Relationships between zinc and meropenem resistance in the natural environment and experimental bioreactors

Catherine Lauren Hands BSc (Hons), MSc

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School of Civil Engineering and Geosciences

Newcastle University

Newcastle upon Tyne

NE1 7RU

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Declaration

I hereby certify that the work presented in this thesis is my original research work. Due reference is given to literature and any research collaborations where appropriate. No part of this thesis has been submitted previously for a degree at this or any university.

Catherine Lauren Hands

Abstract

The efficacy of antibiotics is being challenged by the emergence of bacteria resistant to antibiotics (AR), both in natural and clinical settings. Antibiotics and associated AR can be transmitted and dispersed via environmental bacteria, however AR might also be conferred i*n situ*, without antibiotic pressure. For example, release of metal-bearing wastes to natural environments can proliferate AR. An important case is the influence of zinc (Zn) contamination on environmental AR, which increased tetracycline and quinolone resistance in wastewater isolates. However, how Zn might influence AR to therapeutically critical carbapenem antibiotics, including meropenem is unknown, which fuelled this study. Here the percentage of total isolates resistant to Zn, meropenem and-or both, were compared and assessed in varied microbial communities from natural environments and bioreactors. Overall, Zn levels, and Zn and meropenem resistant isolates correlated in all settings. For example, the abundance of combined meropenem plus Zn resistant isolates was significantly higher in high Zn (South Tyne) versus low Zn (North Tyne) sediments, and correlated with soluble and total Zn levels (p-value < 0.010 and p-value < 0.050, respectively), implying that acquired Zn resistance might confer meropenem resistance to isolates. In parallel, batch reactors seeded with North and South Tyne sediments, and amended with 2.00 mg/L (low) and 100 mg/L (high) Zn (2 x 2 design), showed increased relative percent meropenem resistant isolates in reactors with high (South Tyne: 21.0%; North Tyne: 31.0%) versus low Zn (South Tyne: 17.0%; North Tyne: 14.0%), whereas, sediment source (South vs North Tyne) was not important. Further, sediment soluble Zn levels significantly correlated with meropenem resistant isolates in all reactors, suggesting that the observed meropenem resistance was a possible "side effect" of cellular defence against Zn toxicity. Similar results were seen in Zn and meropenem-amended rotating tubular reactors treating domestic wastewater. Reactors dosed with 2.00 mg/L meropenem plus 100 mg/L Zn showed combined resistance in 51.0% of reactor effluent isolates, whereas only 24.0% displayed combined resistance with amendments of 2.00 mg/L meropenem and 20.0 mg/L Zn. Overall, elevated Zn levels significantly increased Zn, meropenem and combined resistance in isolates from sediments, batch reactors and tubular reactors. Therefore, one can conclude Zn levels impact meropenem resistance, although evidence suggest meropenem resistance is most apparent when Zn is present and resistance can be lost when Zn is removed, suggesting cross resistance mechanisms.

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List of Abbreviations

AMR: Antimicrobial resistance

ANOSIM: Analysis of similarity

ANOVA: Analysis of variance

AR: Antibiotic resistance

ARG: Antibiotic resistance genes

ASRIT: Activated Sludge Respiration Inhibition Test

ATP: Adenosine triphosphate

CDF: Cation diffusion facilitator

CM: Cytoplasmic membrane

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxyribonucleic acid

DO: Dissolved oxygen

DOC: Dissolved organic carbon

EPS: Extracellular polymeric substance

ESBL: Extended-spectrum beta-lactamase

GLP: Good laboratory practice

HGT: Horizontal gene transfer

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometer

MALDI-TOF: Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry

MATE: Multi drug and toxic compound extrusion

MBL: Metallo-β-lactamases

MDR: Multi-drug resistance

MDS: Multi-dimensional scaling

MFS: Major facilitator

MIC: Minimum inhibitory concentration

MIT: Metal inorganic transport

MR: Metal resistance

MRG: Metal resistance genes

MT: Metallothionein

NDM$_{-1}$: New Delhi metallo-β-lactamase (*bla*-NDM-1)

PCR: Polymerase chain reaction

QPCR: Quantitative polymerase chain reaction

OECD: Organisation for Economic Co-operation and Development

OM: Outer membrane

PBP: penicillin binding protein

RND: Resistance-nodulation division

SMR: Small multidrug resistance

UK: United Kingdom

USA: United States of America

VIM: Verona integron encoded metallo-β-lactamase

WWTP: Wastewater treatment plant

Chapter 1 Introduction

Since the discovery of the first antibiotics, over 60 years ago, human mortality and morbidity has been substantially reduced (Davies and Davies, 2010). However, the efficacy of antibiotics is now being challenged by the emergence of bacteria resistant to antibiotics, both in natural and clinical settings due to antimicrobial resistance (AMR) (Baker-Austin *et al.*, 2006; Knapp *et al.*, 2011). The most relevant definition is that of the World Health Organisation, where "Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it" (Walsh, 2013). Microorganisms, including bacteria, fungi, viruses and parasites can gain resistance, allowing them to withstand any attack by antimicrobial drugs, antifungals, antivirals and antimalarials. The emergence of multi-resistance in bacteria, especially in strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* gram-negative bacilli means successful treatment of infections caused by such bacteria are becoming ineffective, causing infections to persist (Wellington *et al.*, 2013; WHO, 2015).

Bacteria exhibit resistance to antimicrobials (in this case antibiotics) by chromosomal or mobile genetic elements (plasmids) using four main strategies: (i) the reduction of the membrane permeability to antibiotics, (ii) drug inactivation due to porin loss and alterations in the penicillin binding proteins (PBPs), (iii) expression of efflux pumps causing rapid efflux of the antibiotic and (iv) mutation of cellular targets (Baker-Austin *et al.*, 2006) (Section 2.4). After the introduction of semi-synthetic penicillins and the use of β-lactamase inhibitors to combat the first instance of resistance, the development of resistance to these combinations by plasmid encoded β-lactamases, meant that aminoglycosides and third generation cephalosporins and quinolones were increasingly used for treatment (Wellington *et al.*, 2013). However this led to the emergence of plasmid mediated aminoglycoside resistance which meant that third generation cephalosporins, such as cefotaxime and ceftazidime and quinolones, such as ciprofloxacin were substantially used to treat most infections (Wellington *et al.*, 2013). Since the 2000s, there has been a large increase in the presence of extended spectrum β-lactamases (ESBLs), which hydrolyse cephalosporins making third generation cephalosporins and quinolones less effective (Wellington *et al.*, 2013). This had led to a shift towards the use of carbapenems, currently described as 'last resort drugs' (Bradley *et al.*, 1999; Papp-Wallace *et al.*, 2011) such as imipenem and meropenem.

