Heavy metal pollution and co-selection for antibiotic resistance: a microbial palaeontology approach

Submitted by Andrew William Dickinson to the University of Exeter

as a thesis for the degree of

MSc by Research in Biological Sciences (Cornwall)

In October 2017

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: …………………………………………………

Abstract

Heavy metal pollution and co-selection for antibiotic resistance: a microbial palaeontology approach

The introduction of heavy metals into the environment can modify the microbial community and their activity. Due to the persistence of metals in soils and sediments, it is implicated that increased tolerance to heavy metals may be a driving factor in the maintenance of antibiotic resistance in the environment due to co-selective mechanisms of resistance. This study marks the first attempt to address the effects of industrial heavy metal contaminants on both the cultivable and the total microbial population of a sediment core that covers a time scale spanning approximately a century, through both phenotypic analysis and with the use of high throughput sequencing technologies, providing a deeper understanding of the heavy metal driven response in microbial diversity through time. We assess the selective pressure of zinc as a driving force for the maintenance and proliferation of antibiotic resistance through phenotypic analysis of resistance in microbial isolates and the quantification of a gene conferring resistance to both metals and antibiotics (*IntI1*). We demonstrate a positive correlation between the level of zinc resistance in the microbial population and zinc in the environment, and the proportion of zinc resistant isolates with proportion of resistance to three clinically relevant antibiotics (oxacillin, cefotaxime and trimethoprim). We assess the effects of selective pressures imposed by heavy metals on total microbial community composition with the use of high-throughput sequencing technologies. These data show that zinc plays a significant role in shaping the structure of community diversity over time and is driving the population towards higher levels of resistance to zinc, resulting in the co-selection of antibiotic resistant organisms in the environment.

Acknowledgements

The completion of this Masters by Research could not have been achieved without the advice, encouragement and guidance of my supervisors: I would like to express my sincere appreciation to my principal supervisor, Dr Michiel Vos and co-supervisor Professor Britt Koskella, without whom this work would not have been possible.

I would like to express my thanks to Dr Ann Power and Dr Richard Jones at The University Exeter Biosciences and Geography departments respectively for their work in core collection and their kind words and guidance for the duration of my studies. I want to thank Dr Karen Moore and Paul O'Neill at the Exeter Sequencing Service for their work in the preparation, sequencing and analysis of the DNA samples used for this work. I would like to thank Professor Kristian Koefoed Brandt and Mette Guldborg Hansen at The University of Copenhagen for their work in the bioavailability analysis. I would like to express my gratitude to Professor Peter Appleby for his work on dating the sediment layers of the core. A special thanks to Dr Pawel Sieroncinski for giving up his time to help in teaching me how to use bioinformatics software.

A number of people were there to help me find my way in the lab, particularly Dr Lihong Zhang, who was not only an incomparable help but a genuine pleasure to know and work alongside. Additionally, Dr Aimee Murray, who provided a great source of knowledge as well as being a great friend and colleague. Special thanks for Dr Uli Klumper for his valued advice and his continued friendship.

A very special thanks to Daniela Farina for welcoming me into the group with such open arms, teaching me the basics, and more, of laboratory etiquette and for her continued concern in the general running of the day-to-day activities of the lab.

Finally, I would like to thank my partner, Emma Brindle, for her support over the years, for being by my side through times of stress, providing emotional support and whom without, I would not be the person I am today.

Table of contents

Page

List of figures and tables

Chapter 4:

Chapter 1

General introduction

Microbial metal resistance

Heavy metals are typically defined as a group of metals with an atomic density greater than $5g/cm^3$, of which there are approximately fifty found in nature that are of special significant concern due to their toxicological effects to humans and other organisms (Nies 1999, Järup 2003, Khatun, Bera et al. 2012, Samanta, Bera et al. 2012, Oves, Saghir Khan et al. 2016). Here we will use heavy metals and metals interchangeably to include elements referred to as metals or metalloids. Some heavy metals have an integral role in biological processes, with metals such as calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni) and zinc (Zn) being required as essential nutrients [Bruins et al. 2000; Nies, 1999]. Although all metal ions are toxic at certain levels, those that are required are only so at low concentrations where they are essential to organisms as micro-nutrients (Bruins, Kapil et al. 2000, Valls and De Lorenzo 2002, Khatun, Bera et al. 2012). These essential metals play varied roles in stabilising of protein structures and bacterial cell walls, redox processes, stabilisers of a variety of enzymes and DNA via electrostatic forces, the regulation of intracellular osmotic pressure, and acting as a catalyst for biochemical reactions (Nies 1992, Ji and Silver 1995, Bruins, Kapil et al. 2000). Intracellular concentrations of these essential metals are required to be adjusted though homeostasis in order to avoid metal deprivation or toxicity (Nies 1999, Bruins, Kapil et al. 2000, Choudhury and Srivastava 2001, Lee, Barrett et al. 2005). Heavy metals such as aluminium (Al), cadmium (Cd), gold

(Au), lead (Pb), mercury (Hg) and silver (Ag) do not play roles in metabolism and are therefore non-essential for life and always considered toxic (Bruins, Kapil et al. 2000).

Selective pressure from environmental metal pollution has resulted in the evolution of resistance mechanisms in microorganisms to almost all toxic metals (Ji and Silver 1995, Rouch, Lee et al. 1995). These resistance systems are mostly plasmid-mediated and are present in all bacterial groups (Hoostal, Bidart-Bouzat et al. 2008), which may be a result of these mechanisms having developed shortly after the appearance of prokaryotic life in a metal polluted environment (Ji and Silver 1995). Plasmid-encoded metal resistance systems usually consist of efflux mechanisms, whereas resistance to essential metal ions is usually chromosome-based and more complex than plasmid-encoded systems (Bruins, Kapil et al. 2000). Six mechanisms of heavy metal resistance in microorganisms have been recognised (Silver and Walderhaug 1992, Rouch, Lee et al. 1995, Bruins, Kapil et al. 2000): (1) Exclusion by permeability barrier, by which cell wall, membrane or envelope alterations allow for the microorganisms attempt to protect essential cellular components that are metal-sensitive. (2) Exclusion by active transport of the metal away from the cell via efflux systems in order to export metals that are toxic to the cell from their cytoplasm. (3) Intracellular physical sequestration of metal by protein binding, preventing damaging the metal-sensitive cellular targets by accumulating the metal ions within the cytoplasm to prevents its exposure to essential cellular components. (4) Extracellular sequestration by the production of sufficient levels of a sequestering substance (5) Enzymatic detoxification, whereby chemical modification of the metal renders it less active. (6) Altering the sensitivity of essential cellular components to reduce metal sensitivity of cellular targets or

increasing the production of a specific cellular component in order to stay ahead of metal inactivation (Rouch, Lee et al. 1995, Silver and Phung 1996, Gilotra and Srivastava 1997, Nies 1999, Bruins, Kapil et al. 2000, Nies 2000, Choudhury and Srivastava 2001, François, Lombard et al. 2012, Oves, Saghir Khan et al. 2016).

Heavy metal pollution in soil environments

Heavy metals can enter into the soil environment both through natural causes and anthropogenic activities (Duruibe, Ogwuegbu et al. 2007). The introduction of heavy metals into the environment can modify the microbial community composition and their activity (Aleem, Isar et al. 2003). Due to their effects on the growth, morphology and biochemistry of microorganisms, heavy metals have driven the microbial population to the result of decreased biomass and diversity, leading to the development of these resistance mechanisms in order to tolerate the presence of heavy metals (Samanta, Bera et al. 2012, Mutuku, Okemo et al. 2014). This has been observed in artificial, mercury-polluted aqueous microcosms where there an increase in resistance to mercury lead to a drastic reduction of bacterial diversity (Rasmussen and Sørensen 1998). The pollution of soils by heavy metals has become a severe problem in many parts of the world (Facchinelli, Sacchi et al. 2001, Solgi, Esmaili-Sari et al. 2012) and the analysis of soils for levels of heavy metal contamination has become important in assessing the impact on microbial communities (Oves, Saghir Khan et al. 2016).

Heavy metals in soil are not degraded or destroyed, persisting in the environment for long periods of time they are considered a significant environmental issue that is affecting ecosystems and threatening public health

(Peltier, Vincent et al. 2010, Kawane 2012, Samanta, Bera et al. 2012, Seiler and Berendonk 2012, Garhwal, Vaghela et al. 2014, Laghlimi, Baghdad et al. 2015). Natural sources of heavy metals in the environment include emissions from volcanoes and the resulting transportation of the continental dusts (Oves, Saghir Khan et al. 2016) and through the weathering of metal-enriched rocks where they exist as their ores in varied chemical forms (Duruibe, Ogwuegbu et al. 2007, Oves, Saghir Khan et al. 2016). Anthropogenic activities such as metal forging, agricultural practices, the manufacture of alkaline batteries, smelting and mining introduce heavy metals into the environment (François, Lombard et al. 2012, Samanta, Bera et al. 2012, Oves, Saghir Khan et al. 2016) and a number of studies have shown that pollution by heavy metals in the environment are mostly due to these activities. Wei and Yang (2010) identified the main sources of heavy metal pollution as being a result of industrial practices in North West China: Ordonez, Loredo et al. (2003) recognise a positive correlation between metal pollution and proximity to industrial practices in Northern Spain. Anthropogenic pollution of the environment is dramatically increasing on a global scale, among which are heavy metals being released into the soil environment (Ahemad and Malik 2011). Waste from industrial activities includes heavy metals such as cadmium, chromium, copper, nickel, mercury, lead and zinc which are hazardous if drained into the environment (Mansoorian, Mahvi et al. 2014). With the growth of industrial practices there has been an increase in the release of waste into the environment leading to the accumulation of heavy metals in soils which, due to their persistence in the environment, have long-lasting effects on soil ecosystems (Dixit, Malaviya et al. 2015).

Industrial zinc pollution and zinc resistance

Of all the metals introduced into the environment through industrial activities zinc is a particular concern, and based on the high levels of pollution zinc is considered to be among the class one toxic pollutants (Mansoorian, Mahvi et al. 2014). Taking into consideration all root sources it has been estimated that the total release of zinc worldwide is 1,350,000 tons (Oves, Saghir Khan et al. 2016). Nicholson, Smith et al. (2003) report that zinc was the metal deposited on soil in the largest quantities from atmospheric deposition in England and Wales, and that zinc pollution may be subject to long-range, trans-boundary transport.

The knowledge that zinc is pivotal in a diverse number of basic biological functions, such as the role in zinc finger motifs in transcription factors that aid in gene regulation (Berg and Shi 1996, Nies 1999), 300 zinc containing proteins (Lee, Barrett et al. 2005), over 200 enzymes with zinc as a major component (Choudhury and Srivastava 2001), in DNA replication, cell division and cell activation (Choudhury and Srivastava 2001), illustrates that it is clear that no life would be possible without zinc (Nies 1999). However, the toxicity level of zinc is found to be lower than that of other heavy metals such as cadmium, copper, nickel and lead (Choudhury and Srivastava 2001) and high concentrations of zinc can affect many of the crucial functions in organisms. A toxicity level can be defined as that which causes a cessation of cellular function and propagation. Zinc is an essential metal ion and occurs exclusively as the divalent cation Zn^{2+}, with completely filled d orbitals, and is not able to undertake redox changes under biological conditions (Choudhury and Srivastava 2001, Kelly, Häggblom et al. 2003). Zn^{2+} is therefore important when

redox reactions are not required as it is used to form complex polypeptide chains (Nies 1999).

At higher than required concentrations of zinc, bacterial growth is inhibited and the community reduced leaving only the limited number of resistant bacteria to survive (Choudhury and Srivastava 2001, Kelly, Häggblom et al. 2003). Resistance to levels of zinc higher than that required by the cell as an essential micronutrient can be due to sequestration by metallothioneins (Morby, Turner et al. 1993), extracellular accumulation (Choudhury and Srivastava 2001), intracellular sequestration (Ledin 2000), by means of efflux based mechanisms (Nies 1992, Choudhury and Srivastava 2001), reduction in permeability and alteration of cellular targets (Baker-Austin, Wright et al. 2006). Metallothioneins are specific metal binding proteins that facilitate the sequestration of toxic metals inside the cell (Naik and Dubey 2013). Extracellular accumulation of zinc has been reported in resistant bacteria isolated from industrially polluted soils in New Delhi, India, which accumulate high levels of zinc located on the outer membrane of the cell (Bruins, Kapil et al. 2000). Intracellular sequestration, the prevention of exposure of metals to essential cellular components by accumulating the metal within the cytoplasm, is commonly used to prevent exposure to Cd^{2+}, Cu^{2+} and Zn^{2+} (Bruins, Kapil et al. 2000). Efflux based mechanisms of resistance use active transport systems to export metals from the cells cytoplasm and represents the biggest method of heavy metal resistance systems in microorganisms (Bruins, Kapil et al. 2000, Dixit, Malaviya et al. 2015). Examples of efflux based resistance mechanisms include the Zn^{2+} resistance mediated by *ZntA* in *Escherichia coli* (Beard, Hashim et al. 1997) and Pb^{2+} resistance mediated by *CadA* in *Staphylococcus aureus* (Nies 1992, Ji and Silver 1995). These mechanisms of resistance against Zn^{2+}

aim to ultimately reduce the intracellular concentration of Zn^{2+} in efforts to defend cellular targets. There is likely to be selection for inducible control of a physiologically required metal such as zinc in order to avoid over-expression and also under-expression of resistance to maintain homeostasis (Rouch, Lee et al. 1995).

Co-selection of metal- and antibiotic resistance

Heavy metal contamination may serve as a long term selective pressure for the selection of metal- and antibiotic-resistant microorganisms (Stepanauskas, Glenn et al. 2005). It has been suggested that exposure to metals indirectly selects for microbial resistance to unrelated toxicants such as antibiotics (Alonso, Sanchez et al. 2001, Peltier, Vincent et al. 2010, Garhwal, Vaghela et al. 2014). Indeed, due to the nature of resistance mechanisms and the knowledge that antibiotic compounds exist in nature, it is thought that resistance to antibiotics might provide tolerance to other toxic compounds such as heavy metals (Allen, Donato et al. 2010). If heavy metals are shown to indirectly select for antibiotic resistance in environmental settings such as soils this would have very serious public health implications (Alonso, Sanchez et al. 2001).

Sediment microhabitats are of particular concern as bacteria occupying these habitats are capable of reaching much higher densities relative to other microhabitats as a result of the availability of organic matter (Wright, Peltier et al. 2006). In particular, heavy metal contaminated sediments have been shown to contain a higher number of antibiotic-resistant strains with more plasmids than non-contaminated sites (Wright, Peltier et al. 2006). Industrially contaminated sediments have been demonstrated to have isolates that are resistant to multiple antibiotics, and it is suggested that among environmental

bacterial populations, increased mobilisation under metal selective conditions may increase the mobilisation of antibiotic resistance genes (Baker-Austin, Wright et al. 2006, Wright, Peltier et al. 2006). This association is of particular interest as anthropogenic levels of heavy metal pollution are much higher than environmental concentrations of antibiotics, and there is potential for metal contamination to maintain antibiotic resistance genes within the environment (Wright, Peltier et al. 2006, Gillings, Gaze et al. 2015). Heavy metal tolerance has been associated with antibiotic resistance in bacteria due to resistance to certain antibiotics and metals being mediated by the same plasmid (Bruins, Kapil et al. 2000, Samanta, Bera et al. 2012). When genes that specify for resistant phenotypes are located on the same genetic element, such as an integron, plasmid or transposon, there is evidence of co-resistance and this particular linkage allows for the co-selection of other genes located on the same genetic element (Chapman 2003, Stepanauskas, Glenn et al. 2005). It has been known for decades that the antibiotic- and metal-resistance genes can share this physical linkage, especially on plasmids and there are many studies that demonstrate the correlation between resistance to multiple heavy metals and resistance to antibiotics as a result of the transfer of integral elements (Baker-Austin, Wright et al. 2006).

One mechanism in the selection and proliferation of resistance to antimicrobial agents is cross-resistance, which occurs when antimicrobials share a common pathway to cell death, the same route of access to their cellular target or attack the same cellular target, leading to resistance to antimicrobial agents such as heavy metals or antibiotics (Chapman 2003). For example, multi drug efflux pumps, which represent the biggest method of heavy metal resistance systems in microorganisms (Bruins, Kapil et al. 2000, Dixit,

Malaviya et al. 2015), mediate decreased susceptibility to heavy metals and antibiotics by extrusion of the toxins out of the cell (Martinez, Sánchez et al. 2009). Efflux based mechanisms have been seen to be capable of exporting both metals and antibiotics from the cell (Mata, Baquero et al. 2000) and in isolates containing the DsbA-DsbA system, which is involved in the formation of metal-antibiotic efflux, that were more resistant to multiple antibiotics and metals, including zinc, than mutants without this system (Hayashi, Abe et al. 2000). Co-regulation as a mechanism of metal- and antibiotic-resistance is due to a number of responses, both transcriptional and translational, to exposure to metals or antibiotics that integrate as a response to either stress (Baker-Austin, Wright et al. 2006). Co-regulation of resistance genes responsible for decreased susceptibility to antibiotics are known to be a result of environmental pollution by heavy metals (Seiler and Berendonk 2012). As a result of this co-regulation of resistance mechanisms, there has been found to be resistance to multiple antibiotics and zinc in isolates from both clinical settings (Conejo, García et al. 2003) and the soil environment (Perron, Caille et al. 2004).

It has been demonstrated that bacterial isolates that are more resistant to zinc also exhibit resistance to multiple antibiotics where, in contrast, metal-sensitive isolates did not (Calomiris, Armstrong et al. 1984). One mechanism involved in the selection of antibiotic resistance is co-resistance, which occurs when the gene specifying resistance phenotypes are located on the same mobile genetic element such as plasmids and integrons (Chapman 2003, Stepanauskas, Glenn et al. 2005). Integrons are genetic elements that are capable of acquiring and transferring gene cassettes, and class 1 integrons are linked with co-selection as they frequently contain gene cassettes conferring antibiotic resistance and are found in contaminated areas (Seiler and

Berendonk 2012). The class 1 integron-integrase gene, *intI1,* is commonly linked to genes conferring resistance to both heavy metals and antibiotics and could be used as a proxy that exhibits rapid responses to environmental pressures and could serve as a potential genetic marker for anthropogenic pollutants (Partridge, Brown et al. 2001). Indeed, it is suggested that the co-selection of antibiotic resistance genes and integrons takes place in heavy metal polluted environments and this co-selection is caused by the location of class-1-integrons on a number of plasmids and transposons that carry resistance genes for both antibiotics and heavy metals (Hegstad, Langsrud et al. 2010, Seiler and Berendonk 2012). The class 1 integrons, such as *intI1*, are located close to the genes that code for the multi-drug effluc pump, *czcA,* which has been shown to be responsible for the extrusion of Zn^{2+}, Cd^{2+} and Co^{2+} (Seiler and Berendonk 2012). The antibiotic and metal efflux resistance mechanism has been linked with and number of antibiotics including β-lactams (Baker-Austin, Wright et al. 2006). Due to the close proximity of the genes within the mobile genetic element it is feasible that they are subject to combined transmission in the case of horizontal gene transfer (Seiler and Berendonk 2012), allowing for the spread of resistance throughout populations. This mechanism has been previously demonstrated in strains resistant to both metals and antibiotics that became intolerant to both toxicants after plasmid removal (Ghosh, Singh et al. 2000). It is possible for the integral elements accommodating the genetic resistance to be contained within larger elements, which has been shown to be a case with Tn21 and Tn21-like transposons containing heavy metal resistance operons and an integron enclosing multiple antibiotic resistance genes (Wireman, Liebert et al. 1997, Bass, Liebert et al. 1999, Liebert, Hall et al. 1999).

These data suggest that zinc contamination in the environment may be an important factor in the maintenance of antibiotic resistance as a component of co-selection for resistance mechanisms. The effects of pollution from industry and the resulting effects on microbial communities has not been extensively studied, though it is well documented that heavy metal contaminated sites also contain a higher percentage of antibiotic resistant organisms (Alonso, Sanchez et al. 2001). These heavy metal contaminated environments maintain selective pressure on populations of microorganisms for lengthy time periods and ecosystems containing high concentrations of heavy metals occur frequently, and if heavy metals are shown to directly select for antibiotic resistance in environmental setting such as soils, this would have very serious public health implication (Alonso, Sanchez et al. 2001)

The antibiotic resistance crisis

Antibiotics are critical in the treatment of bacterial infections in humans and animals and it is of paramount importance that their efficacy be maintained (Allen, Donato et al. 2010). Antibiotics and their applications are considered one of the major breakthroughs in modern medicine due to their contribution in increasing the life span through altering the outcome of bacterial infections and their vital role in medical practices (Rossolini, Arena et al. 2014). Yet there is an ever increasing worldwide issue in the treatment of infectious diseases caused by antibiotic resistance (Peltier, Vincent et al. 2010, Samanta, Bera et al. 2012, Seiler and Berendonk 2012, O'Neill 2014), and the number of infections caused by resistant bacteria is increasing on a global scale with the threat of untreatable infectious diseases becoming a reality (Blair, Webber et al. 2015).

