respiration rates in the BGR (acidic), PPN (metal/silt) and MNP (basic) soils largely decreased following the addition of Au and Pt at most of the tested concentrations. Respiration increased in FLN soil with Pt amendments at most concentrations tested. Soil respiration rates also increased in JBR soils following amendments with Au but only at 500 and 1,000 mg $kg^{-1}$ concentration. Three different responses in terms of enzyme activities were observed with the addition of Au and Pt in the five different soils: firstly, inhibition or inactivation of the enzyme activities, secondly, enhancement of the enzyme activities and thirdly no response, with no changes in enzyme activity observed. The explanation for these differential results reported in this study may be a combination of specific enzyme sensitivity, metal concentration and soil type (Trasar-Cepeda et al., 2000). The conclusion for this phase of the study was that soil type and concentration of Au and Pt were determinants of metal effects on soil microbial activities.

Finally, the effects of Au and Pt on microbial community structure was determined, this time in four different soil types (BGR, FLN, PPN and MNP) using a Next Generation Sequencing approach. Soil type and metal concentration were observed to be key drivers of Pt and Au effects on soil microbial community structure. Different trends were therefore observed in the bacterial community response to Pt and Au amendments at 1, 25 and 100 mg $kg^{-1}$, with new dominant groups observed only in BGR and PPN soils. The effects of Pt on soil microbial diversity were largely adverse and were pronounced in acidic, basic and metal/silt rich soils. In contrast, the adverse effects of Au were more pronounced in the organic matter rich FLN

soil. It was also observed that in each soil type, Pt and Au amendment caused a detectable shift in community structure that was in most samples, positively correlated with increasing metal concentrations. However, this effect was concentration related; Au appeared to be more toxic to soil bacterial communities than Pt at 25 mg $kg^{-1}$ but Pt was more toxic at 100 mg $kg^{-1}$. Bacterial groups enriched by metal amendment in each soil type were identified. This analysis also allowed for the identification of a few bacterial groups that were only detected in Pt or Au amended soils irrespective of soil type.

This phase of the work was an initial investigation carried out to generate baseline data for future work. Future work should include NGS-based whole genome sequencing of environmental DNA which would provide detailed information on microbial soil function alongside taxonomic data. The effects of the shifts in microbial community at different concentrations on microbial functions can then be properly investigated. Culture based approaches designed to select for Au and Pt resistant bacterial species should be planned as these microorganisms can be used for biotechnological purposes (metal recovery and bioremediation).

## Appendix 1 – Publications

**Chapter 1:** Biological role in the transformation of platinum-group mineral grains

Frank Reith, Carla M. Zammit, Sahar S. Shar, Barbara Etschmann, Ralph Bottrill, Gordon Southam ,Christine Ta, Matthew Kilburn, Thomas Oberthür, Andrew S. Ball& Joël Brugger.

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**Conference .**

Sahar Shar[1], Frank Reith[2], Andrew S. Ball[1] Biomineralization of Platinum by microorganisms.Microbiology 2016 conference on November 28-29, 2016 at Valencia, Spain.

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