Carbapenems are broad antimicrobial spectrum antibiotics that are used to treat serious infections and are active against gram-negative bacteria with ESBLs and AmpC enzymes (Livermore, 1995). They belong to the class of β-lactams, like penicillins and cephalosporins

but they differ from these in their exact chemical structure (Kahan *et al.*, 1979; Papp-Wallace *et al.*, 2011) (Section 2.5). Antibiotics such as imipenem and doripenem are used to target gram-positive bacteria while meropenem and ertapenem target gram-negative bacteria (Bedenic *et al.*, 2014) (Section 2.6). However the now increasing use of carbapenems means that carbapenem resistance is being selected and resistance is accumulating via the spread of carbapenem destroying β-lactamases (Abenavoli and Almasio, 2011). For example, in England, United Kingdom (UK), the total use of carbapenem antibiotics statistically increased by 31.3% between 2010 and 2013 (Public Health England, 2014). Carbapenemases belong to several classes, A, B C and D (Papp-Wallace *et al.*, 2011). Class B, known as metallo-β-lactamases (MBLs) contain zinc (Zn) ions in their active site (Livermore, 1995). Many acquired MBLs encoding genes are located in gene cassettes within integrons, as part of a plasmid or chromosome (Section 2.5). As a lot of β-lactams and carbapenem like compounds are naturally produced from some environmental bacteria and fungi, many other bacteria therefore synthesise β-lactamases in order to gain a selective advantage for survival (Waksman and Woodruff, 1940; Alonso *et al.*, 2001; Martinez, 2009b). However the presence of mobile genetic elements (the mobilome), such as plasmids allow for the conjugative transfer of resistant genes, through horizontal gene transfer (HGT), further increasing the mobility of these genes to other bacteria (Martinez, 2009b).

The use of antibiotics in veterinary process, human medicinal uses and agriculture means that any metabolites that have not been broken down or degraded, as well as compounds that have not been chemically altered, will eventually be released into rivers or will remain in sludge after sewage treatment. Antibiotics could then reach agricultural soils through the use of sludge as a fertiliser and the irrigation of soils with treated wastewaters (Kinney *et al.*, 2006). Once present in the soil, they can be transported to surface and groundwater and therefore be cycled within the environment (Wellington *et al.*, 2013) (Section 2.1). As carbapenem like compounds and other antibiotic producing bacteria can be found naturally in the environment (Martinez, 2009b), the presence of these environmental bacteria and other bacteria containing resistant genes from other sources can provide conditions ideal for the selection of new resistant strains (van Elsas and Bailey, 2002). This means that soils, sediments and water can act as reservoirs for antibiotic resistance genes (ARGs) and HGT (van Elsas and Bailey, 2002).

As mentioned above, there are four main mechanisms by which bacteria can gain resistance to antibiotics. These mechanisms are very similar in structure and function to mechanisms by which bacteria gain resistance to metals, such as Zn (Baker-Austin *et al.*, 2006) (Section 2.7).

In addition studies done by Summers *et al.* (1993) Summers (2002) and Alonso (2001) have suggested that metal contamination in natural environments may be associated with the maintenance and proliferation of AR (Section 2.9). Furthermore, many studies have reported that elevated resistance to antibiotics is often found in environments heavily contaminated with metals (Stepanauskas *et al.*, 2005; Baker-Austin *et al.*, 2006; Stepanauskas *et al.*, 2006; Berg *et al.*, 2010; Berg *et al.*, 2012). Although bacteria have always been exposed to metals, the high concentrations of metals now present in some environments due to industrial and anthropogenic derived sources could cause a long term selection pressure, as they are not able to be degraded (Alonso *et al.*, 2001). Hence it is possible that the presence of metal contamination can co- select for ARGs through the process of co-resistance, cross resistance (Chapman, 2003) or co-regulation (Nakajima *et al.*, 1995; Baranova and Nikaido, 2002; Lee *et al.*, 2005) (Section 2.7). Therefore the presence of chemical stressors such as antibiotics, metals and other organic compounds can all influence resistance in microbial communities to antibiotics (Baker-Austin *et al.*, 2006; Stepanauskas *et al.*, 2006; Berg *et al.*, 2010; Berg *et al.*, 2012). Despite this there has been a lot less work on the intrinsic capacity of these microbial communities in natural environments to retain and promote resistance (Alonso *et al.*, 2001).

This thesis aims to determine whether metal contamination promoted carbapenem resistance in microbial communities with and without the presence of metals and-or antibiotics. This was done by conducting a variety of studies using sediments and water column samples from rivers and, experimental reactors with sediments, wastewater and biofilm communities. The first study described in Chapter 3, aims to looks at the relationship between carbapenem resistance, using the antibiotic meropenem and heavy metal resistance, focusing predominantly on the metal Zn, in natural river systems. Here we used two comparative areas of the River Tyne basins, the South Tyne, which is contaminated with high levels of heavy metal pollutants and the North Tyne, which is metal free to assess whether areas with high Zn levels promoted Zn, meropenem and combined meropenem and Zn resistance (combined resistance) in sediment and water column samples. This study led on to setting up a series of batch reactors, described in Chapter 4 which were seeded with sediment from the North and South Tyne and amended with high and low levels of Zn. Here we assessed whether meropenem resistance in microbial communities varied over time with the extended Zn pressure. In the next study, described in Chapter 5, we focused on the changes in Zn, meropenem and a combined resistance in microbial biofilm communities and effluents of rotating tubular reactors. These were treated with domestic sewage that was dosed with different levels of Zn and meropenem.

Therefore the overall aim of this thesis was to assess and determine whether Zn concentrations increased and promoted relative percent meropenem and combined resistance in microbial communities within rivers and wastewaters.

The following hypothesis were made:

a) Ambient Zn conditions influence levels of meropenem and combined resistance in aquatic systems
b) Zn availability influences meropenem and combined resistance
c) The observed resistance relationships translate from reactor to sediment conditions