Increased resistance to already existing antibacterial agents as a result of widespread use in human medicine and animal treatment is of increasing concern as there is a lack of new antibiotics in development (Blair, Webber et al. 2015). It is common to encounter the development of resistance to a particular antibiotic in pathogens following the use of that antibiotic in a clinical setting, and the use the antibiotic directly affects the frequency of resistance (Allen, Donato et al. 2010). Antibiotic resistance genes are often found on integrons, plasmids or transposons which can be transferred to other bacteria in the surrounding environment through horizontal gene transfer (Witte 1998). A further concern is that resistance to antibiotics has developed from resistance to one class of antibiotic to multidrug resistance (MDR) through the use of antibiotics to treat infections that are already resistant to one or more antibiotic, thereby increasing the challenge in maintaining the effective use of antimicrobial treatment (Marti, Variatza et al. 2014, Tanwar, Das et al. 2014). The increase of resistance to multiple antibiotics has resulted in strains with MDR phenotypes and has greatly narrowed the options of available treatment for some pathogens (Rossolini, Arena et al. 2014).

Since antibiotics were introduced over 75 years ago there has been huge selection pressure exerted through the use of millions of tonnes of antibiotics (Laxminarayan, Duse et al. 2013). In the USA an estimated 94,000 invasive MRSA infections were associated with the death of 19,000 individuals in a 1-year period (Laxminarayan, Duse et al. 2013). A review on antimicrobial resistance conducted in the UK in 2014 revealed approximately 50,000 deaths a year in Europe and The United States of America as a result of antimicrobial-resistant infections (O'Neill 2014). It has been estimated that the number of deaths due to antimicrobial resistance in the next 35 years could be as high as

300 million individuals and could increase to 10 million deaths a year by 2050
(Fig. 1, Fig. 2), and the economic damage could be between 60 - 100 trillion US
Dollars if the crisis is not tackled (O'Neill 2014). One major aspect of the
challenge of antibiotic resistance is the spread of resistance in the environment
due to anthropogenic selection (Wright, Peltier et al. 2006).

There is debate with regards to whether antibiotics in the environment
are selecting for resistance in exposed bacteria or if other factors can be
considered more important in maintaining or spreading resistance, of which
environmental metal pollution is considered as one such factor (Peltier, Vincent
et al. 2010, Bhullar, Waglechner et al. 2012). Incidence of increased resistance
in the environment are most often found in locations in which environmental
pollution has had an effect on the local abundance of resistance traits and little
attention has been given to the potential for these environments to maintain and
increase resistance (Knapp, McCluskey et al. 2011, Wellington, Boxall et al.
2013). Antibiotic resistance genes are also observed in environments and
humans that have had little or no antibiotic exposure, such as those found
within the gut microbiome of the Hadza hunter-gatherers of Tanzania (Rampelli,
Schnorr et al. 2015). The presence of antibiotic resistance genes in the Hadza
people suggest the presence in the human gut may be an innate feature and
extensive use in medicine or exposure to environmental pollutants may act as a
promoter of acquisition on antimicrobial genes.

**Microbial community diversity along a gradient of anthropogenic zinc
pollution**

The soil environment is one that is extremely complex and one within which the
occupying microbial population must tolerate consistently altering chemical

conditions that can disturb microbial community diversity (Daniel 2005, Jacobsen and Hjelmsø 2014, Calderón, Spor et al. 2017). Indeed, soils and sediments potentially harbour the highest species-level diversity on Earth and as a result are one of the most challenging of all natural habitats when regarding the size of the microbial communities therein and the vast diversity of species present (Daniel 2005, Vos, Wolf et al. 2013). The role of soil microorganisms in the stability of the whole ecosystem is essential, such as the decomposition of organic matter, nutrient cycling and promoting plant growth, and any disturbances that may alter microbial diversity could have effects that prove harmful to whole ecosystem functioning (Stefanowicz, Niklińska et al. 2009, Jacobsen and Hjelmsø 2014, Calderón, Spor et al. 2017).

Anthropogenic inputs of heavy metal pollutants into the environment can directly and/or indirectly effect the diversity and metabolic activity of the microbial community and have long-term detrimental effects (Hoostal, Bidart-Bouzat et al. 2008, Sobolev and Begonia 2008, Oves, Khan et al. 2012). It is clear that the introduction of heavy metal pollutants into the soil environment can alter microbial communities, thereby inhabiting that particular ecosystem leading to the appearance of heavy metal resistant organisms (Aleem, Isar et al. 2003, Samanta, Bera et al. 2012). Changes in microbial community composition over time due to metal pollutants have only ever been examined sediment depths in which the highest levels of microbial activity are present or spanning only months or a small number of decades (Bouskill, Barnhart et al. 2007, Kaci, Petit et al. 2016). To fully understand the impacts imposed by industrial heavy metal pollution in altering microbial communities and as a driving force for the proliferation of resistant organisms in the environment it is crucial to examine the relative abundance of microbial communities over a temporal scale

including that which precedes and follows the period in which metal pollutants began to dramatically increase.

Microorganisms are at the core of global environmental processes but due to the complexities of microbial diversity, and the fact that only a small fraction of bacteria can be cultivated, molecular tools are needed to study bacterial community composition (Llirós, Inceoğlu et al. 2014). With advances in the study of 16S rRNA sequencing technologies our understanding of the composition of soil microbial communities has vastly improved (Lozupone and Knight 2007). 16S rRNA amplicon sequencing analysis is a widely applied approach in the study of microbial community composition that has expanded our potential for the study of community dynamics over temporal scales, revealing deeper insights into the diversity of bacterial communities (Lozupone and Knight 2007, Sinclair, Osman et al. 2015). With the use of amplicon sequencing of the 16s subunit rRNA gene with state of the art high-throughput sequencing technologies (Illumina) it is possible to analyse the diversity between microbial communities and the environment in which they persist (Sinclair, Osman et al. 2015). High-throughput sequencing technologies has previously been employed to analyse samples from environments like geothermal hot springs (Pace 1997), and have demonstrated that microbial diversity is far more extensive than can be demonstrated by laboratory culturing methods and reveals that microorganisms represent the majority of phylogenetic diversity of life on Earth (Lozupone and Knight 2005). This technique has expanded our potential for the study of community dynamics over spatial and temporal scales, and has revealed deeper insights into the diversity of bacterial communities, demonstrating how far we are from cataloguing the vast diversity of microorganisms on Earth (Lozupone and

Knight 2007, Sinclair, Osman et al. 2015). Despite huge advances in sequencing technologies, Illumina sequencing methods are unable to reliably classify at a genus or species level and have been shown to produce variation in the number of OTUs from replicate samples, leading to possible incorrect projections of diversity (Poretsky, Rodriguez-R et al. 2014, Schloss, Jenior et al. 2016).

Microbial community tolerance to heavy metal has been frequently studied in a laboratory setting with the use of artificial contamination; however, soils that are subject to industrial pollution for decades are rarely studied (Stefanowicz, Niklińska et al. 2009). In this thesis we examine the relationship between zinc contamination and levels of zinc resistance in the microbial community isolated from a sediment core collected from the area of Runcorn, North West United Kingdom, which has a been a major site of chemical industry for over a century (Hodgson, Nieuwenhuijsen et al. 2004). Data from the UK Environment Agency Pollution Inventory reveals substantial amounts of heavy metals are released annually from over a dozen industrial plants in this area (Fig. 3) (Hodgson, Nieuwenhuijsen et al. 2004). To provide a quantitative measure of heavy metals within the sediment, 50 samples from the Griffin Wood Pond primary core were analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to quantify the levels of a range of heavy metals (Al, Ba, Co, Cr, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Sr, Ti and Zn) in the sediment (Chapter 1. Table 1).

This study marks the first attempt in using a sediment core that covers a time scale spanning the increase in industry with the aim of providing a detailed analysis of the changes in microbial community composition in an area with a long history of industrial activity. In an effort to provide a deeper understanding

of the heavy metal driven response in microbial diversity through time due to anthropogenic pollution we examine the correlation between levels of zinc pollution over time and the level of resistance within the microbial population. We assess the selective pressure of zinc as a driving force for the maintenance and proliferation of antibiotic resistance through a phenotypic analysis of resistance in microbial isolates from the sediment core. To provide further information for the co-selective pressures imposed by zinc on antibiotic resistance we quantify the presence of the *IntI1* gene, which has been observed to be linked with resistance to both heavy metals and antibiotics and may serve as a genetic marker for anthropogenic pollution. Following this, we use sequencing technologies to assess the microbial community within the sediment layers that cover the most significant rise in zinc concentration to provide a detailed insight into changes in community composition as a result of changing levels of zinc in the environment. Thereby providing unique data detailing the effects of anthropogenic pollution on microbial populations and the co-selective mechanisms driving the proliferation of antibiotics in the environment over time.
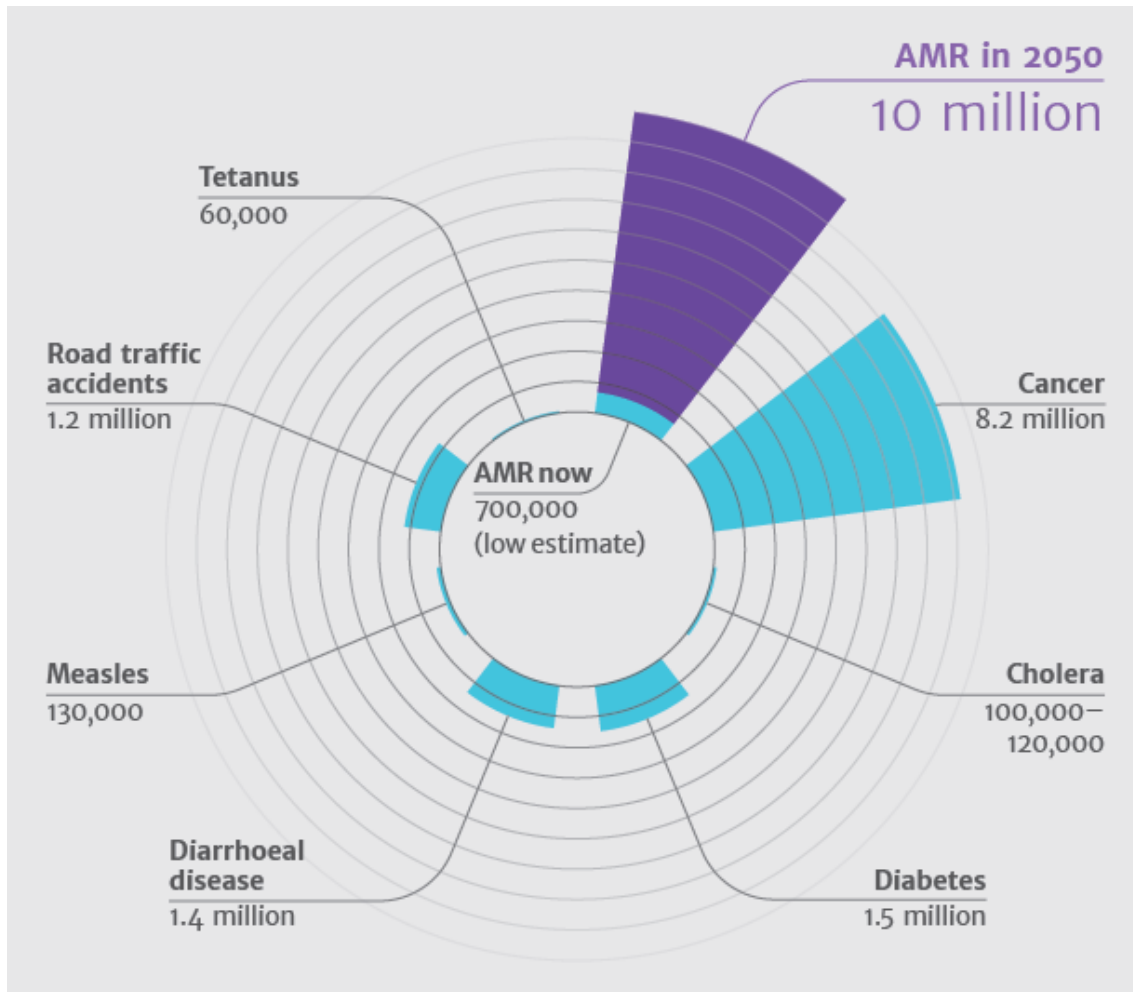
Figures

Figure 1: Number of deaths attributable to antimicrobial resistance every year, compared with other major causes of death, showing an estimated 10 million deaths a year by the year 2050 (O'Neill 2014).
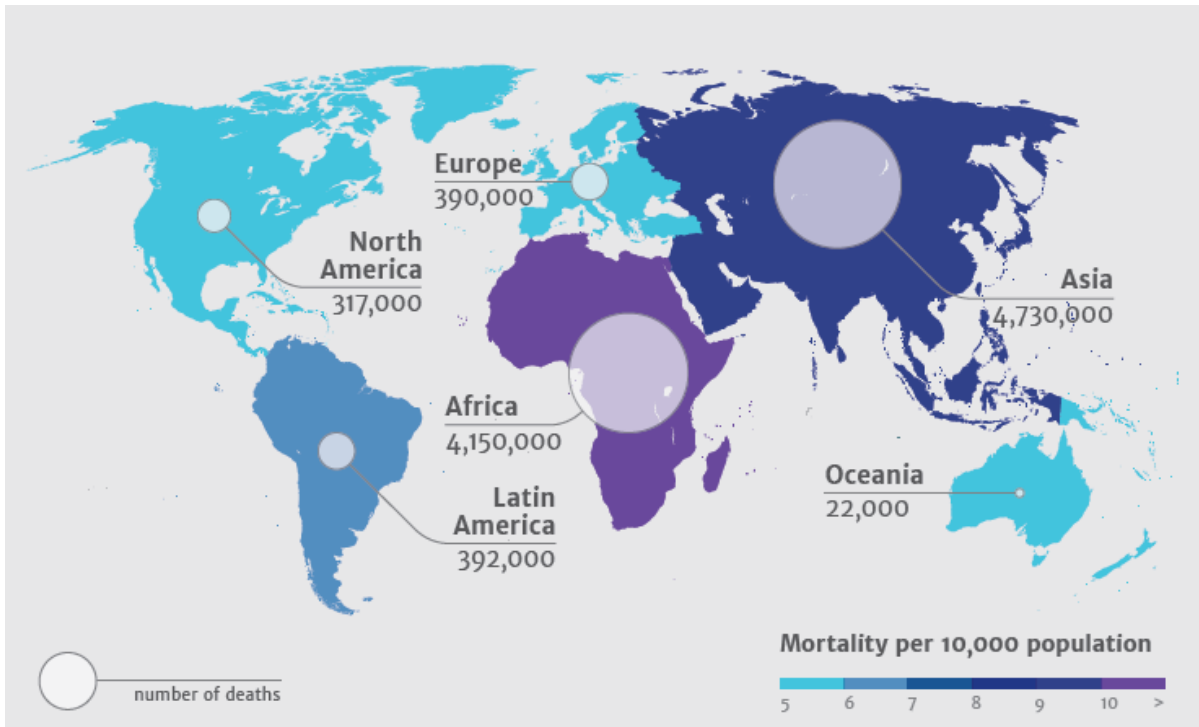
Figure 2: Number of deaths attributable to antimicrobial resistance in different
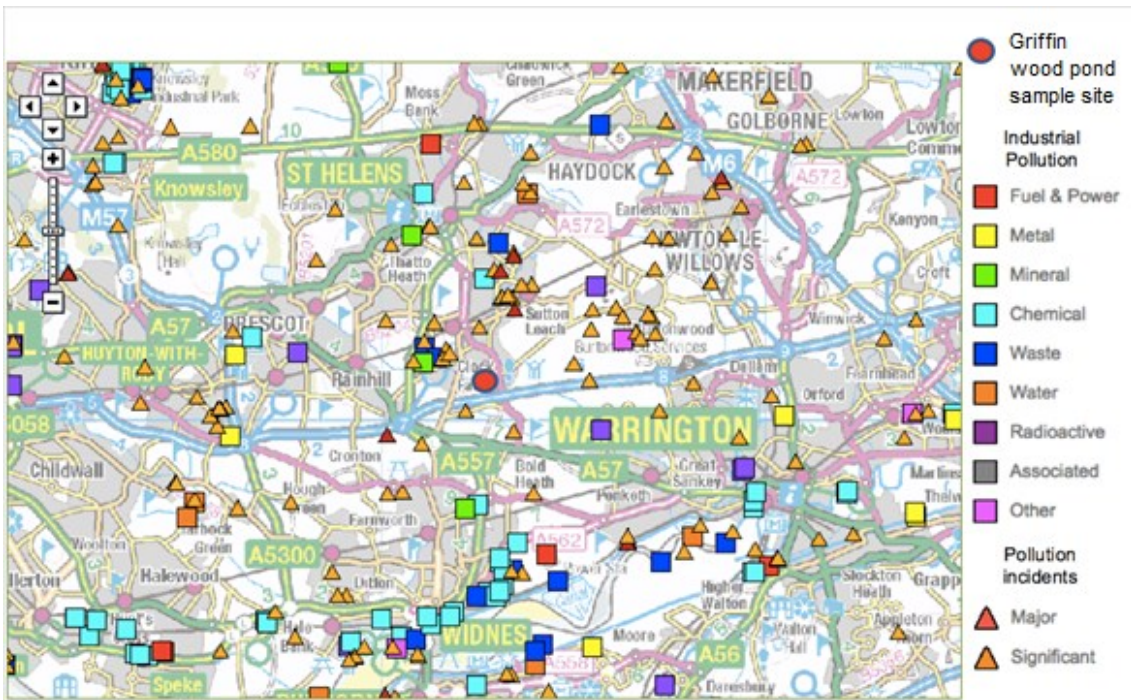parts of the world every year by 2050 (O'Neill 2014).

Figure 3: Map showing pollution incidents and areas of industrial pollution in the area surrounding Griffin Wood Pond sample site. This map is courtesy of the Environmental Agency (https://www.gov.uk/check-local-environmental-data).

Chapter 2

Materials and Methods

Sediment core sampling and processing

A sediment core was extracted from Griffin Wood Pond (GWP) (National grid reference: SJ 53709 90958) approximately 7k East of Widnes and Runcorn, using an Uwitech sediment corer [Uwitec, Mondsee, Austria] deployed from a small inflatable boat into the centre of the pond site. The sediment core extraction took place on 30/04/2014 and was performed by Dr Ann Power and Dr Richard Jones. The core was then transported to the Geography department at the University of Exeter Geography department, Streatham Campus, Exeter, UK, where it was stored at 5°C. Core extrusion was carried out on 12/02/2015 by Andrew Dickinson and Dr Ann Power. The core was vertically extruded and sliced at 1cm intervals to a total of 50 cm depth. Three samples, approximately 0.5g in weight, were isolated from the centre of each sediment slice upon extrusion: one sample was stored in 1.5 mL micro-centrifuge tubes ('Crystal Clear' Micro-centrifuge tube, Starlab Group, Milton Keynes, UK); duplicate samples were stored in 5 mL cryogenic vials (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Samples stored in 1.5 mL micro-centrifuge tubes and one set of the samples stored in the 5 mL cryogenic vials were suspended in 600µl 20% (vol/vol) glycerol solution and stored at -20°C and -80°C respectively. These samples were used for bioavailability and resistance analysis. The remaining sample set in 5 mL cryogenic vials were stored at -80°C without the addition of glycerol for DNA extraction. In the absence of sediment without exposure to heavy metals as a control, these analyses are comparisons of within-sample variations.

Inductively Coupled Plasma Optical Emission Spectroscopy analysis

Samples were taken from the remaining sediment left after core extrusion and sample collection for bioavailability analysis, resistance analysis and DNA extraction. Each sediment layer sample (approx. 0.25 g) was frozen overnight at - 20°C, after which they were freeze dried (ModulyoD Freeze Dryer, Thermo Electron Corp.) at a temperature of -48°C and pressure of less than 0.1 bar. Dried samples were digested in 3 ml concentrated (70%) nitric acid (HNO_3) in a 100 ml beaker and stirred with a glass rod. Each sample was then placed on a hot plate until dry. A further 3 ml concentrated nitric acid and 0.5 ml hydrochloric acid (37%) were added and re-heated until the brown nitrogen dioxide fumes changed to a clear colour and allowed to cool to room temperature. The remaining solution and sediment was filtered (Whatman grade 42 Filters) into a 25 ml flask and topped up to a total of 25 ml with deionized water. This method is in accordance with previously published protocols (Melaku, Dams et al. 2005).

Samples were analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using a Varian VISTA- MPX CCD Simultaneous ICP-OES to identity and quantify the elements present in the sediment core. Results were calibrated using a range of dilution strengths of standard solutions (1000 mg/l of CertiPUR mix containing Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, In, K, Li, Mg, Na, Mn, Pb, Sr, Tl and Zn; Aristar 456262B containing Ti; and Aristar 456102J containing Ta). Samples were measured in duplicate and quality control samples were also measured. This technique was performed on two individual cores from the same site using the same method.