Chapter 2   Review of literature

2.1   Antibiotics and their sources and distribution in natural environments

Antibiotics are one of the most relevant medical inventions and have been a remarkable success since their introduction in the 20th century. However the intensive misuse and overuse of antibiotics has resulted in the development and emergence of antibiotic resistance (AR) in human pathogens (WHO, 2000), making the treatment of bacterial infections increasingly complicated. In addition resistance to antibiotics is emerging faster than the discovery of new antibiotics (Lewis, 2013). There have been no new drugs discovered since 1987, as shown in Figure 2-1, which illustrates the discovery dates of a range of antibiotics (Silver, 2011).
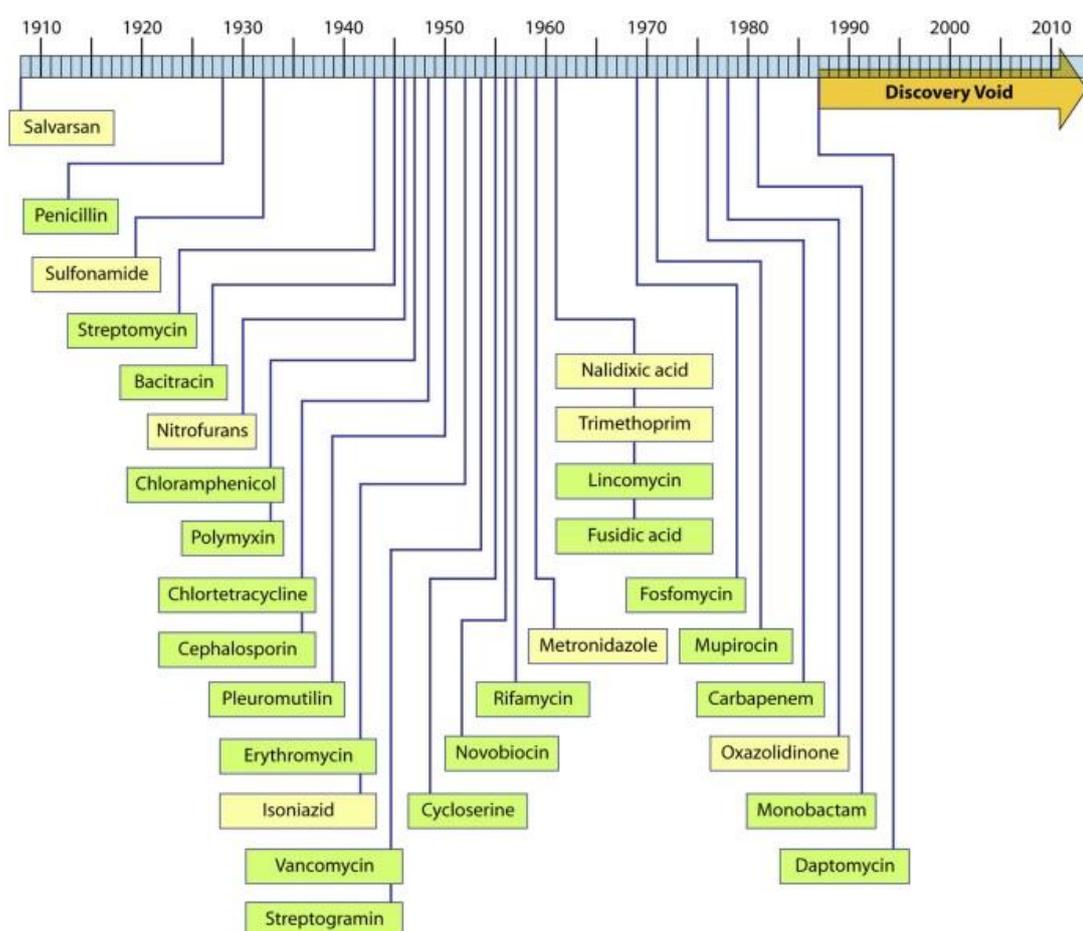
Figure 2-1 Timeline of the discovery of a range of antibiotics (Silver, 2011).

Governments are also now beginning to take AR more seriously, such as the warning given in 2013 by Chief Medical Officer in the UK, Professor Dame Sally Davies, that the rise of AR could cause a national emergency (Sample, 2013). Antibiotics have not only been used for human medicine, but also for veterinary medicine as well as disease prevention and growth promotion in agriculture, aquaculture and horticulture (Laxminarayan *et al.*, 2013). Antibiotics used in livestock will be present in manure that may subsequently be applied to

fields. Therefore, antibiotics could reach the agricultural soils though the use of sludge and irrigation with treated wastewaters (Kinney *et al.*, 2006) (Figure 2-2).
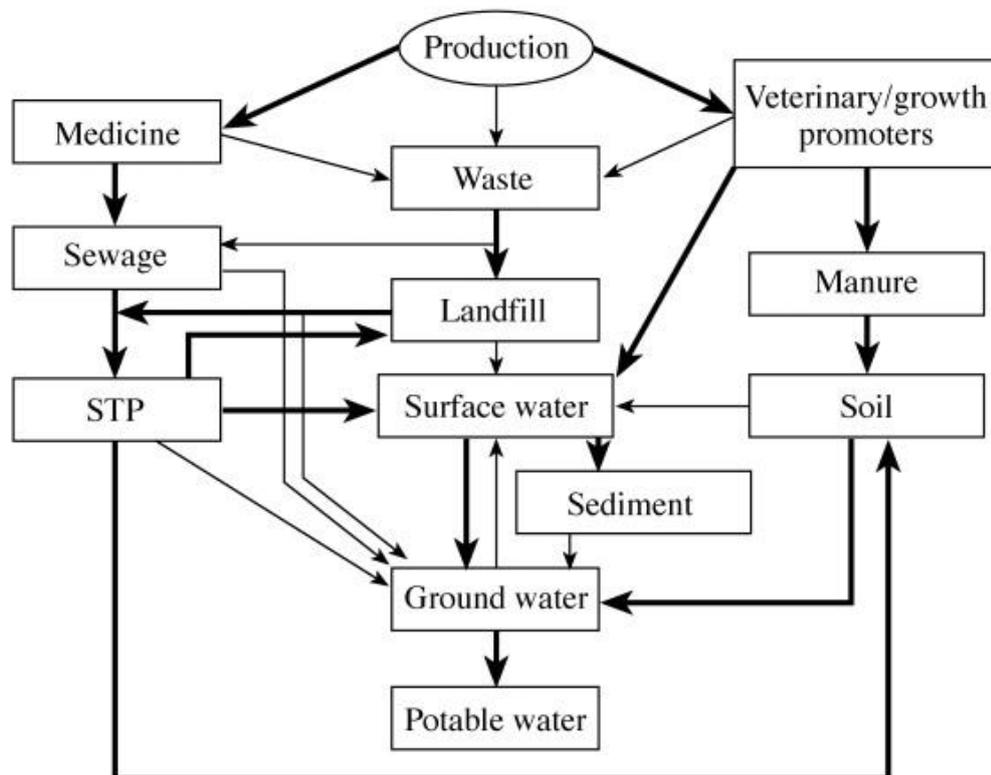
Figure 2-2 Diagram showing the sources and distributions of antibiotics in the environments (STP: sewage treatment plant) (Kümmerer, 2003).

Antibiotics in aquaculture, added directly to waters, may persist in the water compartment and sediments. As most of the compounds used in medicine do not completely degrade, the remaining metabolites will eventually be discharged into wastewater treatment plants (WWTPs) where they could be released into the environment, such as in coastal or river waters (Figure 2-2). As antibiotics are currently not listed as part of the 'Dangerous Substances Directive 76/464/EEC' (EC, 1976), they are not regarded as toxic, persistent or bio-accumulative and so their release into the environment is not controlled (Kümmerer, 2009). However some antibiotics can persist at higher concentrations for long periods and many are not biodegradable, hence the detection of antibiotics in soils, surface water, groundwater and sediments, such as fluoroquinolones and tetracyclines (Hirsch *et al.*, 1999; Kolpin *et al.*, 2002; Kümmerer, 2004). Antibiotics can also enter the environment during their manufacturing process, such as in India, where manufacturing takes places at a large scale and a study found that 2-5 mg/L ciprofloxacin were reported in rivers downstream of a WWTP that received waste from 90 bulk drug manufacturers (Fick *et al.*, 2009). Therefore these

sources, containing concentrations of pharmaceuticals could be one of the main driving force for the selection of resistant bacterial populations (Andersson and Hughes, 2011).

Furthermore humans can be exposed to AR bacteria or ARG in the environment through; (i) crops that have been fertilised with contaminated sludge, manure or slurry, (ii) the consumption of livestock which have accumulated veterinary drugs and resistant bacteria, (iii) fish that have been exposed to antibiotics that were released into waters, (iv) abstracted groundwater and surface water that may contain pharmaceuticals, which could contaminate water drinking supplies and (v) the consumption of shellfish that may have been exposed to antibiotics (Wellington *et al.*, 2013). Therefore the environment is an important factor when addressing the spread of resistance (Davey *et al.*, 2005) and surveillance systems monitoring antibiotic use and resistance need to include both clinical and environmental isolates (Laxminarayan *et al.*, 2013).

2.2   Selection of antibiotic resistance (AR) in natural environments

As antibiotics used in clinical and agricultural settings can persist in soil and aquatic environments, they can act as hotspots for HGT (van Elsas and Bailey, 2002) and can exert a selective pressure, which can promote the evolution spread of resistant genes (Segura *et al.*, 2009). However these antibiotic concentrations in the environment are not the only selective pressure causing AR in natural communities. Studies analysing pre-antibiotic plasmids, showed that resistance genes were present before antibiotics were used (Smith, 1967; Datta and Hughes, 1983; Hughes and Datta, 1983; Houndt and Ochman, 2000), which means that the genes must have been from environmental origin, more precisely from antibiotic producing organisms (Benveniste and Davies, 1973; Webb and Davies, 1993). Therefore their environmental role was to fight off competition and give protection, giving bacteria which naturally produce antibiotics, an ecological advantage against others (Waksman and Woodruff, 1940). These antibiotic producing bacteria therefore carry genes, conferring resistance to their own antibiotics which can be found in the same gene clusters as the antibiotic biosynthesis pathway genes (Benveniste and Davies, 1973; Webb and Davies, 1993). Therefore it is possible that this natural intrinsic resistance, could be exerting selective pressure on other environmental organisms around them, transferring naturally produced ARGs through acquired resistance mechanisms, such as HGT (Alonso *et al.*, 2001; Martínez, 2008).

The selection of ARGs can also occur without antibiotic selective pressure (Alonso *et al.*, 2001). The release of industrial and other waste releases into natural environments can increase AR levels (Kümmerer, 2003; Kümmerer, 2004; Baquero *et al.*, 2008; Martínez, 2008; Knapp *et al.*, 2010). For example, the presence of toxic compounds, including quaternary ammonium compounds, xenobiotic compounds, heavy metals and organic solvents in environments can allow bacteria to select for AR determinants in natural environments (Foster, 1983; Alonso *et al.*, 2001; Stepanauskas *et al.*, 2006). This can take place due to the presence of certain classes of efflux pumps in some bacteria, which can encode for resistance because they use the same mechanism to pump out toxins, i.e., heavy metals, from their cells (Nies, 2003; Poole, 2005). Heavy metal contaminated environments can maintain selective pressure on environmental organisms for long periods of time and many reports suggest that heavy metal polluted environments also contain a higher percentage of AR strains, as well as more plasmids (Rasmussen and Sorensen, 1998; Alonso *et al.*, 2001). This means that antibiotic and metal resistant genes can both exist in the same genetic element or plasmid and this can allow for the selection of AR upon heavy metal selective pressure (Davison, 1999; Alonso *et al.*, 2001). Therefore the increased movement of genes in metal selective conditions could also increase the movement of ARGs among environmental bacteria (Alonso *et al.*, 2001).

2.3   Antibiotic resistance (AR) in rivers, streams and waste treatment systems

The persistence and further exposure of AR bacteria and their resistant genes into natural aquatic environments may firstly, contribute to the resistance seen in clinical settings and secondly, increase the pool of ARGs in environmental bacteria, increasing the probability of resistance transferring into emerging pathogen, through HGT (van Elsas and Bailey, 2002; Segura *et al.*, 2009). Receiving waters, which include rivers, streams and lakes can influence the exposure and transmission of AR bacteria and ARGs and may also function as natural resistance reservoirs (Jones *et al.*, 1986; Auerbach *et al.*, 2007). As described earlier (Section 2.1) antibiotic residues can enter natural aquatic environments, through many pathways (Figure 2-2). An important source is sewage and waste treatment effluents. Before treatment, untreated raw sewage from households, hospitals, industries and many other sources, which contain diverse bacterial species including genes conferring resistance against aminoglycosides (Zhang *et al.*, 2011), β-lactams (Antunes *et al.*, 2006; Yang *et al.*, 2012) and antibiotics including, tetracyclines (Zhang *et al.*, 2009a) and vancomycin (Caplin *et al.*, 2008), enter WWTPs. The interaction of these bacteria with other bacteria already present in the WWTPs, may facilitate and promote the transfer of AR genes, as well the selection of

ARGs within the treatment plant through HGT (Kümmerer, 2004; Schluter *et al.*, 2007; Caplin *et al.*, 2008; Gullberg *et al.*, 2011; Zhang *et al.*, 2011). Therefore WWTPs can act as reservoirs for AR bacteria, ARGs and antibiotic residues and the release of their treated effluents can then influence the dissemination of them into natural aquatic environments.

Antibiotic residues can also be released directly into natural environments (Fick *et al.*, 2009; Li *et al.*, 2009; Li *et al.*, 2010), however in most cases antibiotic levels are normally below known human therapeutic minimum inhibitory concentrations (MICs). Despite these low and sub-inhibitory levels in aquatic systems, such as rivers, there is evidence that they can still select for resistance (Davies *et al.*, 2006; Knapp *et al.*, 2008; Gullberg *et al.*, 2011; Tello *et al.*, 2012). Furthermore, the dispersion rate, sorption to sediments, photolysis and biodegradation can reduce and influence the fate of antibiotics and the dissemination of AR bacteria and ARGs in natural aquatic environments. Despite this AR has been observed in many lakes and rivers, for example a study by Czekalski *et al.* (2012), detected increased levels of ARGs in Lake Geneva, Switzerland. Further Picao *et al.* (2008) also detected plasmid-mediated quinolone resistance genes in *Aeromonas* species isolated from Lake Lugano, Switzerland. Many studies have also reported and conducted studies on AR in rivers (Pei *et al.*, 2006; Li *et al.*, 2009; Li *et al.*, 2010; Ahammad *et al.*, 2014), for example Pei *et al.* (2006), demonstrated that there was a relationship between elevated levels of ARG in Poudre River in northern Colorado and associated human and agricultural activity, which gave further evidence that AR and ARGs are more frequently being detected in receiving waters.

In addition other compartments within these water systems, such as sediments, algae and biofilms may also have an impact on the harbouring and transfer of antibiotic residues and AR bacteria and-or pathogens. Studies have shown that sediments within receiving waters in environments impacted by human activity can harbour high levels of resistance markers, such as AR traits (Pruden *et al.*, 2006; Munoz-Aguayo *et al.*, 2007; Storteboom *et al.*, 2010; Czekalski *et al.*, 2012). Furthermore, ARGs can also be maintained in sediments, algae and biofilms if these environments are being continuously supplied with ARGs (Zhang *et al.*, 2009b). For example ESBLs isolates, including strains with *bla*-TEM, *bla*-SHV and *bla*-CTX-M genes have been detected in urban river sediments (Lu *et al.*, 2010) and water column samples (Machado *et al.*, 2009). Evidence also suggests that ARGs are more likely to be harboured in sediments rather than the water column (Auerbach *et al.*, 2007). Studies have also shown that seasonal factors could also influence the transmission of ARGs through natural systems (Pruden *et al.*, 2012). For example, it has been reported that there were differences in gene abundances present in sediments and lakes, based on seasonal variations (Knapp *et al.*, 2012).