Radiometric dating of sediment core

Dried sediment samples from fifty layers of the Griffin Wood Pond core were analysed for 210Pb, 226Ra, and 137Cs by direct gamma assay using Ortec HPGe GWL series well-type coaxial low background intrinsic germanium detectors (Appleby, Nolan et al. 1986). 210Pb was determined via its gamma emissions at 46.5 keV, and 226Ra by the 295 keV and 352 keV γ - rays emitted by its daughter isotope 214Pb following 3 weeks storage in sealed containers to allow radioactive equilibration. 137Cs was measured by its emissions at 662 keV. The absolute efficiencies of the detectors were determined using calibrated sources and sediment samples of known activity. Corrections were made for the effect of self-absorption of low energy γ - rays within the sample ((Appleby, Richardson et al. 1992). Peter Appleby and Gayane Piliposian at the Environmental Radioactivity Research Centre, University of Liverpool, performed dating analysis.

Zinc bioavailability

Bioavailability analysis was carried out by Mette Guldborg Hansen and Dr Kristian Koefoed Brandt, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. The Zn specific biosensor *Pseudomonas putida* strain KT2440.2431(pDNPcadA1lux) (Hynninen, Tõnismann et al. 2010) and the Cu specific biosensor *Pseudomonas fluorescens* strain DF57-Cu15 (Nybroe, Brandt et al. 2008) were used to determine the Zn and Cu bioavailability, respectively. To correct for matrix effects and nonspecific toxicity, the strain *P. fluorescens* DF57-40E7 was used to determine inhibition of the bioluminescence reaction according to the procedure described in Brandt, Holm et al. (2008). *P. putida* KT2440.2431(pDNPcadA1lux) and *P. fluorescens*

DF57-Cu15/40E7 were grown and harvested as described in Hynninen, Tõnismann et al. (2010) and Nybroe, Brandt et al. (2008) respectively. *P. putida* cell suspensions were made in a medium containing 40 mM 3-N-morpholinopropanesulfonic acid (MOPS, pH7.2), 50 mM KCl, 10 mM NH_3Cl, 0,5mM $MgSO_4$, 0,4% glucose, 1 mM glycerol-2-PO4, 1 µM $FeCl_3$ and 12 µg/ml tetracycline, obtaining an OD_{600} = 0.015-0.020. *P. fluorescens* cell suspensions were made in a medium consisting of 100 mM KCl, 20 mM MOPS (pH 7.2), 7.6 mM $(NH_4)_2SO_4$, 4 mM glycerol-2-phosphate disodium salt, and 0.8% (w/v) glucose, obtaining an OD_{600} = 0.167. The cell suspensions were mixed in a 1:1 ratio with standards or samples in a 96-well microtiter plate. Standard solutions covering a range of 0-1.25 µM $CuSO_4$ and 0-12.8 µM $ZnSO_4$ were prepared in Milli-Q water. Samples were extracted with Milli-Q water in a sediment (wet weight)-water ratio of 1:5.

The plates were subsequently incubated for 90 minutes for *P. fluorescens* DF57-Cu15/40E7 and 3.5 hours for *P. putida* KT2440.2431(pDNPcadA1lux) and bioluminescence was measured using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany). The bioavailable Cu and Zn concentrations were determined by fitting third order polynomial calibration curves from the biosensor response to the standard solutions, using Microsoft Excel (2016).

Zinc resistance and antibiotic resistance

Tryptic Soy Agar (TSA) (Sigma-Aldrich Co. St. Louis, MO, USA) was amended with antibiotics oxacillin (50 µg/mL), trimethoprim (60 µg/mL) (Sigma-Aldrich Co. St. Louis, MO, USA) and cefotaxime (50 µg/mL), (Molekula Limited, Newcastle Upon Tyne, UK) or ZnSO4•7H2O purchased from Sigma-Aldrich Co. St. Louis,

MO, USA. M9 buffer was made using a mix of KH_2PO_4, Na_2HPO_4, NH_4Cl (Sigma-Aldrich Co. St. Louis, MO, USA) and NaCl (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). These antibiotics were chosen as they have been shown to share resistance pathways with a number of heavy metals, most importantly zinc. Preliminary experimental work was performed to determine the minimum inhibitory concentrations (MIC) of each antibiotic using in this study (data not shown).

Sediment from each layer was spread using inoculation spreaders (Starlab Group, Milton Keynes, UK) onto plates containing only TSA and plates with TSA amended with ZnSO4•7H2O at three concentrations (0.031 mg/ml, 0.1787 mg/ml, 0.625 mg/ml). Plates were incubated for 48 hours at 28°C. 27.676 mg sediment from cm 11-35 was spread onto plates containing only TSA and incubated for a period of 48 hours at 28°C. 96 clones were isolated from each TSA plate using a sterile toothpick. Each picked clone was re-suspended into a single 96-well plate well (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) containing 50 µl M9 buffer solution. Clones were then transferred onto TSA plates (control), TSA plates amended with ZnSO4•7H2O (0.625 mg/mL), oxacillin (50 µg/mL), cefotaxime (50 µg/mL) and trimethoprim (60 µg/mL) using a 96-pin replicator (Boekel, Fisher Scientific) and incubated at 28°C for 48 hours.

16s PCR amplification and sequencing

DNA for 16S rRNA amplicon sequencing was extracted from the sediment samples from depths ranging 11 cm to 35 cm using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC. 29525 Fountain Pkwy. Solon, OH 44139) using the standard protocol. The DNA samples were then quantified using Qubit 2.0

Fluorometer (Invitrogen) following the standard protocol, to ensure DNA concentration was at or above 5 μg/μL. Agarose gel electrophoresis was used to assess DNA quality, ensuring high molecular weight and minimal degradation. 0.8% agarose gel (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) was used with the additional of 5 μl ethidium bromide (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) per 100 mL agarose gel and run at 120 volts for 40 minutes.

Soil microbial community profiling was performed using Illumina® HiSeq 2500 using SBS Rapid reagents v2 using Hp10, HP11 and HP12 sequencing primers (Illumina Inc.). The primers sequences used for amplification of the 16s V3-V4 region were 341F Amplicon PCR forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCaG-3') and 785R Amplicon PCR reverse primer (5' TCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATcC-3') (Thijs, De Beeck et al. 2017). These primers have a phosphorothiate oligo (PTO) indicated by the lowercase at the 3' end to limit endonuclease degradation. Primers used for second PCR addition of Illumina NexteraXT barcodes and flowcell binding sequence were; P1 Nextera (i5) index primer: 5'-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC-3' and P2 Nextera (I7) indexing primer: 5'-CAAGCAGAAGACGGCATACGAGA [i7]GTCTCGTGGGCTCGG-3' (Nextera XT DNA Library Prep Kit, Illumina®).

Obtained DNA sequences were assembled and quality checked using the following pipelines; ssequencing reads assembled using Pandaseq (2.3, RDP extended version 1.0.3); filtering and primer removal performed using RDP Tools v2.0.2.; chimera removal was performed with USearch v8.1.1861 using

the RDP gold database; sequencing alignment completed using Infernal v.1.1.1.; clustering and classifying performed using RDP Tool v2.0.2 from database: RDP Release 11, Update 4. Dr Karen Moore and Audrey Farbos, Exeter Sequencing and Welcome Trust Biomedical Informatics Hub, Streatham Campus, Exeter conducted sequencing analysis. The assembly and quality checking was performed by Paul O'Neill at the Exeter Sequencing and Welcome Trust Biomedical Informatics Hub, Streatham Campus, Exeter.

Quantitative real-time PCR of sediment DNA extracts

DNA for qPCR was extracted from sediment samples from depths 11 cm to 35cm using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, 2746 Loker Ave West, Carlsbad, CA 92010) using the standard protocol. qPCR assays for *IntI1* and bacterial 16s rRNA gene fragments were performed in 96-well plates using TaqMan Environmental Master Mix 2.0 (Life Technologies Corp., Applied Biosystems, Woolston, Warrington, UK) which is designed to offer accurate detection in the presence of a high level of PCR inhibitors (Czekalski, Díez et al. 2014). Standard curves were prepared using serial 10-fold dilutions of plasmid DNA containing the respective target gene in a range of 1×10^6 to 10 gene copies. Each 20 µl of reaction mixture consisted of an 18 µl mix of TaqMan Master Mix, primers, probe, Bovine Serum Albumin (BSA) and Dimethyl Sulfoxide (DMSO) and 2 µl of extracted DNA. Primers (Chapter 3: Table 1) (Integrated DNA Technologies, Inc., Leuven, Belgium), Probes (Chapter 3: Table 1) (Life Technologies Corp. Woolston, Warrington, UK), BSA, DMSO and nuclease-free water (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Assays were performed in duplicate for each DNA extract, standard and negative controls using an Applied Biosystems

StepOnePlus real-time PCR system (Applied Biosystems, Woolston, Warrington, UK).

Statistical analysis

Pearson's product-moment correlation and linear regression analyses were performed using RStudio Version 0.99.463 – © 2009-2015 RStudio, Inc. Graphics were made using RStudio Version 0.99.463 – © 2009-2015 RStudio, Inc., MacQiime Version 1.9.1 20150604 OS10.7 and in some cases edited using GNU Image manipulation Program 2.8.18.

MacQiime Version 1.9.1 20150604 OS10.7 was used in the following analyses: beta-diversity using Bray-Curtis dissimilaries index, Principal coordinates analysis plots created using Emperor through Qiime (Vázquez-Baeza, Pirrung et al. 2013); Mantel test (Navas-Molina, Peralta-Sánchez et al. 2013).

Chapter 3

Anthropogenic zinc pollution: cross-resistance between metal- and antibiotic-resistance traits

Introduction

Due to the nature of the persistence of metals within the soil environment, it is typically implicated that increased tolerance to heavy metals may be a driving factor in the maintenance of antibiotic resistance in the environment (Baker-Austin, Wright et al. 2006). Over time scales spanning decades, sediment deposits allow for past levels of contamination to be recorded and dated (Audry, Schäfer et al. 2004, Zhang, Zhang et al. 2015) but to date there have been few studies available, especially not ones that have investigated depths exceeding 20 cm (Kaci, Petit et al. 2016). Assessing zinc pollution over a time scale that covers the introduction of zinc into modern industrial practices, allows us to determine the effects of increased zinc exposure on microbial community composition.

A culture-based approach can provide specific information on the diversity of life and a quantitative output of microbial communities that occupy environmental soils. The phenotypic analysis of the isolates from within a sample extracted from an area under pressure from anthropogenic pollution allows for the investigation of the detrimental effects of zinc on particular microbial inhabitants. Several studies have detected positive correlations between the level of heavy metals and the occurrence of metal resistance genes in the soils (Xiao-Fang, Hong-Bin et al. 2012, Besaury, Bodilis et al.

2013). Previous studies have made aware the connection between metal and antibiotic resistance (McArthur and Tuckfield 2000, Berg, Tom-Petersen et al. 2005, Baker-Austin, Wright et al. 2006) and have demonstrated that in culturable bacterial populations, pre exposure to zinc can increase levels of tolerance towards the antibiotics ciprofloxacin, oxytetracycline and tylosin in wastewater treatment reactors (Peltier, Vincent et al. 2010).

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) has been performed to quantify the heavy metal content of the sediment. In addition, sediment samples were analysed for 210PB, 226Ra and 137cs by direct gamma assay to provide radiometric dating of the sediment core. The combination of data collected through ICP-OES and radiometric dating analysis allows for a more detailed examination of the levels of heavy metal toxicants in sediment and at what period in time increases have occurred. This will provide a direct link between the dates at which levels of industrial activity are known to have dramatically increased and elevated levels heavy metal contamination in the environment. Although ICP-OES for chemical analysis of environmental heavy metal pollution provides highly accurate data on total metal concentrations, it does not provide specific information on the biological availability of the metals (Hynninen, Tõnismann et al. 2010, Oves, Saghir Khan et al. 2016). Metals in soil can vary greatly in mobility, biological availability and chemical behaviors (Oves, Saghir Khan et al. 2016) and bioavailibily and toxicity often correlate poorly with total metal concentration (Nybroe, Brandt et al. 2008). Therefore it is necessary, with the use of a zinc specific biosensor strain, *P. putida* T2440.2431 (pDNPcadA1lux), to produce a quantifiable output in response to a target chemical, in this case zinc, to determine zinc bioavailability (Hynninen, Tõnismann et al. 2010).

An analysis of the phenotypic resistance to zinc in the microbial population occupying the sediment is necessary to gain a further understanding of the impacts of anthropogenic zinc pollution on microbial diversity through time. Through the extraction of isolates that have been cultured from sediment layers that exhibit the greatest increase in zinc concentration (35 cm – 11 cm), and subsequent growth in the presence of zinc and antibiotics, we aim to provide an ecologically relevant framework from which to address the role of zinc exposure as a mechanism for increased incidence of antibiotic resistance.

Prevalence if the *intI1* gene, linked with resistance to both heavy metals and antibiotics, may serve as a potential genetic marker for anthropogenic pollution. (Gillings, Gaze et al. 2015). The quantification of this resistance gene linked with tolerance towards both heavy metals and antibiotics may provide further evidence for the co-selective mechanisms driving antibiotic resistance. With the use of quantitative PCR we aim to provide a measure of *IntI1* gene proliferation over time, thereby establishing a genetic marker for anthropogenic influences in the selection of resistance organisms towards those with increased tolerances to antimicrobials.

In this chapter, we mark the first attempt to use a sediment core that spans a time scale covering the increase in use of heavy metals in industrial practices with the aim to gain a better understanding of the effects of zinc pollution on the microbial population. Specifically, to examine the correlation between the levels of resistance within microbial populations and the increase in concentrations of zinc in the environment. Further to this, we aim to better understand the selective pressures imposed by metal contamination introduced through industry as a significant selective agent driving the maintenance and proliferation of antibiotic resistance. By analysing the correlation of resistance in

isolates from sediment to both zinc and antibiotics, and correlation with a quantifiable marker gene for resistance, I aim to provide further evidence for the co-selective pressures imposed by metal exposure on antibiotic resistance in the environment due to anthropogenic activities.

Results

A temporal gradient of Zinc contamination in an industrially polluted pond sediment

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analysis was used to quantify the concentration of zinc in the sediment from the Griffin Wood Pond preliminary core revealing the highest zinc concentration found to be 0.625 mg/g (Fig. 1). The concentration of zinc found in the preliminary sediment core greatly increases from a depth of 40cm and peaks at a depth of 23cm (0.625 mg/g), after which the concentration declines. Following the analysis of the preliminary core, it was necessary to perform an additional ICP-OES analysis on a second sediment core from the same sample site under optimal sterile conditions. ICP-OES analysis of the Griffin Wood Pond sediment core used for the experimental work in this study (henceforth referred to as the main core) was performed for each depth (1cm to 50cm) to quantify the levels of zinc present in at each depth, showing the highest zinc concentration to be 0.486 mg/g (Fig. 2, table 2). The measure of the strength of a linear association between the decreasing concentration of zinc within the sediment as depth through the sediment core increases shows there is a highly significant negative linear correlation between depth and zinc concentration in the preliminary sediment core (r = -0.729, p < 0.001; Pearson's product-moment correlation coefficient). In the main core, the concentration of zinc found within the

sediment begins to greatly increase from a depth of 30cm and peaks at a depth of 21cm (0.486 mg/g), after which there are moderate fluctuations in zinc concentration before the concentration steeply declines. The concentration of zinc within the sediment decreases significantly with depth (r = -0.679, p < 0.001; Pearson's product-moment correlation coefficient).

In order to determine the bioavailability of zinc in the sediment core samples the zinc specific biosensor *P. putida* strain T2440.2431 (pDNPcadA1lux) was used to prove a quantifiable output. Severe matrix effects due to the presence of toxicant mixtures resulted in the return of data for twelve out of fifty sediment sample. The highest concentration of bioavailable zinc can be seen at a depth of 20 cm (0.042 mg/g), however this represents 8.6% of the total zinc concentration found at that depth, and the highest percentage of bioavailable zinc is found at a depth of 23 cm (9.97%). A Pearson product moment correlation coefficient was computed to evaluate the linear relationship between bioavailable zinc and total zinc concentration found at these depths in the primary sediment core revealing significant positive correlation with the total zinc concentration in the sediment (r =0.674, p < 0.05) (Fig. 3).

Sediment core chronology

The results of the radiometric analyses are given in table 3 and shown graphically in Figure 4.

Lead-210 Activity

Total ^{210}Pb concentrations (Figure 4a) reached equilibrium with the supporting ^{226}Ra at a depth of around 23 cm. Unsupported concentrations (Figure 4b) appear to vary a little irregularly with depth, with a small but possibly significant non-monotonic feature in the 16-17 cm section. The overall decline is however

more or less exponential and some of the apparent irregularities may be due to the relatively large uncertainties caused by the low activities, particularly in the deeper sections. Since the maximum unsupported concentration is just under 60 Bq kg-1 the ^{210}Pb dating horizon is unlikely to be more than around 3 ^{210}Pb half-lives (60-70 years).

Artificial Fallout Radionuclides

The ^{137}Cs concentrations (Figure 4c) have a relatively well-defined peak in the 12-13 cm section that most probably records the 1963 fallout maximum from the atmospheric testing of nuclear weapons.

Core Chronology

^{210}Pb dates calculated using the CRS dating model (ApplebyPG 1978) place 1963 at a depth of 12.5 cm, in good agreement with the depth indicated by the ^{137}Cs record. The results, shown in Figure 5 and given in detail in Table 4, suggest a slightly higher sedimentation rate during the past two decades, with a mean value during that time of 0.25 ± 0.04 g cm-2 y-1 (0.30 cm y-1). Prior to that, from the mid-20th century through to the early 1990s, sedimentation rates appear to have been relatively uniform with a mean value of 0.17 ± 0.03 g cm-2 y-1 (0.19 cm y-1). The non-monotonic feature at 16.5 cm, which may record a brief episode of more rapid accumulation, is dated to the mid-1940s. Sedimentation rates at times earlier than this are highly uncertain and have been assumed similar to those in later years.

Zinc effects on cultivable microbial community

In order to measure the levels of phenotypic resistance in bacterial communities found in the main core, sediment from each layer was spread onto plates amended with ZnSO4•7H2O at the highest, lowest and median concentration of

zinc found in the preliminary core (0.625 mg/ml, 0.031 mg/ml and 0.1787 mg/ml respectively) to correlate zinc resistance with the levels of zinc exposure. Sediment was also plates on plates containing only TSA. After growth, the colonies were counted and the measure of the strength of a linear association between zinc resistance of cultivable bacteria and zinc concentration in sediment layers from a depth of 1cm to 50cm was performed. Resistance was calculated as the percentage of resistant colony-forming units (CFU) of total CFU on zinc free plates. Resistance to zinc at the highest concentration found in the preliminary core (0.625mg/g) has a moderate positive linear correlation with zinc concentration ($r = 0.416$, $p < 0.01$) (Fig. 6). Resistance to zinc at the median concentration found in the preliminary core (0.1787 mg/g) has a weak positive linear correlation with zinc concentration ($r = 0.370$, $p < 0.01$ (Fig. 7)) (Pearson's product-moment correlation coefficient).

Zinc and antibiotic co-selection

To address the role of zinc exposure as a mechanism for increased incidence of antibiotic resistance, sediment layers ranging from a depth of 11 cm to 35 cm were used in analysing the relationship between zinc resistance and antimicrobial resistance as it is between these depths that the most significant changes in zinc concentration can be observed (Fig. 2). 96 clones were randomly isolated per sediment layer to represent the cultivable bacterial community and grown on plates amended with ZnSO4•7H2O (0.625 mg/mL), oxacillin (50 μg/mL), cefotaxime (50 μg/mL), trimethoprim (60 μg/mL) and TSA only plates. The number of isolates growing on each plate were counted to provide a proportional representation of resistant isolates.

A linear regression analysis was performed to assess the relationship between

proportion of zinc resistant isolates and zinc concentration across sediment layers varying in zinc concentration. Zinc concentration has a significant effect on proportion of zinc resistance in the randomly selected isolates ($p < 0.001$, $m = 2.5894$) (Fig. 8a). These results are consistent with our previous analysis of the relationship between zinc resistance and zinc concentration in the total cultivable bacterial population. Linear regression analyses were then performed to test the relationship between the proportion of zinc resistance and proportion of resistance to the antibiotics oxacillin (50µg/mL), cefotaxime (50µg/mL) and trimethoprim (60 µg/mL). In order to reduce the chances of type 1 errors, the extracted p-values from the linear regression analysis (oxacillin = $p < 0.001$, cefotaxime = $p < 0.001$, trimethoprim = $p < 0.001$) were bound together leaving a vector of p-values. Following this, a Bonferroni correction was performed on the extracted *p-values*. We can see from these results that communities isolated from depths of 11cm to 35cm that exhibit increased tolerance to zinc are significantly more resistant to the antibiotics oxacillin, cefotaxime and trimethoprim (all $p < 0.001$) (Fig 8b,c,d). Due to the significant correlation between zinc concentration and depth and the level of zinc resistance is the variable of interest for this study, we ignore depth as an explanatory variable in this analysis.

IntI1 prevalence and zinc resistance

DNA was extracted from depths ranging from 11 cm to 35 cm were used for qPCR analysis of the class 1 integron-integrase gene, *intI1,* which is commonly linked to genes conferring resistance to both heavy metals and antibiotics and could be used as a proxy that exhibits rapid responses to environmental pressures, serving as a potential genetic marker for anthropogenic pollutants

(Gillings, Gaze et al. 2015). *IntI1* prevalence was calculated by dividing the number of target genes by the number of 16S rRNA gene copies. *IntI1* prevalence (frequency) varied across sediment depth (Fig. 9), ranging from 0.12% at a depth of 25 cm to 3.66% at 20 cm depth. A Pearson product moment correlation was computed to assess the relationship between *IntI1* gene prevalence (%) and the concentration of zinc as assessed by ICP-OES (%) showed there to be significant moderate positive linear correlation between total zinc concentration and *IntI1* prevalence ($r = 0.5579, p < 0.01$). The linear relationship between phenotypic zinc resistance (%) and *IntI1* gene prevalence (%) was computed and the results show there is a highly significant positive correlation between resistance (%) and *IntI1* prevalence (%) ($r = 0.779, p < 0.001$) (Fig. 10). Linear regression analyses were performed to test the relationship between the proportion of zinc resistance, the proportion of resistance to the antibiotics oxacillin, cefotaxime, trimethoprim and prevalence of the *IntI1* gene in the communities. In order to reduce the chances of type 1 errors, the extracted p-values from the linear regression analysis (oxacillin = $p < 0.001$, cefotaxime = $p < 0.001$, trimethoprim = $p < 0.001$) were bound together leaving a vector of p-values after which a Bonferroni correction was performed on the extracted *p-values*. These data show that isolates from depths of 11cm to 35cm that exhibit increased tolerance to zinc and cefotaxime were shown to be correlated with *IntI1* gene prevalence in the community ($p < 0.001$ in both cases). Resistance to oxacillin and trimethoprim in isolates did not show to be significantly correlated with *IntI1* gene prevalence in the community (p values not shown).

Discussion

Antibiotic resistance is an issue that raises serious concern across the globe (Laxminarayan, Duse et al. 2013) and despite a plethora of data on the effects of antimicrobials on a variety of organisms, there is a need to further our understanding of how these factors influence microbial tolerances to antibiotics within natural environments. A detailed analysis of a sediment core extruded from a site of major chemical industry in the North West United Kingdom was used to provide data on the environmental contamination of zinc. This study marks the first attempt to study the effects of industrial heavy metal contamination across a temporal gradient spanning the increase in industrial activity and covering the decades before and after that period. Additionally, this is the first study to use that data to directly examine the effects of increased tolerances towards industrial contaminants and a decrease in susceptibility to three clinically relevant antibiotics of different classes (oxacillin, cefotaxime and trimethoprim).

ICP-OES data shows that in the sediment core used in this analysis the concentration of zinc begins to increase towards the surface from a depth of 30cm, peaking at a depth of 21 cm, after which there are moderate fluctuations before the concentration begins a steep decline (Fig. 2). The concentration of zinc within the sediment decreases with depth. Zinc is found naturally in soils and sediments (approximately 70 mg kg $^{-1}$ in crustal rock) but the worlds zinc production is still rising do to anthropogenic activities (Wuana and Okieimen 2011). The increase in zinc concentration over time throughout both sediment cores used in this study may be attributed to the introduction of zinc into industrial practices and the beginning of the modern industrial era. Indeed, natures geochemical cycle is slow when compared with the output from

anthropogenic activities that have drastically accelerated the accumulation of metals in soils (Wuana and Okieimen 2011).

Although the method of chemical analysis through ICP-OES for environmental pollution of zinc provides highly accurate data for total zinc concentration, it does not provide information on the actual bioavailability of zinc to microbial organisms. With the use of whole-cell specific bio-reporters, microorganisms that produce a quantifiable output in response to a target chemical, we can acquire a quantitative analysis of the bioavailable zinc concentration (Hynninen, Tõnismann et al. 2010). Severe matrix effects due to the presence of toxicant mixtures or nutrients resulted in the return of data for twelve layers of sediment sample (Fig. 9). The zinc specific biosensor *P. putida* strain was used to determine zinc bioavailability within the primary sediment core samples. The highest concentration of bioavailable zinc is at a depth of 20 cm, representing 8.6% of the total zinc concentration found at that depth. The depth with the highest percentage of bioavailable zinc is 23 cm at 9.97%. At the depths analysed, there is significant positive correlation with the total zinc concentration in the sediment revealing the potential for increased risk of toxicity towards microbial communities present in the sediment. The concentration of bioavailable zinc is positively correlated with total zinc concentration. This indicates that increased total concentration also increases the amount that is freely available to cross an organism's cellular membrane, resulting in various effects on cellular function (Bruins, Kapil et al. 2000).

In the sediment core, two age markers were identified in the sediment profile. The radionuclide ^{137}Cs in sediment that is used to infer the chronology based on temporal patterns of the resulting fallout from atmospheric nuclear testing, and the naturally occurring fallout radionuclide ^{210}Pb, which is widely

used to date marine sediments based on the radioactive decay law (ApplebyPG 1978, Zhang, Zhang et al. 2015). The results date the sediment core as far back in time as 1926 (20.5 cm depth), with sedimentation rates being uncertain at this time and the period before this, we are unable infer with any confidence the accurate dates for those past 16.5 cm (1945). It is clear, however, that the sediment core used for this study covers a time scale that has not previously been employed.

An analysis of the phenotypic response to zinc concentration in the bacterial communities present in the sediment provides data on how microbes respond to elevated levels of zinc contamination in the environment. This analysis revealed higher incidence of resistance at sediment layers with higher concentrations found in the preliminary core, with positive linear correlation with zinc concentration (Fig. 6). Resistance to zinc at the median concentration found in the preliminary core has a positive linear correlation with zinc concentration, though this linear association is weaker (Fig. 7). At the median zinc concentration there was shown to be phenotypic resistance greater than 100% at shallower depths (Fig. 7), suggesting at this concentration the presence of zinc may prove beneficial in the biological processes of those microbial communities present. These results indicate that there are increased incidences of zinc resistance within the cultivable microbial communities isolated from depths that contain higher concentrations of zinc. In contrast, those microbial communities isolated from depths that have lower levels of zinc present have decreased tolerance to higher zinc concentrations than that found at their respective depths.

It has previously been demonstrated that bacterial isolates that are more resistant to zinc also exhibit resistance to multiple collectively applied antibiotics

where, in contrast, metal-sensitive isolates did not (Calomiris, Armstrong et al. 1984). Indeed, there are commonalities between resistance mechanisms for both zinc and antibiotics (Bruins, Kapil et al. 2000, Choudhury and Srivastava 2001, Baker-Austin, Wright et al. 2006), which suggests that zinc contamination in the environment may be an important factor in the maintenance of antibiotic resistance as a component of co-selection for resistance mechanisms. Therefore, an analysis of the phenotypic response of zinc tolerant communities to antibiotics is necessary in furthering our understanding of the proliferation of antimicrobial resistance due to zinc contamination. We isolated 96 clones for each layer at depths 11 cm and 35 cm where the most significant changes in zinc concentration occurred to test for the potential relationship between zinc resistance and decreased susceptibility to three different antibiotics that are of clinical importance: cefotaxime, oxacillin and trimethoprim. Cefotaxime is a third-generation cephalosporin (Fani, Brotherton et al. 2013) that is used in the treatment of sexually transmitted infections (Golparian, Ohlsson et al. 2014, van Dam, van Ogtrop et al. 2014). Oxacillin is a broad-spectrum β-lactam antibiotic, resistance to which has emerged rapidly over the last several years (Phitaktim, Chomnawang et al. 2016). Trimethoprim is a synthetic antimicrobial that is widely applied due its low cost of production and its efficacy (Ho and Juurlink 2011). Isolates from these depths that exhibit increased tolerance to zinc are significantly more resistant to the antibiotics oxacillin, cefotaxime and trimethoprim (all $p < 0.001$). These data provide vital information in the growing concern that metal contamination functions as a selective agent in the maintenance of antimicrobial resistance in the environment. Though, the naturally occurring concentrations of the antibiotics oxacillin and cefotaxime in

the sediment are unknown, in addition to abiotic factors, that may influence the level of resistance in the microbial communities in the sediment.

It has been proposed that co-selection of metal- and antibiotic-resistance is likely caused by the location of class one integrons on mobile genetic elements (Baker-Austin, Wright et al. 2006), with reports specifying proximity to genes coding for efflux based resistance to Zn^{2+} (Stokes, Nesbø et al. 2006, Gillings, Boucher et al. 2008). It is therefore necessary to assess the prevalence of the class one integron-intergrase gene, *IntI1*, in the microbial community isolated from the sediment core due its linkage with genes conferring resistance to metals and antibiotics (Gillings, Gaze et al. 2015). *IntI1* gene prevalence varied across sediment depth (Fig. 9), ranging from 0.12% at a depth of 25 cm to 3.66% at 20 cm depth. Correlation analysis reveals significant linear correlation between zinc concentration and *IntI1* prevalence and zinc resistance and *IntI1* prevalence. A Linear regression analysis of the proportion of zinc-antibiotic-resistance and *IntI1* gene prevalence shows there to increased gene prevalence among isolates with increased resistance to zinc and cefotaxime (p < 0.001 in both cases), where proportion of resistance to oxacillin and trimethoprim did show to be significantly correlated with *IntI1* gene prevalence (p values not shown).

These data provide vital information in the growing concern that metal contamination functions as a selective agent in the maintenance of antimicrobial resistance in the environment. Showing there to be a correlation between increased resistance to zinc and increased resistance to three clinically relevant antibiotics (cefotaxime, oxacillin and trimethoprim). Additionally, giving some evidence for the relationship between resistance heavy metal pollutants and clinically relevant antibiotics (cefotaxime, oxacillin and trimethoprim) being

mediated by the same gene linked to resistance to both, though it does not provide significant data regarding oxacillin and trimethoprim. This may be due to the location of the resistance determinants for oxacillin and trimethoprim not being located on the same genetic element as that which confers resistance to zinc. Indicating that although the *IntI1* gene may act as a measure of the general level of resistance determinants for individual antimicrobials, its applicability as a target marker for selective pressure imposed by anthropogenic pollution on the maintenance of antibiotic resistance may not have relevance in all cases.
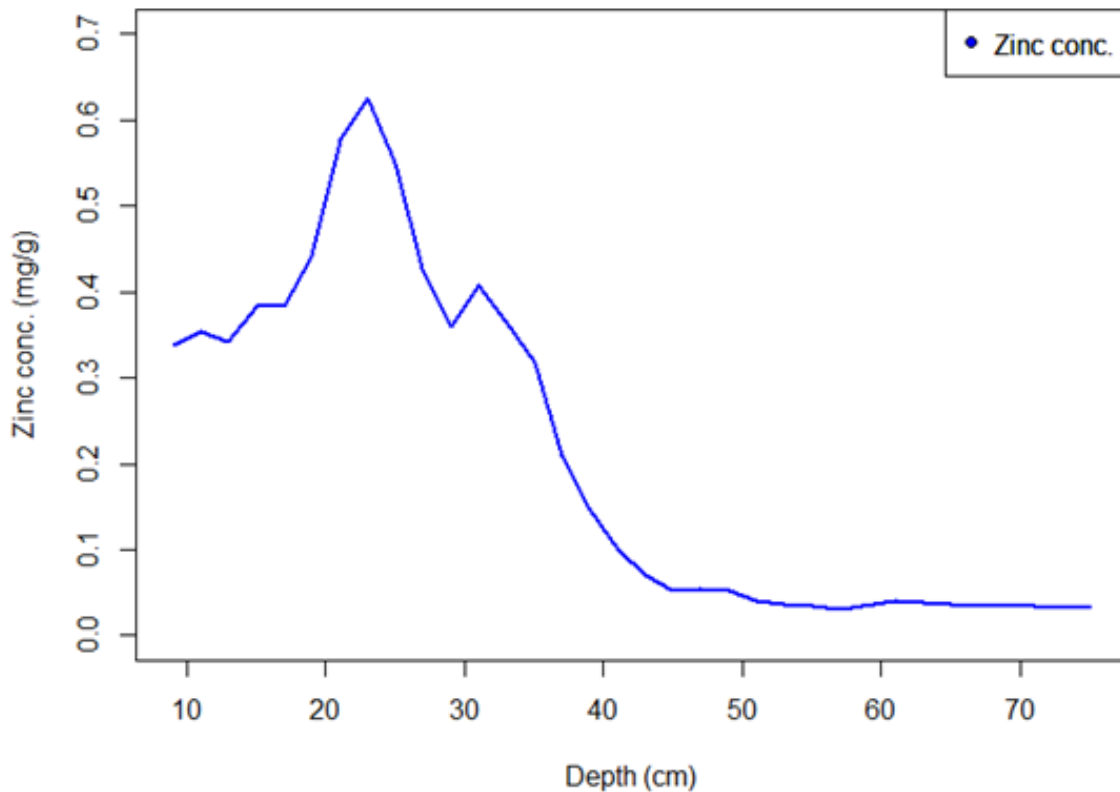
Figures

Figure 1: Concentration of zinc found within the preliminary sediment core (mg/g) from Inductively Coupled Plasma Optical Emission Spectroscopy analysis. The concentration of zinc found within the sediment core greatly increases towards the surface from a depth of 40cm. The highest concentration of zinc (0.625 mg/g) found at a depth of 23cm, after which the concentration begins to decrease towards to surface. There is a significant strong negative linear correlation between depth and zinc concentration in the preliminary sediment core (r = -0.729, p < 0.001), showing that the concentration of zinc within the sediment decreases as depth through the sediment core increases.
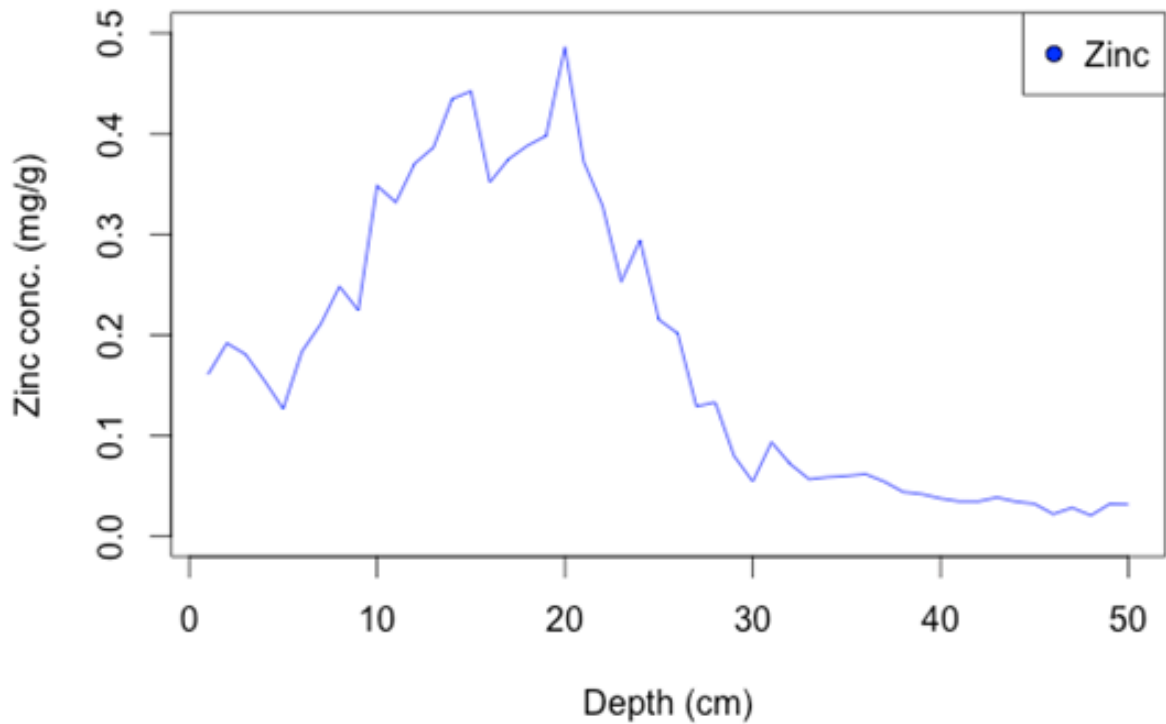
Figure 2: Concentration of zinc found within the primary sediment core (mg/g)
from ICP-OES analysis. The concentration of zinc found within the sediment
core greatly increases from a depth of 30cm towards the surface. The highest
concentration of zinc (0.486 mg/g) found at a depth of 21cm and the lowest
concentration found is 0.0208 mg/g at a depth of 48cm. There is a significant
strong negative linear correlation between depth and zinc (r = -0.679, p <
0.001).

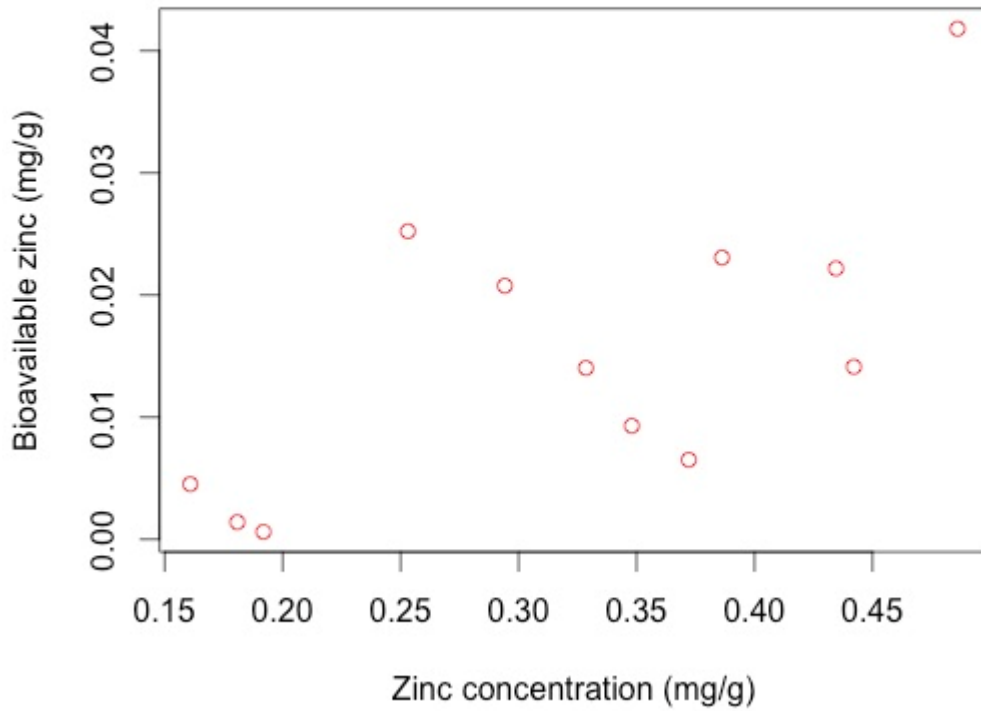**Zinc concentration and bioavailability (mg/g)**

Figure 3: Concentration of bioavailable zinc (mg/g) measured using zinc specific biosensor *P. putida* strain T2440.2431 (pDNPcadA1lux) in twelve sediment samples. There is a significant positive correlation between total zinc measured by ICP-OES and bioavailable zinc concentration in the sediment (r =0.674, *p < 0.05*).