In addition, the selection of ARGs can also occur without antibiotic selective pressure, for example, heavy metals or other chemicals can select for AR determinants and traits in natural environments (Foster, 1983; Alonso *et al.*, 2001; Stepanauskas *et al.*, 2006). This has been shown in waste treatment systems, where high Zn concentrations also resulted in observed high AR (Peltier *et al.*, 2010).

2.4   Mechanisms of antibiotic resistance (AR)

Bacteria can confer resistance to antibiotics due to intrinsic or acquired resistance. Intrinsic resistance is when a bacterial species is able to resist the action of antibiotics due to inherent structural or functional characteristics (Blair *et al.*, 2015) and does not depend on antibiotic selective pressure and HGT. Intrinsic resistance is normally due to the gram-negative outer membranes (OMs), the expression of multi drug resistance (MDR) efflux pumps (Nikaido, 1994) and more recently revealed additional chromosomally encoded elements (Fajardo *et al.*, 2008; Girgis *et al.*, 2009; Blake and O'Neill, 2013; Cox and Wright, 2013). As well as bacterial pathogens, environmental organisms also show intrinsic resistance traits to many classes of antibiotics. For example soil bacteria, naturally produce antibiotics independent of the selective pressure caused from the use of clinical antibiotics (D'Costa *et al.*, 2006; Bhullar *et al.*, 2012). These intrinsic resistant traits could be a threat to human health as these traits could be transferred to pathogenic bacteria, through the exchange of genetic information encoding ARGs (Forsberg *et al.*, 2012).

As mentioned above one mechanism of intrinsic resistance in gram-negative bacteria is the OM. The external environment and the cytoplasm of the bacteria is separated by a cytoplasmic membrane (CM). However bacteria also have other additional structures which serve as a permeability barrier to protect them from toxic substances. In gram-positive bacteria this peptidoglycan layer has a larger permeability threshold and can allow smaller molecules to penetrate through, allowing them to be intrinsically susceptible to various antibiotics (Randall *et al.*, 2013) . In contrast though gram-negative bacteria have a much finer molecular sieve, the OM, which surrounds the peptidoglycan layer and makes them intrinsically insusceptible to many antibiotics (Vaara, 1992) (Figure 2-3).
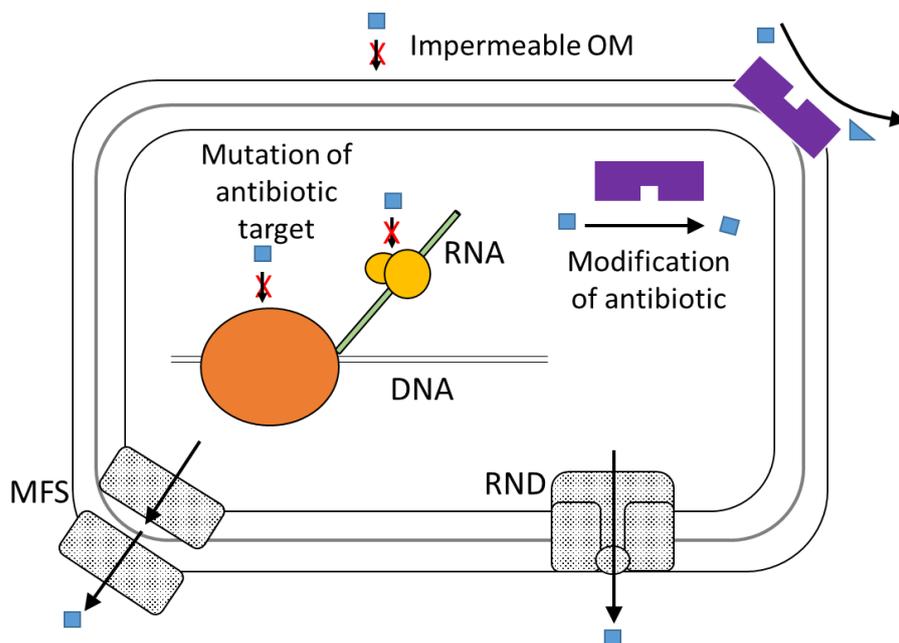
Figure 2-3 Mechanisms in gram-negative bacteria involved in AR (Allen et al., 2010).

Another contribution of intrinsic resistance is the presence of porins, which are present on the OM to allow the uptake of essential nutrients due to the membrane's impermeability (Ochs *et al.*, 1999; Ruiz *et al.*, 2003; Olesky *et al.*, 2006). However the OM, will slow down permeability but will not completely cause resistance to the antibiotic. Therefore the presence of additional resistance mechanisms, such as active efflux pumps as well as OM, means that they synergistically work together to produce intrinsic resistance (Cox and Wright, 2013).

Efflux pumps can be described as transport proteins that extrude toxic substances from bacterial cells into the external environment (Webber and Piddock, 2003). They are present in the chromosome of many environmental organisms (Van Bambeke *et al.*, 2000; Fajardo *et al.*, 2008) and may have originated from natural bacteria that export toxic molecules that have been produced by the host as a mechanism of survival for bacteria in natural environments (Piddock, 2006). Efflux pumps can export one particular molecular or, a broad range of molecules (Piddock, 2006). There are five different types of efflux proteins in the bacteria membrane that are associated with MDR (Saier, 1998; Webber and Piddock, 2003; Piddock, 2006) (Table 2-1). These are the Adenosine triphosphate (ATP) binding cassette (ABC) superfamily (Higgins *et al.*, 1982; Fath and Kolter, 1993), the major facilitator superfamily (MFS) (Henderson and Maiden, 1990; Griffith *et al.*, 1992; Marger and Saier, 1993; Goswitz and Brooker, 1995), the multi drug and toxic- compound extrusion (MATE) family (Morita *et al.*, 1998), the small multidrug resistance (SMR) family (Paulsen *et al.*, 1996) and the resistance-nodulation division family (RND) (Dinh *et al.*, 1994) (Table 2-1).

Table 2-1 Families of efflux pump proteins associated with MDR (Webber and Piddock, 2003; Piddock, 2006).

Efflux proteins	Energy source	Antibiotic and other substrates effluxed
ATP binding cassette (ABC) superfamily (Higgins *et al.*, 1982; Fath and Kolter, 1993)	ATP	Gram-positive : Numerous antibiotics Gram-negative : Macrolides
Major facilitator superfamily (MFS) (Henderson and Maiden, 1990; Griffith *et al.*, 1992; Marger and Saier, 1993; Goswitz and Brooker, 1995)	Proton motive forces*	Gram-positive: Acriflavine, benzalkonium, betrimide, centrimide, chlorohexidine, pentamidine Gram-negative: Nalidixic acid, novobiocin
Multi drug and toxic- compound extrusion (MATE) family (Morita *et al.*, 1998)	Proton motive forces*	Gram-positive: Aminoglycosides, fluroquinolones, cationic drugs
Small multidrug resistance (SMR) family (Paulsen *et al.*, 1996)	Proton motive forces*	Gram-positive: Acriflavine, benzalkonium, betrimide,
Resistance-nodulation division family (RND) (Dinh *et al.*, 1994; Saier Jr *et al.*, 1994)	Proton motive forces*	Gram-negative: Numerous antibiotics

*Proton motive force: an electrochemical gradient where the movement of hydrogen ions drives the substrate transport

In gram-negative bacteria it is the RND family of efflux pumps (Figure 2-3) that confers intrinsic AR (Dinh *et al.*, 1994) and some examples of clinically relevant bacteria that use the pumps are *P. aeruginosa* and *E. coli* (Table 2-1). The clinically relevant efflux pumps for gram-positive bacteria are MFS pumps, which are expressed in bacteria such as *Staphylococcus aureus* and *Streptococcus pneumonia* (Piddock, 2006) (Table 2-1). Acquired resistance occurs when bacteria have acquired genes, thorough HGT (Davies, 1997) or through the occurrence of mutations within genes located on the chromosomes of bacteria which can then be transmitted vertically when the bacteria replicates (Martinez and Baquero, 2000).