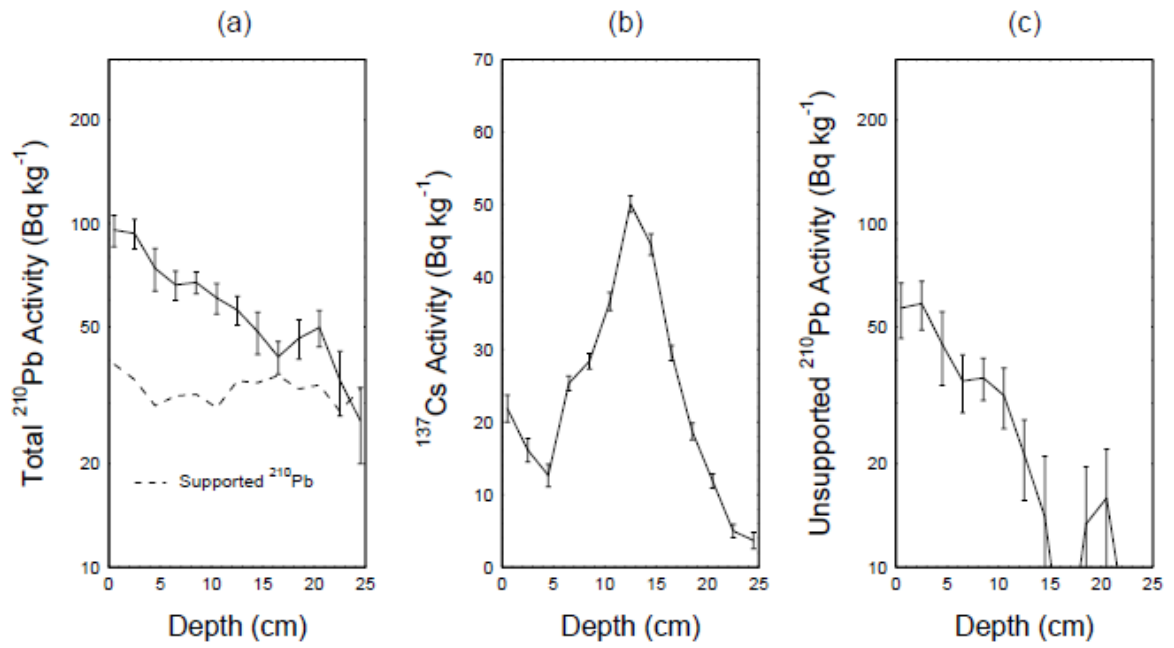Figure 4: Fallout radionuclides in the Griffin Wood Pond sediment core showing (a) total and supported ^{210}Pb, (b) unsupported ^{210}Pb and (c) ^{137}Cs concentrations versus depth.
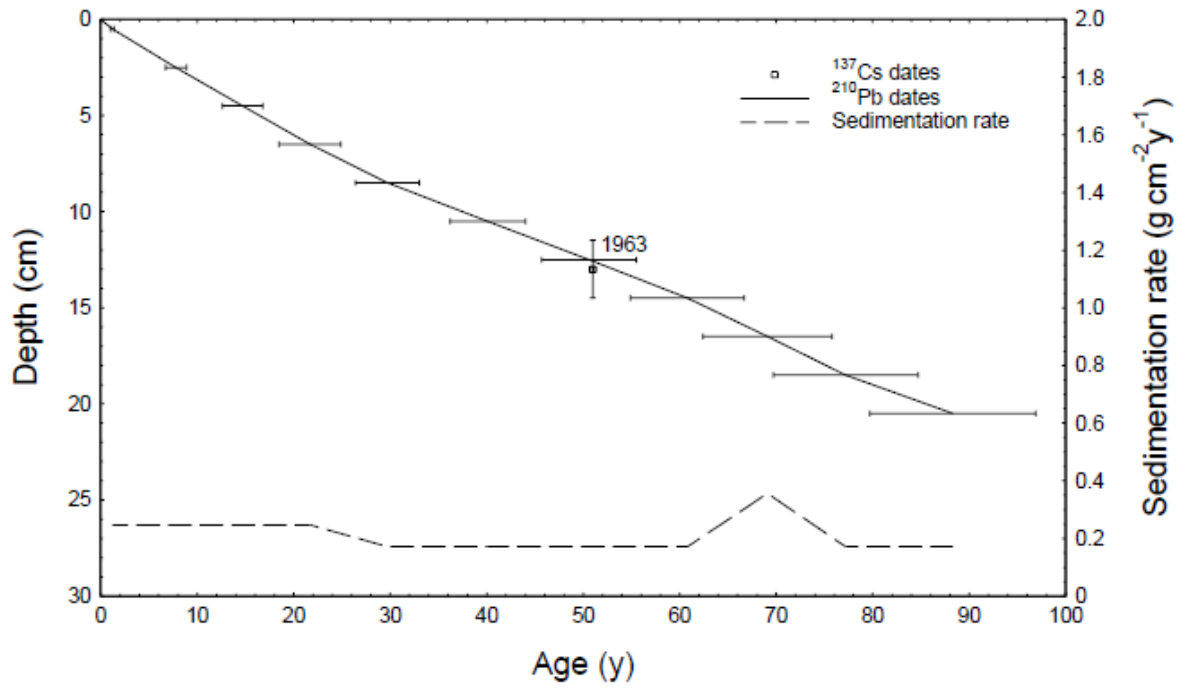
Figure 5: Radiometric chronology of the Griffin Wood Pond sediment core showing the ^{210}Pb dates and sedimentation rates and the 1963 depth suggested by the ^{137}Cs record.
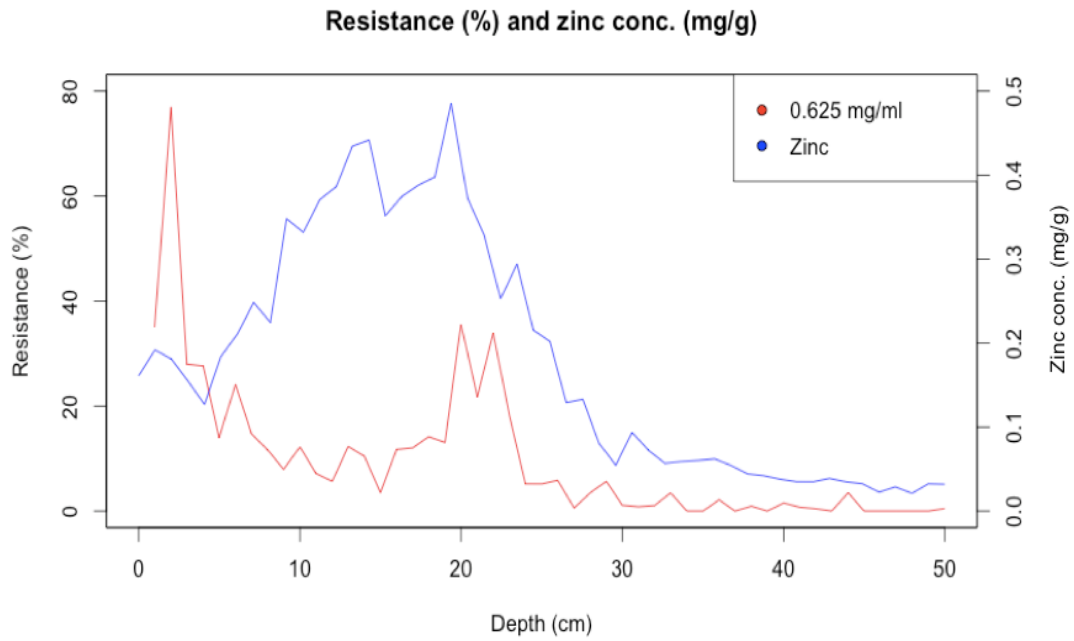
Figure 6: Bacterial community resistance (percentage of resistant CFU of total CFU on zinc free plates) on agar amended with 0.625 mg/ml formula (highest concentration of zinc (mg/g) found throughout the preliminary sediment core) and zinc concentration (mg/g). Resistance to zinc has a moderate positive linear correlation with zinc concentration (r = 0.416, p < 0.01).
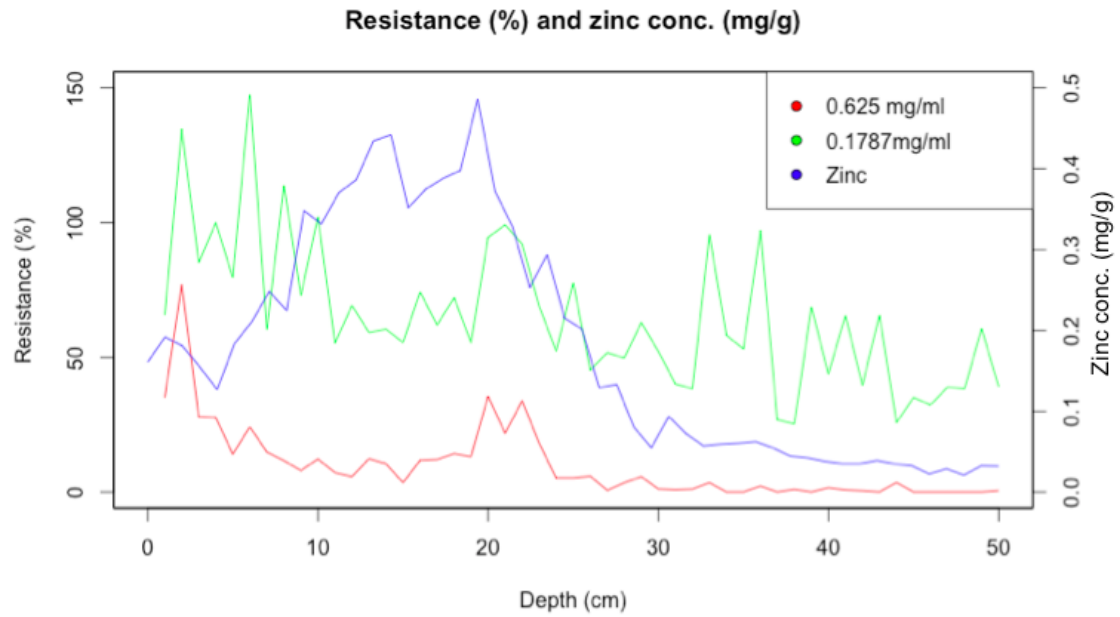
Figure 7: Bacterial community resistance (percentage of resistant CFU of total CFU on zinc free plates) on agar amended with 0.625 mg/ml and 0.1787 mg/ml formula (highest and median concentration of zinc (mg/g) found throughout the preliminary sediment core) and zinc concentration (mg/g). Resistance to zinc at 0.1787 mg/ml has a weak positive linear correlation with zinc concentration (r = 0.370, p < 0.01).
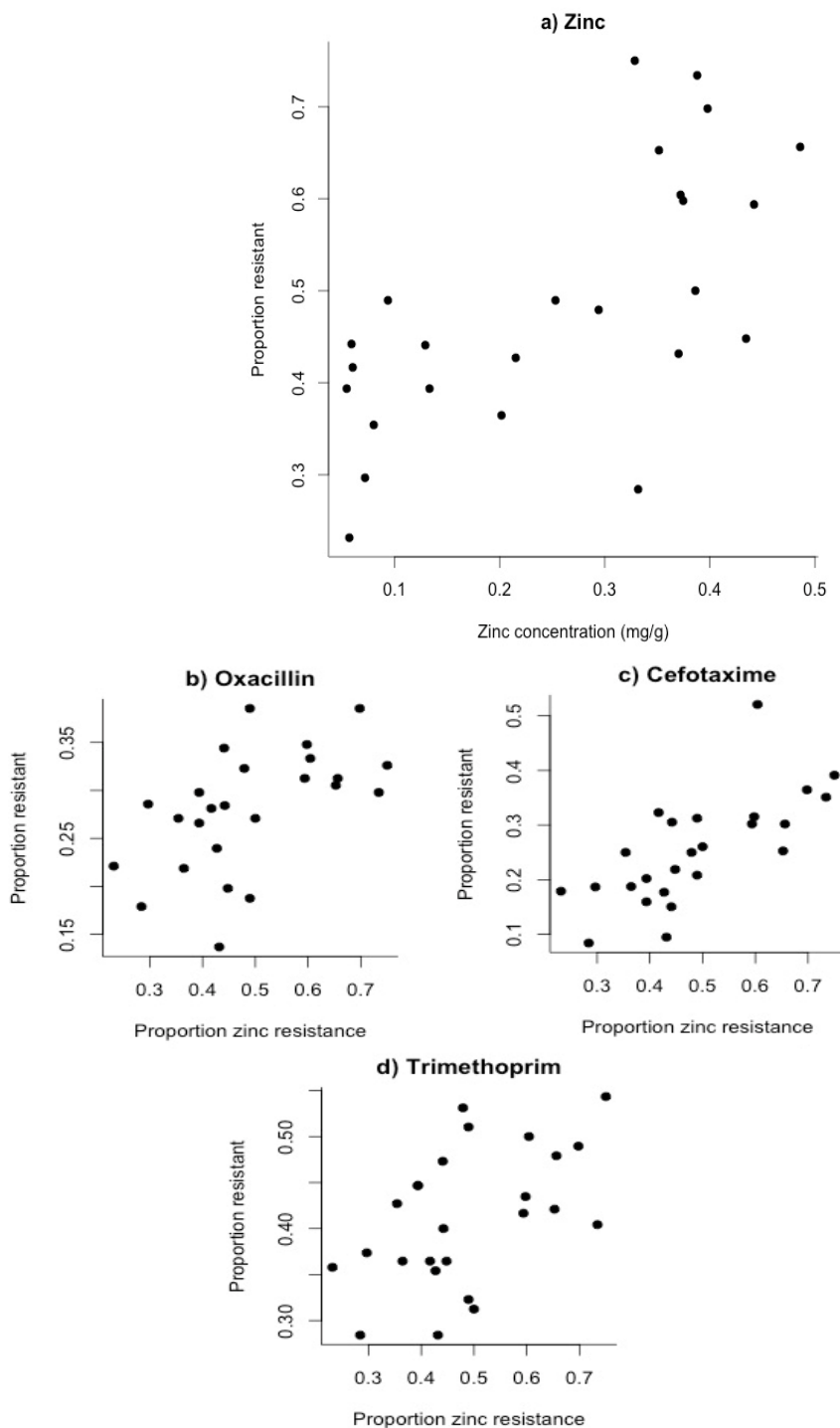
Figure 8: Proportion of resistant CFU (%) to (a) zinc *p < 0.001* (b) oxacillin

(50μg/mL) *p < 0.001*, (c) cefotaxime (50μg/mL) *p < 0.001* and (d) trimethoprim

(60μg/mL) *p < 0.001*. Zinc concentration has a significant effect on proportion of

zinc resistance ($p < .001$, $m = 2.5894$). Bonferroni correction on extracted *P-*

values (b) *P < 0.001* (c) *P < 0.001* (d) *P < 0.001* indicate a significant increase

in tolerance to these antibiotics in isolates that exhibit resistance to zinc.
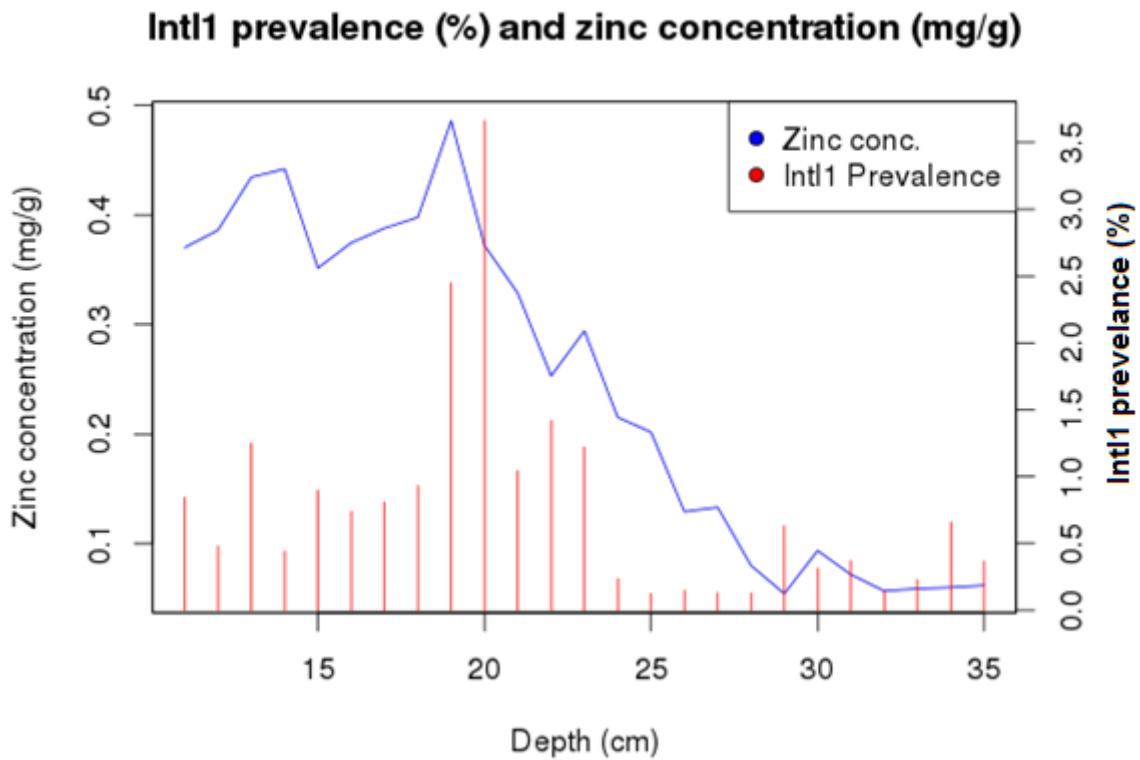
Figure 9: Prevalence of the *Intl1* gene (%) against levels of zinc found in the primary sediment core (mg/g) 11 – 35 cm depths. There is significant moderate positive linear correlation between zinc concentration and *Intl1* prevalence (*r = 0.557, P < 0.01*)

Intl1 prevalence (%) and zinc resistance (%)
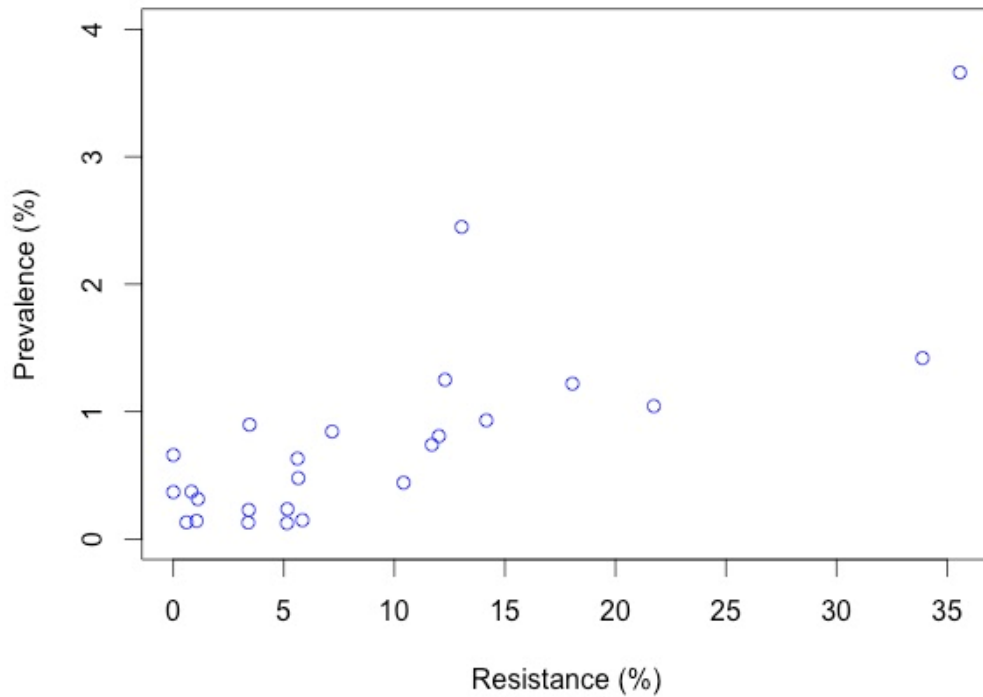
Figure 10: Prevalence (%) of the *Intl1* gene plotted as a function of zinc resistance (%) within samples 11 – 35. There is a significant strong positive linear correlation between resistance (%) and *Intl1* prevalence (%) (*r = 0.779, P < 0.001*).

Tables

Table 1. Primers and probes used in *Intl1* analysis		
Primer/probe name	Sequence (5' - 3')	Size (bp)
***Intl1* Primers**		
Intl1-LC1	GCCTTGATGTTACCCGAGAG	196
Intl1-LC5	GATCGGTCGAATGCGTGT	
16s Primers		
1369F	CGGTGAATACGTTCYCGG	124
1492R	GGWTACCTTGTTACGACT	
***Intl1* Probe**		
Intl1-probe	ATTCCTGGCCGTGGTTCTGGGTTTT	
16s Probe		
TM1389F	CTTGTACACACCGCCCGTC	

Table 2: Maximum, minimum, mean and range of heavy metal concentrations (mg/g) found throughout the sediment core from Griffin Wood Pond sample site by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

Metal	Max	Min	Mean	Range
Al	12.511	3.660	8.879	8.850
Ba	0.213	0.056	0.123	0.157
Co	0.013	0.002	0.007	0.011
Cr	0.026	0.006	0.019	0.020
Cu	0.699	0.007	0.169	0.692
Fe	20.707	9.584	15.712	11.123
Ga	0.181	0.067	0.123	0.113
K	1.234	0.419	0.758	0.814
Mn	0.650	0.119	0.297	0.530
Mg	2.741	1.801	2.315	0.939
Na	0.262	0.052	0.139	0.209
Ni	0.044	0.011	0.027	0.033
Pb	0.219	0.006	0.081	0.212
Sr	0.034	0.002	0.010	0.032
Ta	0.010	0.006	0.007	0.004
Ti	0.011	0.0001	0.005	0.010
Zn	**0.486**	**0.020**	**0.178**	**0.465**

Table 3: Fallout radionuclide concentrations in the Griffin Wood Pond core.

Depth		Total		Unsupported		Supported		^{137}Cs	
cm	g cm^{-2}	Bq kg^{-1}	±	Bq kg^{-1}	±	Bq kg^{-1}	±	Bq kg^{-1}	±
0.5	0.3	95.7	10.1	56.8	10.4	39.0	2.6	21.9	1.9
2.5	1.9	93.5	9.2	58.5	9.4	35.1	2.3	16.1	1.6
4.5	3.6	74.1	10.6	44.6	10.8	29.5	2.3	12.6	1.6
6.5	5.3	66.3	6.5	34.8	6.7	31.5	1.4	25.3	1.0
8.5	7.0	67.3	4.8	35.5	5.0	31.8	1.2	28.4	1.1
10.5	8.8	60.7	6.2	31.6	6.3	29.1	1.4	36.6	1.3
12.5	10.6	56.0	5.4	21.2	5.6	34.8	1.2	50.1	1.1
14.5	12.4	48.4	6.8	14.0	7.0	34.4	1.5	44.5	1.4
16.5	14.2	40.9	4.5	4.8	4.6	36.1	1.1	29.5	1.1
18.5	15.9	46.3	6.1	13.4	6.2	33.0	1.4	18.7	1.1
20.5	17.8	49.8	6.0	15.9	6.2	33.9	1.4	11.9	1.0
22.5	19.7	35.0	7.4	6.6	7.6	28.4	1.7	5.0	0.9
24.5	21.6	26.6	6.6	-6.4	6.8	33.1	1.7	3.7	1.1

Table 4: ^{210}Pb chronology of the Griffin Wood Pond core

Depth		Chronology			Sedimentation Rate		
		Date	Age				
cm	g cm^{-2}	AD	y	±	g cm^{-2} y^{-1}	cm y^{-1}	± (%)
0.0	0.0	2014	0	0			
0.5	0.3	2013	1	0	0.25	0.32	14.6
2.5	1.9	2006	8	1	0.25	0.30	14.6
4.5	3.6	1999	15	2	0.25	0.29	14.6
6.5	5.3	1992	22	3	0.25	0.27	14.6
8.5	7.0	1984	30	3	0.17	0.22	12.8
10.5	8.8	1974	40	4	0.17	0.19	12.8
12.5	10.6	1963	51	5	0.17	0.19	12.8
14.5	12.4	1953	61	6	0.17	0.22	12.8
16.5	14.2	1945	69	7	~0.36	~0.40	
18.5	15.9	1937	77	8	~0.17	~0.21	
20.5	17.8	1926	88	9	~0.17	~0.18	

Chapter 4

Microbial community diversity across a gradient of anthropogenic zinc pollution

Introduction

With the use of amplicon sequencing of the 16s subunit rRNA gene with high-throughput sequencing technologies, it is now possible to analyse the structure-function relationship between microbial communities and the environment in which they persist (Sinclair, Osman et al. 2015). The assessment of soils and sediments for levels of heavy metal contamination has become important in evaluating the impact on microbial communities (Oves, Saghir Khan et al. 2016). The introduction of heavy metals into the environment can modify the microbial community and their activities (Aleem, Isar et al. 2003) and long-term heavy metal exposure in soils can have damaging effects on soil microbial activity, such as enzyme inactivation or by inhibiting the use of metabolites (Sobolev and Begonia 2008). Microbial communities are composed of a number of metabolically interdependent species and metal content may be a crucial selective agent in the evolution of bacterial communities (Giller, Witter et al. 1998, Hoostal, Bidart-Bouzat et al. 2008).

Microbial community tolerance to heavy metal has been frequently studied with the use of artificially contaminated in the laboratory, however soils that are subject to industrial pollution for decades are not studied (Stefanowicz, Niklińska et al. 2009). The changes in community composition under pressure from anthropogenic pollutants has only previously been evaluated as over spatial scales, covering several locations within an area of industrial activity,

over temporal scales spanning only several months to approximately four decades or down to depths not exceeding the 20 cm zone in which the highest microbial activity is present (Bouskill, Barnhart et al. 2007, Berg, Brandt et al. 2012, Kaci, Petit et al. 2016). By assessing the changes in the microbial community diversity over sediment temporal scales that span the rise of industry and covering the decades previous, we are better able to evaluate the impact of anthropogenic pressure on sediment microbial populations.

In this chapter, we analyse the microbial community composition of a sediment core extracted from an area known to be a major site of chemical industry for over a century (Hodgson, Nieuwenhuijsen et al. 2004) in the area of Runcorn, North West United Kingdom. This will allow for a further understanding of the heavy metal driven response in microbial diversity through time due to anthropogenic pollution. High-throughput Illumina sequencing of the 16S rRNA gene was performed using DNA extracted from the sediment samples from the depths of the core in which the concentration of metals were shown to vary the greatest. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was performed to quantify the heavy metal content of the sediment (see Chapter 3) and to compare this with the sequence data and visualise the relative abundance of taxa throughout the core with respect to metal concentration. Relative abundance was assessed to provide a quantitative composition of commonness of phyla over time. The taxonomic level at which these analyses were performed was that of phyla, due to the sequence data being unable to provide any lower taxonomic classification. A measure of dissimilarities between the relative abundance of taxa (Bray-Curtis) was used to explore and visualise the distances between the microbial compositions of each of the twenty-five layers of sediment with the use of

principal coordinates analysis (PCoA). Following this, a mantel test was performed between beta (ß) -diversity and the concentration of heavy metals (mg/g)) to assess the effects of heavy metals on microbial communities. ß-diversity was analysed in this study as it provides information on the variation in community composition between samples, which is preferable to alpha (α)-diversity for which the output describes the diversity in species between samples (Legendre, Borcard et al. 2005)

The aim of this study is to gain a deeper insight into the effects of anthropogenic heavy metal contamination on microbial community composition. By using a sediment core that covers a time scale that spans approximately a century (see Chapter 3) we are able to make a first attempt in testing the effects of heavy metal exposure on microbial community composition before, during and after an increase in industrial activities in the UK.

Results

Community composition and diversity of a metal-polluted sediment over time

High-throughput Illumina sequencing of the 16S rRNA gene was performed using DNA extracted from the sediment samples from depths ranging 11 cm to 35 cm (depths 31cm and 35 cm missing due to insufficient reads) to characterise bacteria community composition over a temporal metal gradient. Figure 1 provides a phylum-level phylogenetic classification overview of the relative abundance (%) of the taxa found in the sediment. Between depths of 11 cm and 34 cm the sequences that account for 25% to 40% of the total relative abundance could not be assigned to any known bacterial taxa. *Proteobacteria* were the dominant phylum at depths of 11 cm and 12 cm, representing

approximately 30% and 31% of total relative abundance respectively. *Chloroflexi* account for around 8% of sequences at depth of 11 cm which increases through the core to around 16% at 34 cm, and *Bacteroidetes* decrease in relative abundance with depth as they account for around 8% of sequences at 11 cm and fall to around 4% at 34 cm. *Acidobacteria*, *Firmicutes*, *Aminicenantes* and *Spirochaetes* account for 1% to 3% of sequences and *Planctomycetes*, *Verrucomircobria* and *Euryarchaeota* all account for 1% or less of total relative abundance.

Using the Bray-Curtis dissimilarities method to provide a distance matrix to quantify the compositional dissimilarities in the relative abundance of taxa between samples a mapping of the dissimilarities values for pairwise comparison (ß-diversity) was obtained. A principal coordinate analysis (PCoA) was computed to explore and visualise the measures of phylogenetic dissimilarity found in each depth (cm), and showed that principal component 1 (PC1) accounted for 49.96% of variation in community composition and principal component 2 (PC2) accounted for 20.94% of variation (Fig. 2).

To test the effects of heavy metal pollution on microbial community composition through the sediment core, ICP-OES was used to measure metal concentrations (Al, Ba, Co, Cr, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Sr, Ti and Zn) over the length of the core (Figure 3) (see Chapter 3. Table 2). If differences in community composition within varying depths of a sediment core are a result of environmental factors, beta- (ß) diversity should be analysed with respect to the hypothesised forcing variable (Legendre, Borcard et al. 2005). Therefore, a Mantel test of the correlation coefficient between two distance matrices (ß-diversity and the concentration of heavy metals (mg/g)) was performed to formally asses the effects of heavy metal contamination on microbial

communities (Mantel 1967). There is a significant positive linear correlation between the level of aluminium ($r = 0.315, P < 0.001$), barium ($r = 0.542, P < 0.001$), cobalt ($r = 0.438, P < 0.001$), copper ($r = 0.293, P < 0.01$), potassium ($r = 0.317, P < 0.01$), magnesium ($r = 0.253, P < 0.01$), manganese ($r = 0.223, P < 0.01$), nickel ($r = 0.346, P < 0.001$), lead ($r = 0.586, P < 0.001$) and strontium ($r = 0.279, P < 0.01$) in the sediment and ß-diversity, with strong positive linear correlation between the level zinc ($r = 0.800, P < 0.01$) and ß-diversity (Table 1). These data show there are consistent changes within the microbial community present over time through the core and suggest there are a number of metals that have a significant effect on microbial community composition, with the strongest significant influence from zinc.

Discussion

Due to the complexity of bacterial diversity and the fact that only a small fraction of bacteria can be cultivated in the laboratory, it is crucial to get a clearer understanding of the full scale of the bacterial community composition (Llirós, Inceoğlu et al. 2014). It is therefore appropriate to analyse the structure-function relationship between microbial communities and the environment in which they persist. Sequencing of the 16S rRNA gene ('16') from environmental samples has shown that microbial diversity is more extensive than can be demonstrated with the use of culturing techniques, expanded the potential for the study of community dynamics over temporal scales, and has revealed deeper insights into the diversity of bacterial communities (Lozupone and Knight 2005, Sinclair, Osman et al. 2015).

One of the challenges facing the study of soil microbial ecology is in determining the complex mosaic of selective gradients that drive the high levels

of biodiversity found in soils (Vos, Wolf et al. 2013). The present study aims to gain further insight into how environmental heavy metal contamination might alter bacterial community composition in sediment extruded from a site of major chemical industry in the North West United Kingdom. 16S rRNA gene amplicon illumina sequencing (Hiseq) and multivariate analysis techniques were used to assess the variation in microbial diversity through the sediment samples and the potential selective effects of heavy metals on diversity.

The resulting data acquired via the use of Hiseq technology must then be interpreted as a function of environmental parameters in order to further our understanding of resulting response in microbial diversity (Ramette 2007). It is important to summarise and explore the data using the appropriate statistic method, however Ramette (2007) demonstrate clearly that the use of multivariate analysis techniques such as Principal Coordinates Analysis (PCoA) or Mantel tests are rarely used despite their usefulness. The microbial communities present within the sediment samples were shown to be consistent with members of phyla that make up the majority typically found within soil samples worldwide (Fig. 1) (Youssef and Elshahed 2009).

Using the Bray-Curtis dissimilarities to provide a distance matrix to explore differences in the relative abundance of taxa along the length of the core, a Principal Coordinate Analysis (PCoA) was employed to compare the communities across a temporal gradient and display these dissimilarities (Fig. 2) (Ramette 2007, Ligi, Oopkaup et al. 2014). Each point in the PCoA plot represents a bacterial community from a layer of sediment (11cm to 34cm) and shows that as depth increases through the core, the variation in the relative abundance of bacterial communities differs. Principal component 1 explains 49.96% of total variation and principal component 2 explains 20.94% of total

variation between communities occupying each depth (cm). These data show a consistent change in community composition over time through the core with each depth varying in diversity a few percent with the depths both above and beneath, leading to greater differences in microbial composition between the sediment found at shallower depths and those found deeper in the core.

The mantel test is used for the formal assessment of comparing the distance matrices of species dissimilarity and environmental dissimilarity for each sample (Ramette 2007, Hoostal, Bidart-Bouzat et al. 2008, Haller, Tonolla et al. 2011). This is achieved by calculating the correlation coefficient between the corresponding positions within the two matrices and evaluating the significance based on shifts in the objects in one of the assigned matrices (Ramette 2007). Heavy metal content may be a crucial selective agent in the evolution of bacterial communities (Hoostal, Bidart-Bouzat et al. 2008). In order to understand the effects of heavy metals on the microbial community abundance within the sediment core, concentrations of individual heavy metal types were separated into distance matrices, one for each heavy metal detected within the sediment. The microbial composition of the sediment was correlated with both depth and heavy metal concentration showing zinc to have the strongest positive correlation (*r = 0.800, P = 0.001)*, as shown by the Mantel correlation test (Table 1). These data suggest that the diversity of microbial communities is most effected by environmental zinc pollution. Further analysis into the combined influence of heavy metals may be necessary to gain a broader understanding of how metal pollution alters the structural composition of microbial community populations within a soil environment. Additionally, other abiotic factors such as soil pH, dissolved oxygen, sediment size, salinity, temperature and nutrient availability can influence microbial community

composition and were not taken into consideration in this study. In future research assessing the effects of heavy metal contamination on microbial community composition other abiotic factors should be considered.
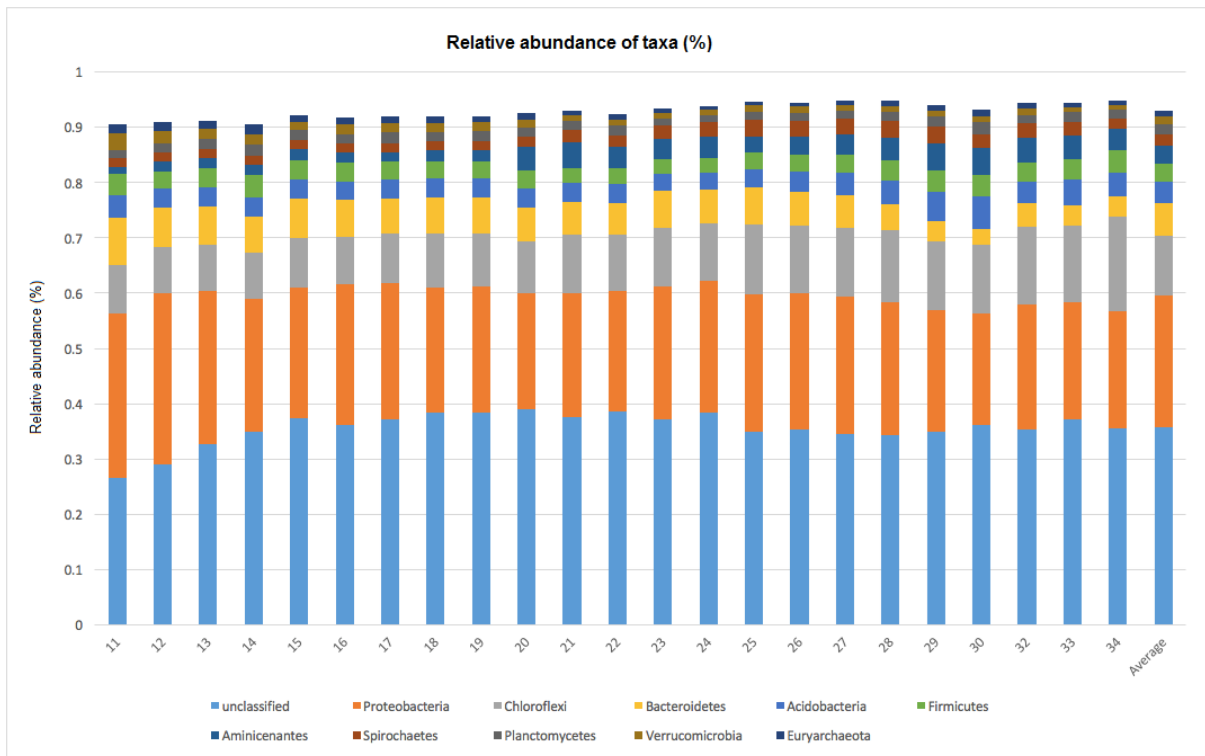
Figures

Figure 1: Relative abundance (%) of the taxa found in the sediment layers 11

cm – 34 cm displayed from the Griffin Wood Pond primary core. Analysis
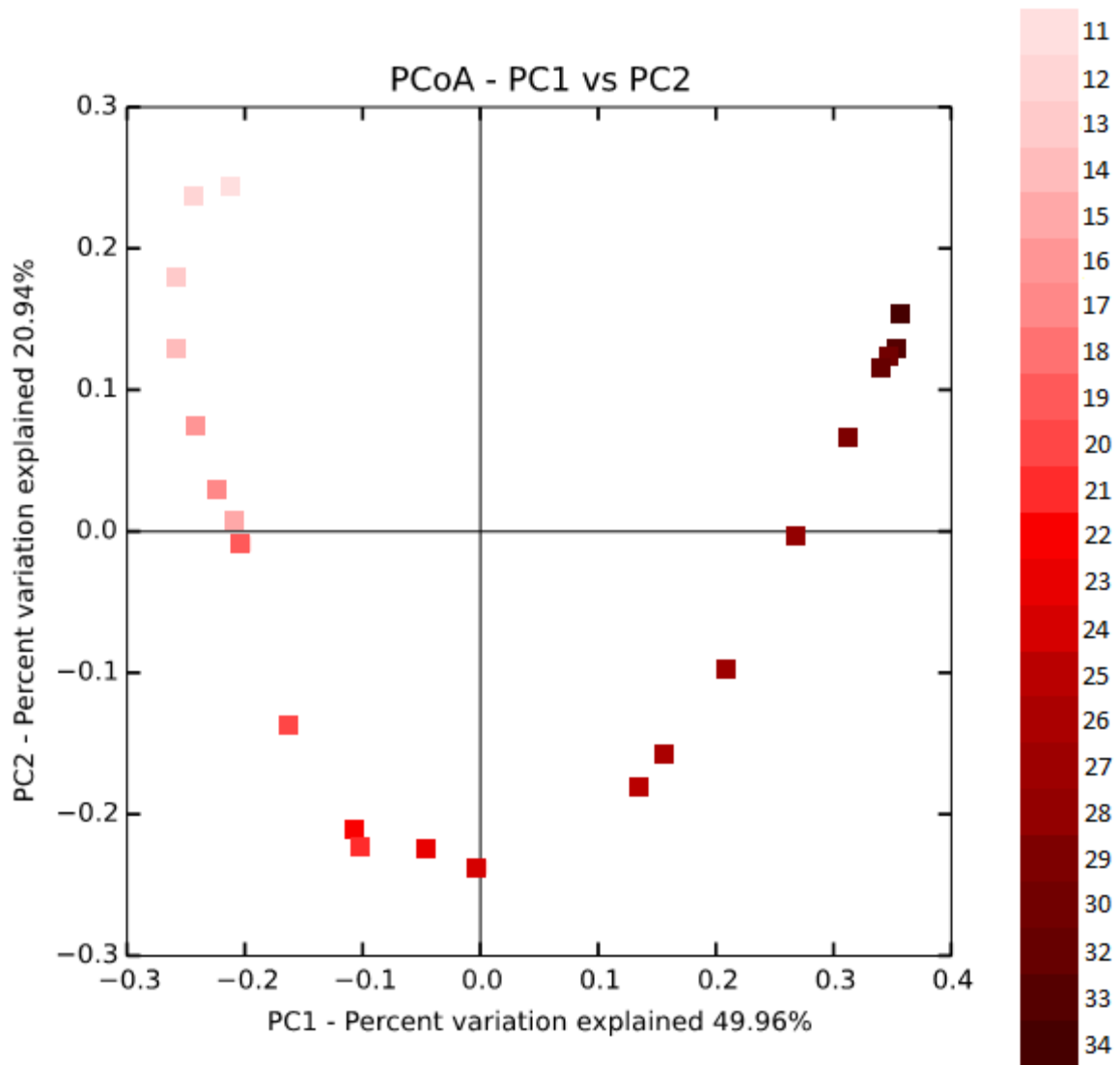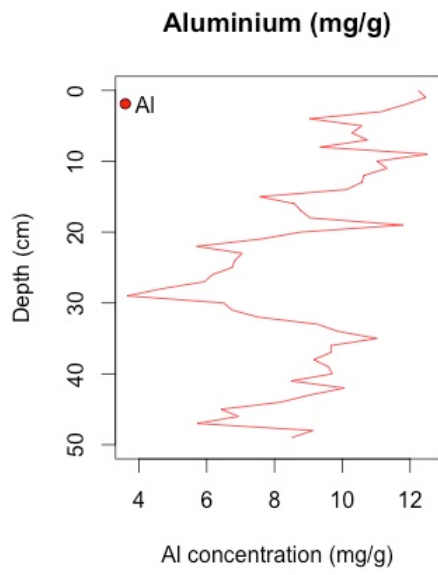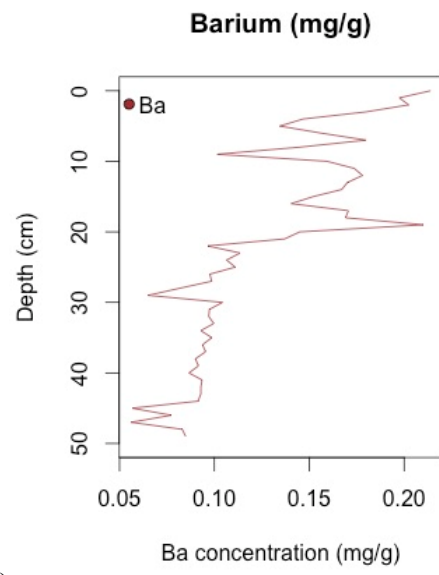
performed at phylum level.