There are three ways in which bacteria can acquire resistance; the first is by minimising the concentrations of antibiotics in the cell due to poor penetration into the cell or antibiotic efflux. As discussed above the membranes of gram-negative bacteria are intrinsically less permeable to many antibiotics due to the OM porin proteins that form an extra permeability barrier (Vaara, 1992). Therefore antibiotic entry is reduced by down regulating porin expression or replacing the porins with more selective channels (Ochs *et al.*, 1999; Ruiz *et al.*, 2003; Olesky *et al.*, 2006). However selective pressure exerted by antibiotics could favour the emergence of mutations in porin genes, and also in genes that regulate the production of porins (Lavigne *et al.*, 2013; Tangden *et al.*, 2013). This has been seen in carbapenems and cephalosporins, where resistance is normally due to the presence of degrading enzymes (Tamber and Hancock, 2003; Baroud *et al.*, 2013; Lavigne *et al.*, 2013). In addition the overexpression of efflux pumps can increase resistance levels to certain antibiotics. This overexpression is often due to the mutation of regulatory genes that express efflux pumps (Blair *et al.*, 2015).

The second mechanism consists of modifying the antibiotic target by genetic mutation or post translational modification of the target (Figure 2-3). Normally an antibiotic binds tightly to its target protein or site, preventing the normal activity of the target. However a single point mutation in the gene that encodes an antibiotic target, can confer resistance, where the antibiotic is not efficiently bound to the target, allowing the target to still carry out its normal activity (Blair *et al.*, 2015). Further, transformation, which is the uptake of deoxyribonucleic acid (DNA) from the environment can confer AR, when the target protein is modified through the formation of 'mosaic genes' that have formed by the uptake of DNA from closely related environmental species. An example of this is penicillin resistance in *S. pneumonia*, where resistance is conferred due to PBPs which have arisen from closely related species known as *Streptococcus mitis* (Blair et al., 2015*)*. In addition the modification of a target, by the

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addition of a chemical group can also confer resistance. The addition of a chemical group prevents antibiotic binding but does not change the protein sequence of the target (Blair *et al.*, 2015).

The third mechanism is the inactivation or destruction of the antibiotic by hydrolysis or modification, therefore causing bacteria to resist the action (Figure 2-3). This use of enzymatic activity to destroy antibiotics was first seen with the discovery of penicillinases (which are β-lactamases), that conferred resistance to penicillin through hydrolysis (Abraham and Chain, 1988). Since then many more enzymes have been identified, which can degrade and modify antibiotics from a range of classes. There are also sub-classes of these enzymes which act on different antibiotics within the same class, for example, different β-lactamases can degrade β-lactam antibiotics such as penicillins and cephalosporins. The large increase in ESBLs, which hydrolyse cephalosporins, since the 2000s has meant that more carbapenem antibiotics are now being used. However this has also led to the emergence of carbapenemases, β-lactamases which specifically hydrolyse carbapenems and these are mostly plasmid mediated (Queenan and Bush, 2007; Queenan *et al.*, 2010; Abenavoli and Almasio, 2011).

2.5   Carbapenems and resistance

The increase in the presence of ESBLs, making third generation cephalosporins and quinolones less effective, has led to more carbapenem antibiotics, such as meropenem being used (Bradley *et al.*, 1999; Papp-Wallace *et al.*, 2011). Carbapenems are broad spectrum β-lactam antibiotics, active against both gram-positive and gram-negative bacteria. More importantly they are still active against gram-negative bacteria with ESBLs and AmpC enzymes (Livermore, 1995). Carbapenems act by binding to PBPs, inhibiting the growth of the cell wall and eventually causing the cell wall to lyse, resulting in the bacterium death (Papp-Wallace *et al.*, 2011). Antibiotics such as imipenem and doripenem are more effective against gram-positive bacteria (Rodloff *et al.*, 2006; Bassetti *et al.*, 2009; Queenan *et al.*, 2010), while meropenem and ertapenem are slightly more potent against gram-negative organisms (Nix *et al.*, 2004; Bassetti *et al.*, 2009). However some of these antibiotics are more effective than others depending on the specific bacteria, for example meropenem is not as effective as imipenem or doripenem against *Acinetobacter baumannii* (Oliver *et al.*, 2004). In addition some of these carbapenems can be combined with other antibiotic classes to treat serious infections that are becoming harder to treat due to the emergence of MDR pathogens (Sheikh *et al.*, 1993; Balke *et al.*, 2006; Drusano *et al.*, 2009).

Resistance to carbapenems can either be chromosomal or acquired. In gram-negative bacteria, carbapenem resistance is conferred by the production of carbapenemases or a combination of structural mutations and other β-lactamase enzymes (Logan, 2012). Carbapenemases which hydrolyse carbapenems are also able to break down other β-lactam antibiotics, such as penicillins and cephalosporins. In addition other enzymes such as ESBLs and AmpC cephalosporins are able to resist carbapenems due to the alteration or loss of porins that are present on the OM of gram-negative bacteria (Charrel *et al.*, 1996). As mentioned earlier carbapenemases belong to several classes, A, B and D (Papp-Wallace *et al.*, 2011). Class B carbapenemases, known as MBLs have a Zn ion in their active site. They are also the only carbapenem class where carbapenemases are prevalent (Papp-Wallace *et al.*, 2011). These carbapenemases are dependent on the Zn ion for catalytic activity (Bush *et al.*, 1995).

Acquired carbapenemases can also be found in classes A and D, where serine is required at the active site. An example of a class A carbapenemases are *K. pneumonia carbapenemases* (KPC), which can be carried with other β-lactams on mobile plasmids or transposons (Yigit *et al.*, 2001). An example of a class D enzyme, is OXA, which are commonly seen in *P. aeruginosa* and *Actinobacter* species and have oxacillin-hydrolysing abilities (Queenan and Bush, 2007; Patel and Bonomo, 2011). The first described acquired class B MBLs, which were plasmid mediated IMP-type carbapenemases were found in 1991 in Japan and are still present (Walsh, 2010). At present in Europe, Verona integron encoded metallo-β-lactamase (VIM) enzymes are increasing and now spreading globally causing resistance to primarily *P. aeruginosa* and now also Enterobacteriaceae (Psichogiou *et al.*, 2008; Walsh, 2010; Canton *et al.*, 2012). Another MBL, the New Delhi metallo-β-lactamase (*bla-*NDM-1) arose in India in 2008 and is emerging over the India sub-continent (Kumarasamy *et al.*, 2010; Poirel *et al.*, 2010; Walsh *et al.*, 2011; Ahammad *et al.*, 2014). Since the first appearance of *bla-*NDM-1 in the Indian subcontinent, NDM-producing bacteria have been reported worldwide (Dortet *et al.*, 2014). NDM-producing *Acinetobacter* have also been recovered from environmental samples in China (Zhang *et al.*, 2013) and eight other NDM variants have since been described and published (NDM-1 to -8) (Dortet *et al.*, 2014).

2.6   Meropenem

Meropenem is a broad spectrum, β-lactam injectable carbapenem antibiotic, active against both gram-positive and gram-negative bacteria and has been in clinical use since 1994 (Turner *et al.*, 1999). It exerts its action by interfering with bacterial cell wall synthesis and causing bacterial cell lysis by binding to PBPs (Zhanel *et al.*, 1998). It is active against a broad spectrum of bacteria due to its high level stability to serine β-lactamases, its similarity to

PBPs and its ease in penetrating bacteria cell walls (MHRA, 2010). In addition meropenem produces time-dependent bactericidal activity, therefore concentrations must be kept above the minimum inhibitory concentration (MIC) for a suitable percentage of time (Kollef, 2008; Nicolau, 2008). Its main excretion route after treatment is via urine. The total use of carbapenem antibiotics in England, UK statistically increased by 31.3% between 2010 and 2013, with meropenem accounting for 89.0% of use (Public Health England, 2014). Figure 2-4 shows the consumption of different carbapenems in England from 2010 to 2013.
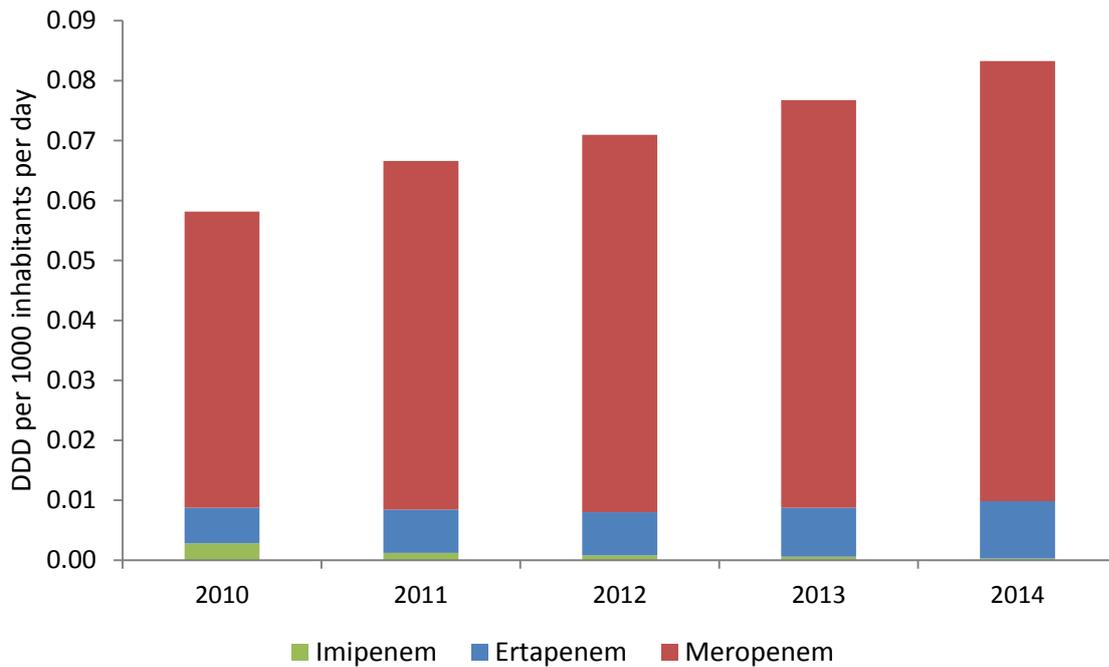
Figure 2-4 The consumption of different carbapenems in England from 2010 to 2014, expressed as defined daily dose (DDD) per 1000 inhabitants per day (Public Health England, 2014).

In the UK meropenem is found under the trademark Merrem/Meronem IV, and is developed jointly by AstraZeneca and Sumitomo Pharmaceuticals Co. and marketed by AstraZeneca (AstraZenenca, 2000). It is available as 500 mg or 1.00 g powder for solution, and administered by injection or infusion (Table 2-2).

Table 2-2 Chemical and molecular properties and structure of Merrem® I.V. (MHRA, 2014)

Name of product	Active substance	Chemical name	Molecular formula	Molecular Mass	Chemical structure
Merrem® I.V. 500 mg/ 1 g Powder for Solution for Injection or Infusion	Meropenem trihydrate	(4R,5S,6S)-3-[[(3S,5S)-5-(Dimethylcarbamoyl)-3-pyrrolidinyl](Smith *et al.*, 1998)thio]-6-[(1R)-1hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid trihydrate	$C_{17}H_{25}N_3O_5S \cdot 3H_2O$	437.52 (trihydrate)	
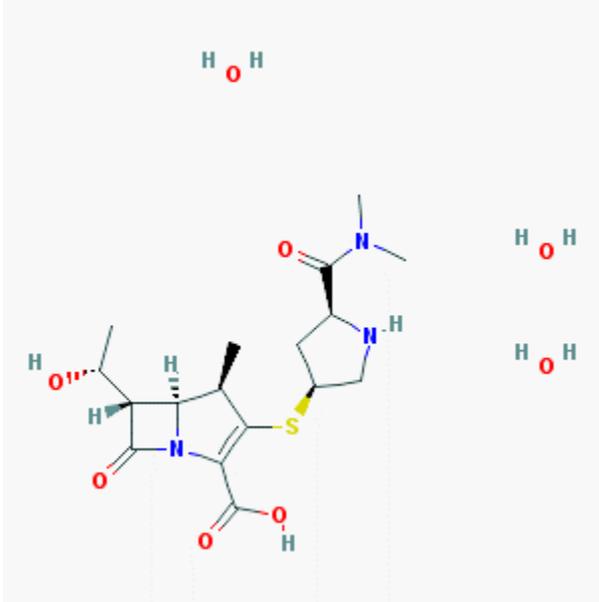
Meropenem has a similar spectrum of activity to imipenem and cilastatin, with meropenem having more of an effect on *P. aeruginosa* and Enterobacteriaceae. Meropenem is also active against *Pseudomonas* and *Acinetobacter*, methicillin susceptible *staphylococci*, *Enterococcus faecalis*, *streptococci* and anaerobes including *Bacteroides* species (Hawkey and Livermore, 2012). It is generally used to treat nosocomial pneumonia, broncho-pulmonary infections in cystic fibrosis, complicated skin and soft tissue infection, complicated intra-abdominal infections, complicated urinary tract infections and acute bacterial meningitis (Hawkey and Livermore, 2012; MHRA, 2014). Eco-toxicity tests revealed that the predicted environmental concentration (PEC) of meropenem was 8.8×10^{-2} µg/L and the predicted no effect concentration (PNEC) was 1.5 µg/L, giving a PEC/PNEC of 5.9×10^{-2} (AstraZenenca, 2010). Therefore meropenem does not present a significant risk to the environment, is not readily biodegradable and is not predicted to bio-accumulate in aquatic organisms.