Figure 2: Ordination plot showing grouping of soil samples based on their microbial community structure (ß-diversity). Each point represents a bacterial community from a layer of sediment with the shade of red darkening as depth increases. Principal component 1 (PC1) accounted for 49.96% of variation in community composition and principal component 2 (PC2) accounted for 20.94% of variation.
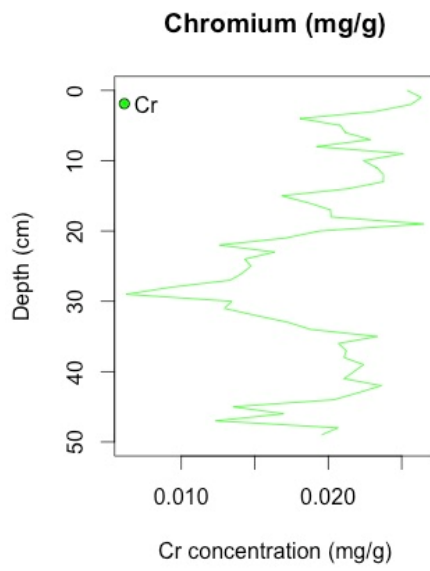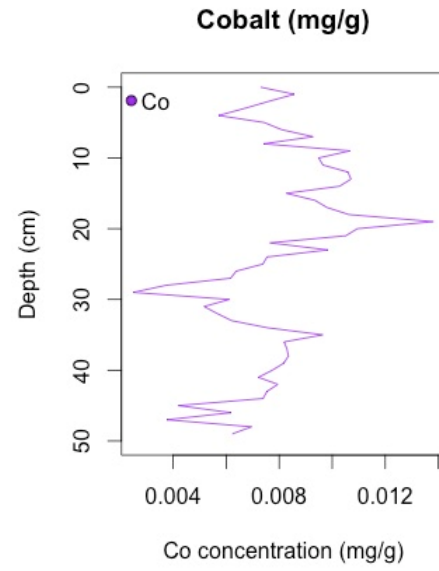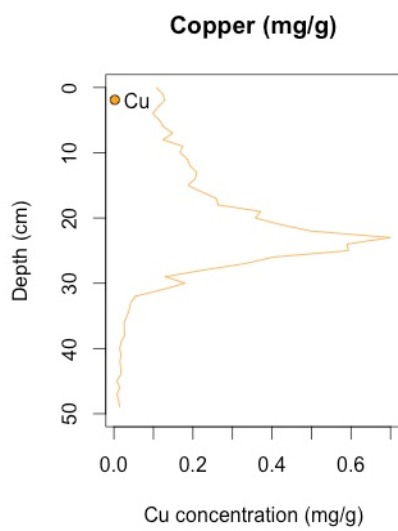
a)

b)

c)

d)

e)

f)

Iron (mg/g)

g)

Lead (mg/g)

h)

Magnesium (mg/g)

i)

Manganese (mg/g)

j)

Nickel (mg/g)

k)

Potassium (mg/g)

l)

Sodium (mg/g)

Strontium (mg/g)

m)

n)

Titanium (mg/g)

Zinc (mg/g)
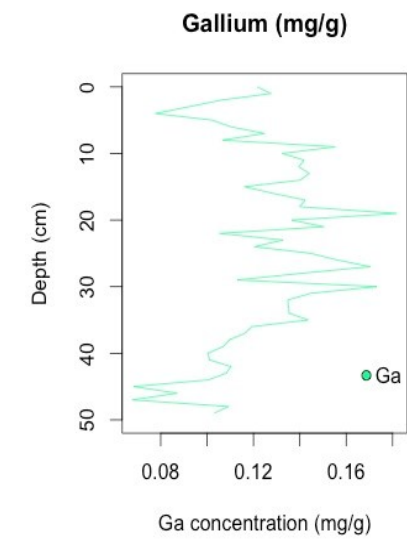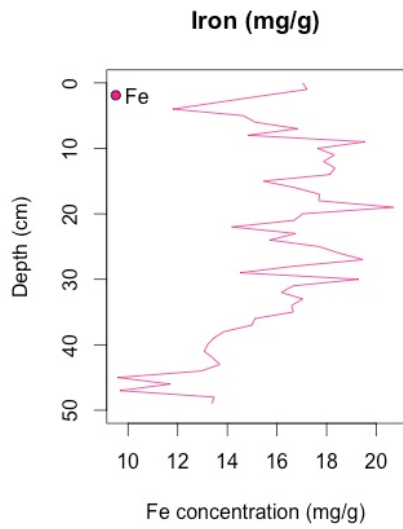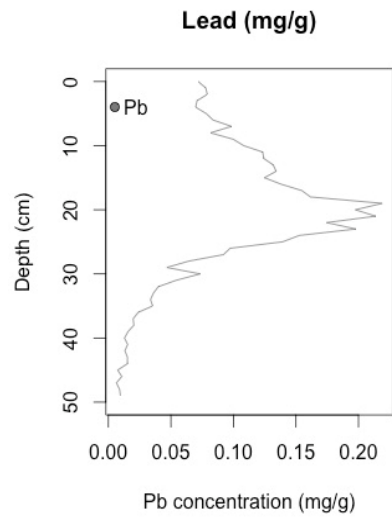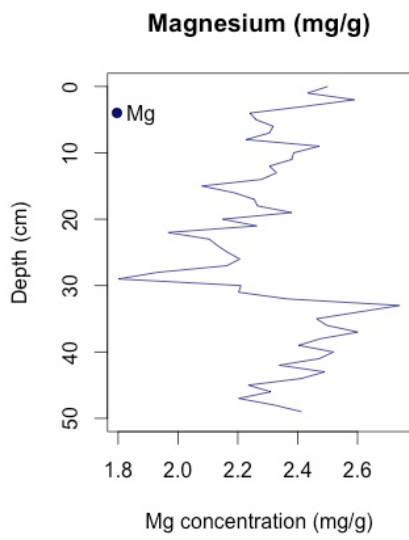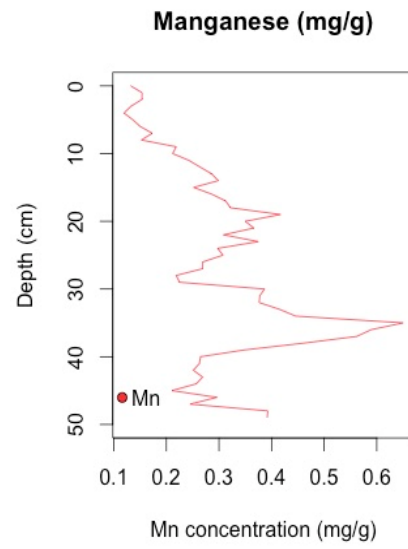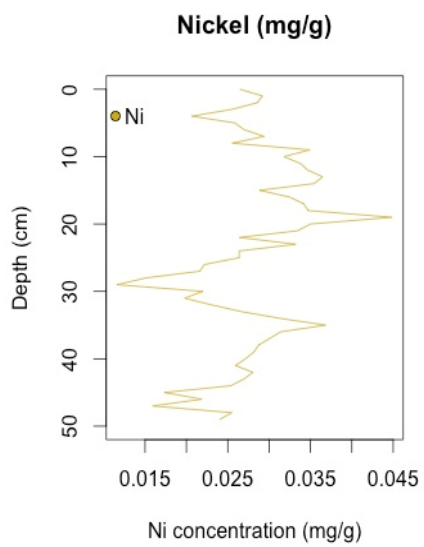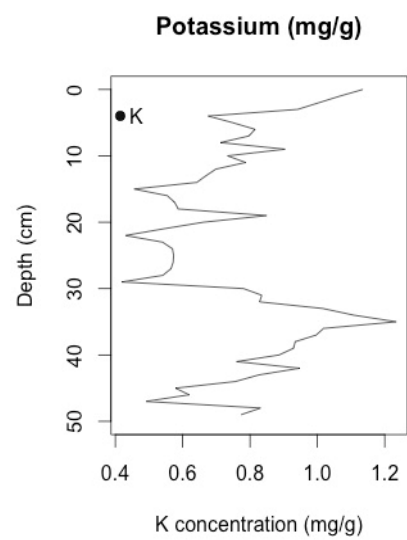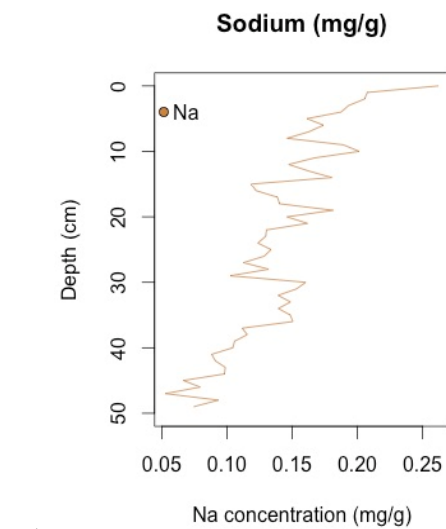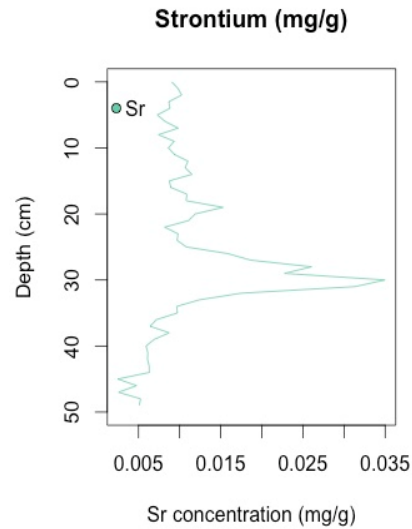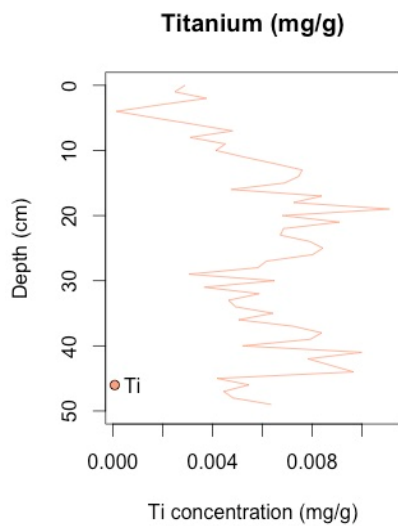
o)
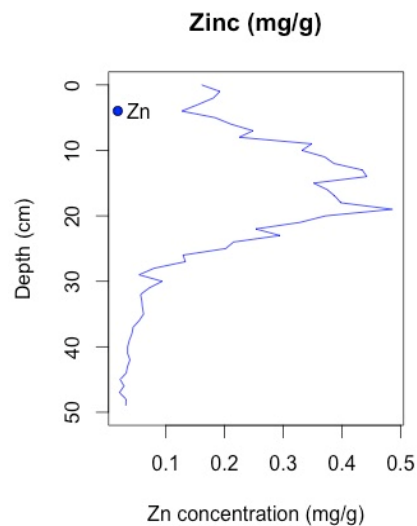
p)

Figure 3: Metal concentrations (mg/g) found throughout the core for all metals detected by ICP-OES analysis: a) Aluminium, b) Barium, c) Chromium, d) Cobalt, e) Copper, f) Gallium, g) Iron, h) Lead, i) Magnesium, j) Manganese, k) Nickel, l) Potassium, m) Sodium, n) Strontium, o) Titanium and p) Zinc.

Tables

Table 1: Mantel test of the correlation between two distance matrices: beta diversity and the concentration of heavy metals (mg/g) present in the sediment core. From these data we can see that there is a significant strong positive linear correlation between the level of zinc in the sediment and beta diversity (*r = 0.80069, P < 0.01*)

Beta Diversity	Metal	*r*	*p-value*
β	Al	0.31458	0.001
β	Ba	0.54281	0.001
β	Co	0.43823	0.001
β	Cr	0.34444	0.001
β	Cu	0.29328	0.006
β	Fe	-0.04093	0.611
β	Ga	-0.08041	0.308
β	K	0.31765	0.002
β	Mg	0.25356	0.007
β	Mn	0.22372	0.01
β	Na	0.04114	0.619
β	Ni	0.34687	0.001
β	Pb	0.58627	0.001
β	Sr	0.27943	0.006
β	Ta	0.16119	0.051
β	Ti	0.11045	0.175
β	**Zn**	**0.80069**	**0.001**
β	Depth	0.89686	0.001

Chapter 5

General discussion

This study is the first of its kind to address the effects of industrial heavy metal contaminants on both the cultivable and the total microbial population of a sediment core that covers a time scale spanning approximately a century through both phenotypic analysis and with the use of high throughput sequencing technologies. Previous studies have examined changes in microbial diversity over spatial scales or over limited temporal scales, only including the depths at which microbial activity is highest (Bouskill, Barnhart et al. 2007, Berg, Brandt et al. 2012, Kaci, Petit et al. 2016). By assessing the effects of heavy metals on microbial diversity across a temporal gradient, covering the rise of industry and the decades previous and proceeding, we are able to gain a much deeper understanding of how anthropogenic contaminants effect the microbial diversity of sediments. The results presented in this thesis show that the pre-exposure of microbial communities to increased concentrations of zinc and other metals in the environment could result in increased incidence of resistance among cultivable populations in sediment extracted from an area of elevated contaminants. Additionally, providing further insight into the association of metal exposure and co-selection for antibiotic resistance through phenotypic analysis of isolates and the quantification of a gene that is known the confer resistance to both heavy metals and antibiotics.

The analysis was performed using a sediment core from an area of high industrial activity to provide information regarding the impacts of levels of heavy metals over time, more specifically zinc, from human industrial practices on microbial community diversity, heavy metal tolerance and the persistence and

proliferation of antibiotic resistance in the environment. Zinc was the target

metal in this study because despite its pivotal role in a number of essential

biological processes, it is considered to be among the class one toxic pollutants

due it to its comparatively low toxicity level (Choudhury and Srivastava 2001).

Additionally, the high levels of zinc introduced into the environment through

industrial sources has resulted in the classification of zinc as a class one toxic

pollutant (Mansoorian, Mahvi et al. 2014). ICP-OES analysis of a sediment core

provided heavy metal content data through time and revealed marked increases

in concentrations of a variety of metals consistent with those used commonly in

industrial practices (Nicholson, Smith et al. 2003) (Chapter 4: Fig. 3). The

concentration of zinc was shown to correlate positively with depth through the

core [Chapter 3: Fig. 2], indicating that levels of zinc contamination in the

environment began to increase significantly after its introduction into industrial

practices. Dating of the sediment core shows that the peak of zinc concentration

in soil is dated to be from approximately 1926, with a dramatic decline through

the lower depths leading to much lower concentrations in sediment further back

in time.

Total concentrations of metals often correlate poorly with metal

bioavailability and toxicity, and information regarding metal availability is very

limited (Nybroe, Brandt et al. 2008). It was therefore necessary to acquire

information regarding zinc bioavailability with the use of whole-cell specific bio-

reporters, which has previously been demonstrated to quantify the levels of

bioavailable zinc in environmental samples (Hynninen, Tõnismann et al. 2010).

Unfortunately, due to the presence of nutrients or toxicants in the sediment and

the resulting effects on the soil matrix, only twelve of the twenty-five sediment

samples used for the zinc bioavailability analysis provided results for use in this

study. However limited, the results obtained were shown to be positively correlated with total zinc concentration in the sediment, therefore corroborating the ICP-OES results available for a wider range of samples.

By assessing the levels of phenotypic zinc resistance in bacterial communities in the sediment, we are able to determine whether exposure to high levels of zinc in the environment increases incidence of resistance within populations. Through phenotypic analysis of the response in microbes exposed to the highest measured concentration of zinc in the sediment, we were able to assess whether those that had been pre-exposed to higher concentrations were more resistant than those pre-exposed to lower concentrations of zinc. The resulting data demonstrates conclusively that the level of zinc present throughout the core has a significant selective effect on the level of phenotypic zinc resistance in microbial isolates.

Heavy metals in the environment may serve to indirectly select for increased tolerances towards other antimicrobial agents such as antibiotics (Alonso, Sanchez et al. 2001, Peltier, Vincent et al. 2010, Garhwal, Vaghela et al. 2014). One objective of this study was to assess how zinc tolerance varied along a temporal gradient of zinc contamination, and if those population that exhibit increased tolerance also expressed increase resistance to antibiotics. By analysing isolates from the layers of sediment in which the most significant increase in zinc concentration was observed, we were able to conclusively demonstrate the relationship between increased zinc tolerance and decreased susceptibility to the clinically important antibiotics oxacillin, cefotaxime and trimethoprim. These data strongly indicate that levels of zinc in the environment is a driving force in the microbial population towards increased antibiotic resistance due to the co-selective nature of heavy metals and antibiotics. This

pattern has also been previously demonstrated with copper amended soils in which isolates that expressed increased tolerance to copper also had a higher occurrence of antibiotic tolerances (Berg, Tom-Petersen et al. 2005). These data suggest the metal and antibiotic resistance traits are associated and the broad categorisation of resistance mechanisms, and determining which mechanism in play between antimicrobial co-selection, is made difficult by the host of pathways used by microorganisms (Alonso, Sanchez et al. 2001). Further research into the identification of the specific resistance determinants involved in specific metal-antibiotic co-selection would prove valuable in a greater understanding of the proliferation of antimicrobial resistance. There is data linking the shared resistance systems of drug- metal-efflux and alteration of cellular targets used for resistance to zinc, β-lactams and trimethoprim (Baker-Austin, Wright et al. 2006). Further detailed sequencing analysis of the specific resistance genes for these mechanisms would prove invaluable in providing a cohesive understanding the role of metal contaminants in the persistence of antibiotic resistance.

To further assess the relationship between metal- antibiotic co-resistance we quantified the presence of the class one integron-intergrase gene *IntI1* in the microbial isolates from the sediment, which is a proposed link between genes conferring resistance to both metals and antibiotics (Gillings, Gaze et al. 2015). The prevalence of the *IntI1* gene in microbial isolates was shown to significantly correlate with zinc concentration and the level of zinc resistant microbes, indicating a clear increase in this particular resistance gene as a result of selective pressure from levels of zinc in the environment. The same linear analysis with proportion of resistance to cefotaxime, oxacillin, trimethoprim in isolates and prevalence of the *IntI1* gene. These data show that isolates with

increased resistance to zinc and cefotaxime had an increase in *IntI1* gene prevalence, however, oxacillin and trimethoprim resistance were not shown to have significant correlation with *IntI1* gene prevalence. Though this resistance gene is said to act a genetic marker for anthropogenic pollution and confers resistance to both heavy metals and antibiotic, that has not been shown to be the case in this study. This may be a result of the mechanism of resistance towards oxacillin and trimpethoprim not being mediated by the same genetic element as that containing the *IntI1* gene. The location of *IntI1* gene has had a previously reported proximity to genes coding for efflux based resistance to Zn^{2+} (Stokes, Nesbø et al. 2006, Gillings, Boucher et al. 2008), which is a known mechanism of resistance to β-lactams. Further analysis of *IntI1* gene prevalence in metal- antibiotic-resistant microbes from industrially polluted environments could prove useful in furthering our understanding of this gene as a potential genetic marker for anthropogenic pollution effects on microbial population.

The effects of selective pressure imposed by metals on total microbial community composition through time were assessed with the use of high-throughput sequencing. Through extracting DNA and sequencing of the 16S rRNA gene, the communities present within the sediment layers with the most significant zinc gradient, we were able to quantify the relative abundance of taxa within the sediment and assess the effects of metals on microbial composition. The taxa present within the sediment are consistent with those typically found within soil samples worldwide (Chapter 4: Fig. 1) (Youssef and Elshahed 2009). In order to visualise changes in community composition throughout the sediment an analysis of dissimilarities between communities present in each layer and subsequent Principal Coordinate Analysis was

performed. A Bray-Curtis dissimilarities measure of communities between layers shows that as the depth through the sediment increases, as does the relative abundance of taxa throughout the sediment (Chapter 4: Fig. 2).

In order to assess the impact of heavy metals as a crucial selective agent in the structure of communities between layers, a Mantel test was performed using the Bray-Curtis dissimilarities measure (ß-diversity) and heavy metal concentrations in the sediment. The Mantel test shows the effects of heavy metals on the microbial community diversity throughout twenty-four layers of the extracted sediment, revealing several metals to have a significant impact on diversity. There significant positive linear correlation between the level of aluminium, barium, cobalt, copper, potassium, magnesium, manganese, nickel, lead, strontium and zinc with ß-diversity (Chapter 4: Table 1). Of the metals in this analysis, zinc has had the most significant impact on microbial community composition and collectively, these data demonstrate that the level of zinc present in the sediment is having a significant effect on microbial tolerance and shaping the structure of community diversity within the sediment to that of a population with increased levels of zinc resistance.

To our knowledge, no previous studies have assessed the effects of heavy metals on microbial populations over a period that spans the time which industrial activity began to increase, as well as the decades preceding and following. In addition, studies that address co-selection within microbial populations/communities and at a community level do so through culture dependent phenotypic analysis of isolated microbes and do not include culture independent analysis. This study demonstrates a novel approach into investigating the levels of metal resistance in the population through phenotypic analysis, in addition to changes in the microbial community composition through

time as a result of increased exposure to heavy metals. The results of this study show the effects of increased zinc exposure over time in microbial population and revealed increased bacterial community tolerance to zinc present in soil, with the tolerance increase being correlated with the pollution level. The increase in zinc resistance also revealed increased resistance to antibiotics. The occurrence of antibiotic resistant microorganisms is of global concern, and a further analysis of the proliferation of resistance would help gain a better understanding of the maintenance of antibiotic resistance due to anthropogenic heavy metal pollution. Only one sediment core extruded from the area was used in the resistance and community composition analyses without the use of uncontaminated sediment as a control. Though an additional core was assessed for zinc concentration, these data are not necessarily representative of the area as a whole. Abiotic factors including soil pH, dissolved oxygen, sediment size, salinity, temperature and nutrient availability of the sediment are not known, thereby limiting our interpretation of the results collected. Further research should consider the use of multiple sediment cores extruded from an industrial area in addition to taking variations over time of other physicochemical properties within the sediment into consideration.

References

Ahemad, M. and A. Malik (2011). "Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater." Bacteriol J **2**: 12-21.

Aleem, A., et al. (2003). "Impact of long-term application of industrial wastewater on the emergence of resistance traits in Azotobacter chroococcum isolated from rhizospheric soil." Bioresource Technology **86**(1): 7-13.

Allen, H. K., et al. (2010). "Call of the wild: antibiotic resistance genes in natural environments." Nature Reviews Microbiology **8**(4): 251-259.

Alonso, A., et al. (2001). "Environmental selection of antibiotic resistance genes." Environmental microbiology **3**(1): 1-9.

Appleby, P., et al. (1986). "210Pb dating by low background gamma counting." Hydrobiologia **143**(1): 21-27.

Appleby, P., et al. (1992). "Self-absorption corrections for well-type germanium detectors." Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms **71**(2): 228-233.

ApplebyPG, O. (1978). "Thecalculationof210Pbdatesassuminga constantrateofsupplyofunsupported210Pbtothesediment." catena **5**(1): 1-8.

Audry, S., et al. (2004). "Fifty-year sedimentary record of heavy metal pollution (Cd, Zn, Cu, Pb) in the Lot River reservoirs (France)." Environmental pollution **132**(3): 413-426.

Baker-Austin, C., et al. (2006). "Co-selection of antibiotic and metal resistance." Trends in microbiology **14**(4): 176-182.

Bass, L., et al. (1999). "Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avianEscherichia coli." Antimicrobial agents and chemotherapy **43**(12): 2925-2929.

Beard, S. J., et al. (1997). "Zinc (II) tolerance in Escherichia coli K-12: evidence that the zntA gene (o732) encodes a cation transport ATPase." Molecular microbiology **25**(5): 883-891.

Berg, J., et al. (2012). "Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term Cu exposure." Applied and Environmental Microbiology **78**(20): 7438-7446.

Berg, J., et al. (2005). "Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field." Letters in Applied Microbiology **40**(2): 146-151.

Berg, J. M. and Y. Shi (1996). "The galvanization of biology: a growing appreciation for the roles of zinc." Science **271**(5252): 1081.

Besaury, L., et al. (2013). "Abundance and diversity of copper resistance genes cusA and copA in microbial communities in relation to the impact of copper on Chilean marine sediments." Marine pollution bulletin **67**(1): 16-25.

Bhullar, K., et al. (2012). "Antibiotic resistance is prevalent in an isolated cave microbiome." PloS one **7**(4): e34953.

Blair, J. M., et al. (2015). "Molecular mechanisms of antibiotic resistance." Nature Reviews Microbiology **13**(1): 42-51.

Bouskill, N. J., et al. (2007). "Quantification of changing Pseudomonas aeruginosa sodA, htpX and mt gene abundance in response to trace metal toxicity: a potential in situ biomarker of environmental health." FEMS microbiology ecology **60**(2): 276-286.

Brandt, K. K., et al. (2008). "Evidence for Bioavailable Copper– Dissolved Organic Matter Complexes and Transiently Increased Copper Bioavailability in Manure-Amended Soils as Determined by Bioluminescent Bacterial Biosensors." Environmental science & technology **42**(8): 3102-3108.

Bruins, M. R., et al. (2000). "Microbial resistance to metals in the environment." Ecotoxicology and environmental safety **45**(3): 198-207.

Calderón, K., et al. (2017). "Effectiveness of ecological rescue for altered soil microbial communities and functions." The ISME journal **11**(1): 272-283.

Calomiris, J. J., et al. (1984). "Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water." Applied and Environmental Microbiology **47**(6): 1238-1242.

Chapman, J. S. (2003). "Disinfectant resistance mechanisms, cross-resistance, and co-resistance." International Biodeterioration & Biodegradation **51**(4): 271-276.

Choudhury, R. and S. Srivastava (2001). "Mechanism of zinc resistance in Pseudomonas putida strain S4." World Journal of Microbiology and Biotechnology **17**(2): 149-153.

Choudhury, R. and S. Srivastava (2001). "Zinc resistance mechanisms in bacteria." Current Science: 768-775.

Conejo, M. C., et al. (2003). "Zinc eluted from siliconized latex urinary catheters decreases OprD expression, causing carbapenem resistance in Pseudomonas aeruginosa." Antimicrobial agents and chemotherapy **47**(7): 2313-2315.

Czekalski, N., et al. (2014). "Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake." The ISME journal **8**(7): 1381.

Daniel, R. (2005). "The metagenomics of soil." Nature reviews. Microbiology **3**(6): 470.

Dixit, R., et al. (2015). "Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes." Sustainability **7**(2): 2189-2212.

Duruibe, J., et al. (2007). "Heavy metal pollution and human biotoxic effects." International Journal of Physical Sciences **2**(5): 112-118.

Facchinelli, A., et al. (2001). "Multivariate statistical and GIS-based approach to identify heavy metal sources in soils." Environmental pollution **114**(3): 313-324.

Fani, F., et al. (2013). "Genomic analysis and reconstruction of cefotaxime resistance in Streptococcus pneumoniae." Journal of Antimicrobial Chemotherapy **68**(8): 1718-1727.

François, F., et al. (2012). "Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury." Applied and Environmental Microbiology **78**(4): 1097-1106.

Garhwal, D., et al. (2014). "Lead tolerance capacity of clinical bacterial isolates and change in their antibiotic susceptibility pattern after exposure to a heavy metal." International Journal of Medicine and Public Health **4**(3).

Ghosh, A., et al. (2000). "Characterization of large plasmids encoding resistance to toxic heavy metals in Salmonella abortus equi." Biochemical and biophysical research communications **272**(1): 6-11.

Giller, K. E., et al. (1998). "Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review." Soil Biology and Biochemistry **30**(10): 1389-1414.

Gillings, M., et al. (2008). "The evolution of class 1 integrons and the rise of antibiotic resistance." Journal of bacteriology **190**(14): 5095-5100.

Gillings, M. R., et al. (2015). "Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution." The ISME journal **9**(6): 1269-1279.

Gilotra, U. and S. Srivastava (1997). "Plasmid-encoded sequestration of copper by Pseudomonas pickettii strain US321." Current microbiology **34**(6): 378-381.

Golparian, D., et al. (2014). "Four treatment failures of pharyngeal gonorrhoea with ceftriaxone (500 mg) or cefotaxime (500 mg), Sweden, 2013 and 2014." Euro Surveill **19**(30): 20862.

Haller, L., et al. (2011). "Composition of bacterial and archaeal communities in freshwater sediments with different contamination levels (Lake Geneva, Switzerland)." water research **45**(3): 1213-1228.

Hayashi, S., et al. (2000). "The DsbA-DsbB Disulfide Bond Formation System of Burkholderia cepacia Is Involved in the Production of Protease and Alkaline

Phosphatase, Motility, Metal Resistance, and Multi-Drug Resistance." <u>Microbiology and immunology</u> **44**(1): 41-50.

Hegstad, K., et al. (2010). "Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health?" <u>Microbial drug resistance</u> **16**(2): 91-104.

Ho, J. M.-W. and D. N. Juurlink (2011). "Considerations when prescribing trimethoprim–sulfamethoxazole." <u>Canadian Medical Association Journal</u> **183**(16): 1851-1858.

Hodgson, S., et al. (2004). "Excess risk of kidney disease in a population living near industrial plants." <u>Occupational and environmental medicine</u> **61**(8): 717-719.

Hoostal, M. J., et al. (2008). "Local adaptation of microbial communities to heavy metal stress in polluted sediments of Lake Erie." <u>FEMS microbiology ecology</u> **65**(1): 156-168.

Hynninen, A., et al. (2010). "Improving the sensitivity of bacterial bioreporters for heavy metals." <u>Bioengineered bugs</u> **1**(2): 132-138.

Jacobsen, C. S. and M. H. Hjelmsø (2014). "Agricultural soils, pesticides and microbial diversity." <u>Current Opinion in Biotechnology</u> **27**: 15-20.

Järup, L. (2003). "Hazards of heavy metal contamination." <u>British medical bulletin</u> **68**(1): 167-182.

Ji, G. and S. Silver (1995). "Bacterial resistance mechanisms for heavy metals of environmental concern." <u>Journal of Industrial Microbiology & Biotechnology</u> **14**(2): 61-75.

Kaci, A., et al. (2016). "Diversity of active microbial communities subjected to long-term exposure to chemical contaminants along a 40-year-old sediment core." <u>Environmental Science and Pollution Research</u> **23**(5): 4095-4110.

Kawane, R. (2012). "Studies on antibiotics and heavy metal resistance profiling of Escherichia coli from drinking water and clinical specimens." <u>Biosci. Disc</u> **3**(3): 292-295.

Kelly, J. J., et al. (2003). "Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipid fatty acid profiles." <u>Biology and Fertility of Soils</u> **38**(2): 65-71.

Khatun, M., et al. (2012). "Estimation of heavy metal tolerance and antibiotic susceptibility of Bacillus cereus isolated from municipal solid waste." <u>Int J Pharma Bio Sci</u> **3**: 819-829.

Knapp, C. W., et al. (2011). "Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils." <u>PloS one</u> **6**(11): e27300.

Laghlimi, M., et al. (2015). "Characterization of soil Heavy Metal Contamination in the Abandoned mine of Zaida (High Moulouya, Morocco)." Int. Res. J. Earth Sci **3**(3): 1-3.

Laxminarayan, R., et al. (2013). "Antibiotic resistance—the need for global solutions." The Lancet infectious diseases **13**(12): 1057-1098.

Ledin, M. (2000). "Accumulation of metals by microorganisms—processes and importance for soil systems." Earth-Science Reviews **51**(1): 1-31.

Lee, L. J., et al. (2005). "Genome-wide transcriptional response of chemostat-cultured Escherichia coli to zinc." Journal of bacteriology **187**(3): 1124-1134.

Legendre, P., et al. (2005). "Analyzing beta diversity: partitioning the spatial variation of community composition data." Ecological Monographs **75**(4): 435-450.

Liebert, C. A., et al. (1999). "Transposon Tn21, flagship of the floating genome." Microbiology and Molecular Biology Reviews **63**(3): 507-522.

Ligi, T., et al. (2014). "Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing." Ecological Engineering **72**: 56-66.

Llirós, M., et al. (2014). "Bacterial community composition in three freshwater reservoirs of different alkalinity and trophic status." PloS one **9**(12): e116145.

Lozupone, C. and R. Knight (2005). "UniFrac: a new phylogenetic method for comparing microbial communities." Applied and Environmental Microbiology **71**(12): 8228-8235.

Lozupone, C. A. and R. Knight (2007). "Global patterns in bacterial diversity." Proceedings of the National Academy of Sciences **104**(27): 11436-11440.

Mansoorian, H. J., et al. (2014). "Removal of lead and zinc from battery industry wastewater using electrocoagulation process: influence of direct and alternating current by using iron and stainless steel rod electrodes." Separation and Purification Technology **135**: 165-175.

Mantel, N. (1967). "The detection of disease clustering and a generalized regression approach." Cancer research **27**(2 Part 1): 209-220.

Marti, E., et al. (2014). "The role of aquatic ecosystems as reservoirs of antibiotic resistance." Trends in microbiology **22**(1): 36-41.

Martinez, J. L., et al. (2009). "Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems." FEMS Microbiology Reviews **33**(2): 430-449.

Mata, M., et al. (2000). "A multidrug efflux transporter in Listeria monocytogenes." FEMS Microbiology Letters **187**(2): 185-188.

McArthur, J. V. and R. C. Tuckfield (2000). "Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution." Applied and Environmental Microbiology **66**(9): 3722-3726.

Melaku, S., et al. (2005). "Determination of trace elements in agricultural soil samples by inductively coupled plasma-mass spectrometry: microwave acid digestion versus aqua regia extraction." Analytica Chimica Acta **543**(1): 117-123.

Morby, A. P., et al. (1993). "SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene smtA: identification of a Zn inhibited DNA-protein complex." Nucleic acids research **21**(4): 921-925.

Mutuku, C., et al. (2014). "Metal pollutants distribution within Lake Victoria basin and their influence on the native and transient microbial flora." Journal of Agricultural and Biological Science **9**(4): 1990-6145.

Naik, M. M. and S. K. Dubey (2013). "Lead resistant bacteria: lead resistance mechanisms, their applications in lead bioremediation and biomonitoring." Ecotoxicology and environmental safety **98**: 1-7.

Navas-Molina, J. A., et al. (2013). "Advancing our understanding of the human microbiome using QIIME." Methods in enzymology **531**: 371.

Nicholson, F., et al. (2003). "An inventory of heavy metals inputs to agricultural soils in England and Wales." Science of the Total Environment **311**(1): 205-219.

Nies, D. H. (1992). "Resistance to cadmium, cobalt, zinc, and nickel in microbes." Plasmid **27**(1): 17-28.

Nies, D. H. (1999). "Microbial heavy-metal resistance." Applied microbiology and biotechnology **51**(6): 730-750.

Nies, D. H. (2000). "Heavy metal-resistant bacteria as extremophiles: molecular physiology and biotechnological use of Ralstonia sp. CH34." Extremophiles **4**(2): 77-82.

Nybroe, O., et al. (2008). "Differential bioavailability of copper complexes to bioluminescent Pseudomonas fluorescens reporter strains." Environmental toxicology and chemistry **27**(11): 2246-2252.

O'Neill, J. (2014). "Antimicrobial resistance: tackling a crisis for the health and wealth of nations." Review on antimicrobial resistance: 1-16.

Ordonez, A., et al. (2003). "Distribution of heavy metals in the street dusts and soils of an industrial city in Northern Spain." Archives of Environmental Contamination and Toxicology **44**(2): 0160-0170.

Oves, M., et al. (2012). Soil contamination, nutritive value, and human health risk assessment of heavy metals: an overview. Toxicity of heavy metals to legumes and bioremediation, Springer**:** 1-27.

Oves, M., et al. (2016). "Heavy metals: biological importance and detoxification strategies." J Bioremed Biodeg **7**(334): 2.

Pace, N. R. (1997). "A molecular view of microbial diversity and the biosphere." Science **276**(5313): 734-740.

Partridge, S. R., et al. (2001). "Transposons Tn1696 and Tn21and their integrons In4 and In2 have independent origins." Antimicrobial agents and chemotherapy **45**(4): 1263-1270.

Peltier, E., et al. (2010). "Zinc-induced antibiotic resistance in activated sludge bioreactors." water research **44**(13): 3829-3836.

Perron, K., et al. (2004). "CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in Pseudomonas aeruginosa." Journal of Biological Chemistry **279**(10): 8761-8768.

Phitaktim, S., et al. (2016). "Synergism and the mechanism of action of the combination of α-mangostin isolated from Garcinia mangostana L. and oxacillin against an oxacillin-resistant Staphylococcus saprophyticus." BMC microbiology **16**(1): 195.

Poretsky, R., et al. (2014). "Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics." PloS one **9**(4): e93827.

Ramette, A. (2007). "Multivariate analyses in microbial ecology." FEMS microbiology ecology **62**(2): 142-160.

Rampelli, S., et al. (2015). "Metagenome sequencing of the Hadza hunter-gatherer gut microbiota." Current Biology **25**(13): 1682-1693.

Rasmussen, L. D. and S. J. Sørensen (1998). "The effect of longterm exposure to mercury on the bacterial community in marine sediment." Current microbiology **36**(5): 291-297.

Rossolini, G. M., et al. (2014). "Update on the antibiotic resistance crisis." Current opinion in pharmacology **18**: 56-60.

Rouch, D. A., et al. (1995). "Understanding cellular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance." Journal of Industrial Microbiology & Biotechnology **14**(2): 132-141.

Samanta, A., et al. (2012). "An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain Bacillus sp. isolated from municipal waste." Journal of Microbiology and Biotechnology Research **2**(1): 178-189.

Schloss, P. D., et al. (2016). "Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system." PeerJ **4**: e1869.

Seiler, C. and T. U. Berendonk (2012). "Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture." Frontiers in microbiology **3**.

Silver, S. and L. T. Phung (1996). "Bacterial heavy metal resistance: new surprises." Annual Reviews in Microbiology **50**(1): 753-789.

Silver, S. and M. Walderhaug (1992). "Gene regulation of plasmid-and chromosome-determined inorganic ion transport in bacteria." Microbiological reviews **56**(1): 195-228.

Sinclair, L., et al. (2015). "Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform." PloS one **10**(2): e0116955.

Sobolev, D. and M. Begonia (2008). "Effects of heavy metal contamination upon soil microbes: lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers." International journal of environmental research and public health **5**(5): 450-456.

Solgi, E., et al. (2012). "Soil contamination of metals in the three industrial estates, Arak, Iran." Bulletin of environmental contamination and toxicology **88**(4): 634-638.

Stefanowicz, A. M., et al. (2009). "Pollution-induced tolerance of soil bacterial communities in meadow and forest ecosystems polluted with heavy metals." European Journal of Soil Biology **45**(4): 363-369.

Stepanauskas, R., et al. (2005). "Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments." Environmental science & technology **39**(10): 3671-3678.

Stokes, H. W., et al. (2006). "Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community." Journal of bacteriology **188**(16): 5722-5730.

Tanwar, J., et al. (2014). "Multidrug resistance: an emerging crisis." Interdisciplinary perspectives on infectious diseases **2014**.

Thijs, S., et al. (2017). "Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys." Frontiers in microbiology **8**.

Valls, M. and V. De Lorenzo (2002). "Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution." FEMS Microbiology Reviews **26**(4): 327-338.

van Dam, A. P., et al. (2014). "Verified clinical failure with cefotaxime 1g for treatment of gonorrhoea in the Netherlands: a case report." Sex Transm Infect: sextrans-2014-051552.

Vázquez-Baeza, Y., et al. (2013). "EMPeror: a tool for visualizing high-throughput microbial community data." Gigascience **2**(1): 16.

Vos, M., et al. (2013). "Micro-scale determinants of bacterial diversity in soil." FEMS Microbiology Reviews **37**(6): 936-954.

Wei, B. and L. Yang (2010). "A review of heavy metal contaminations in urban soils, urban road dusts and agricultural soils from China." Microchemical Journal **94**(2): 99-107.

Wellington, E. M., et al. (2013). "The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria." The Lancet infectious diseases **13**(2): 155-165.

Wireman, J., et al. (1997). "Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates." Applied and Environmental Microbiology **63**(11): 4494-4503.

Witte, W. (1998). "Medical consequences of antibiotic use in agriculture." Science **279**(5353): 996-997.

Wright, M. S., et al. (2006). "Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams." FEMS microbiology ecology **58**(2): 293-302.

Wuana, R. A. and F. E. Okieimen (2011). "Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation." Isrn Ecology **2011**.

Xiao-Fang, L., et al. (2012). "An attempt to quantify Cu-resistant microorganisms in a paddy soil from Jiaxing, China." Pedosphere **22**(2): 201-205.

Youssef, N. H. and M. S. Elshahed (2009). "Diversity rankings among bacterial lineages in soil." The ISME journal **3**(3): 305.

Zhang, X., et al. (2015). "Dating sediment in a fast sedimentation reservoir using cesium-137 and lead-210." Soil Sci. Soc. Am. J.