2.7   Heavy metal accumulation and resistance mechanisms

Not all heavy metals are biologically important to living organisms, and those that are, can be differentiated by how soluble they are in seawater, a measurement which can be assumed to be similar to the average environment (Weast, 1984). Most divalent cations, that are known as trace metals include metals which are found at a seawater concentration of 100nM and 1µm, such as iron (Fe) and Zn, metals such as nickel (Ni), copper (Cu) and arsenic (As) which are present with concentrations between 10nM and 100 nM, rare elements including metals such as cobalt (Co) and silver (Ag) and elements with concentrations just below 1nM such as cadmium (Cd), chromium (Cr) and lead (Pb) (Weast, 1984). Of these Zn, Ni, Cu, Co are toxic at high concentrations however they are all essential to living organisms at low concentrations, while metals such as Cd and As are not as important at a functional level but are still considered as toxins (Mergeay *et al.*, 1985). Bacteria, like all living organisms can adapt to metal concentrations by using avoidance and sequestration strategies to protect themselves.

There are a variety of efflux pumps which are involved with heavy metal transport and resistance (Table 2-3). Heavy metal ions enter bacterial cells though two main uptake systems. The first one, the metal inorganic transport (MIT) pump, is a fast and unspecific system, which is driven by a chemiosmotic gradient across the cell's cytoplasmic membrane, and brings metal ions into the cell even when they are already in excess (Table 2-3).

Table 2-3 Families of efflux pump proteins associated with heavy metal transport and resistance (Nies, 1999).

Efflux proteins	Type of transport	Energy source	Metal ions
ATP binding cassette (ABC) superfamily (Higgins *et al.*, 1982; Fath and Kolter, 1993)	Uptake	ATP	$Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}$
	Efflux	ATP	?
P-type ATPpases (Fagan and Saier, 1994)	Both	ATP	$Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}$
A-type (Saier, 1994)	Efflux	ATP	Arsenite
Resistance-nodulation division family (RND) (Dinh *et al.*, 1994; Saier Jr *et al.*, 1994; Saier, 1994)	Efflux	Proton motive forces	$Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Ag^{+}$
HoxN (Eitinger and Friedrich, 1991)	Uptake	Chemiosmotic	Co^{2+}, Ni^{2+}
Chromate (CHR) transport (Nies *et al.*, 1998)	Antiport	Chemiosmotic	Chromate
Metal inorganic transport (MIT) (Paulsen *et al.*, 1998)	Uptake	Chemiosmotic	Most cations
Cation- diffusion facilitators (CDF) (Nies and Silver, 1995; Paulsen and Saier, 1997)	Efflux	Chemiosmotic	$Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}$

*Proton motive force: an electrochemical gradient where the movement of hydrogen ions drives the substrate transport

The second uptake system is slower, substrate specific and uses ATP hydrolysis as the energy source (Table 2-3). This system is only used in times of need or starvation by the cell and so is inducible (Nies and Silver, 1995). When a cell is present in an environment with a high concentration of heavy metals, ions such as Zn^{2+}, as well as Ni^{2+}, Co^{2+} and manganese (Mn^{2+}), are accumulated in gram-negative bacteria firstly by fast and unspecific CorA uptake systems. CorA, a chemiosmotic membrane-integral protein, belongs to the family of efflux pumps involved in metal inorganic transport (MIT) (Paulsen *et al.*, 1998) (Table 2-3).

The more specific and slower uptake system uses inducible ATP-binding cassette (ABC) transporters for transporting Zn^{2+}, Ni^{2+} and Mn^{2+} (Fath and Kolter, 1993) and slow and specific chemiosmotic HoxN transporters for Ni^{2+} and Co^{2+} (Eitinger and Friedrich, 1991) (Table 2-3). Once the metals are in the cells, they can bind to sulphide groups or interact with other metal ions, for example Cd^{2+} with Zn^{2+}, which could inhibit the function of the respective cations. Therefore this potential for heavy metal toxicity, means that bacteria use a variety of mechanisms including metal ion homeostasis factors and MR determinants to protect themselves from toxicity (Nies, 1999). As heavy metal ions cannot be degraded or modified like other toxic compounds the following mechanisms are used in bacteria, as well as other microorganisms to gain resistance: (i) a permeability barrier to exclude metals (Gowri and Srivastava, 1996), (ii) active export of metals from the cell (Nies, 1992a; Choudhury and Srivastava, 2001a) (Nies and Silver, 1995), (iii) binding proteins or other ligands by intracellular sequestration to prevent damage to the metal sensitive cellular targets (Gilotra and Srivastava, 1997), (iv) extracellular sequestration (Diels *et al.*, 1995; Saxena and Srivastava, 1998; Nies, 2000) and, (v) transformation and detoxification (Ji and Silver, 1995; Srivastava *et al.*, 1998; Srivastava *et al.*, 1999).

2.8   Zn resistance mechanisms

Zn is a trace metal essential to all living organisms at low concentrations (Ji and Silver, 1995; Srivastava *et al.*, 1998; Srivastava *et al.*, 1999) and it is important for development and growth of cells (MacDonald, 2000). In addition Zn is also needed for basic cellular functions such as DNA replication, transcription, cell division and cell activation and is also a constituent of DNA binding proteins (Chou *et al.*, 1998). High concentrations of Zn are toxic to living organisms and so survival is only possible due to cooperation between resistant mechanisms and the cell metabolism, to maintain the required amount of Zn in cells and also manage the excess not needed.

Zn is accumulated in cells by unspecific magnesium transport systems, including the CorA MIT transporter present in many bacteria and archaea (Smith and Maguire, 1995) or the MgtE system (Smith *et al.*, 1995) (Figure 2-5).
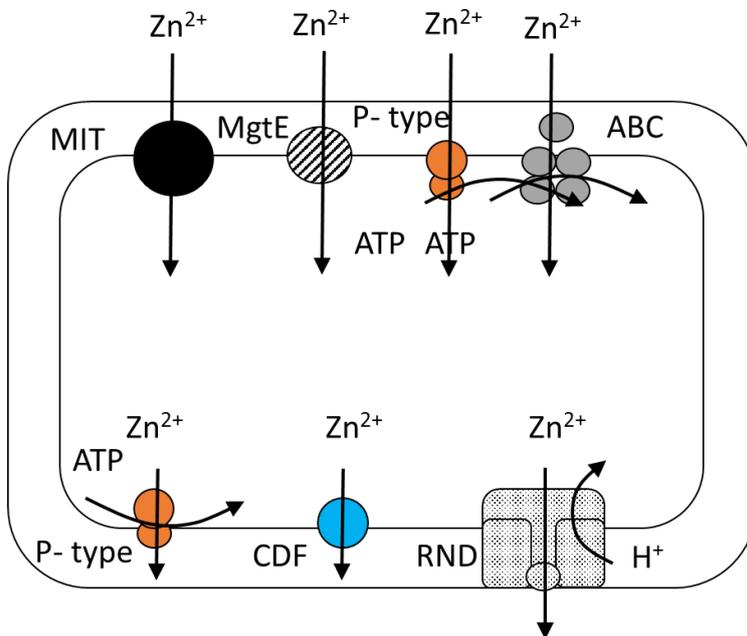
Figure 2-5. Protein families that are involved in Zn uptake and efflux by bacteria (Nies, 1999).

Slow and specific ABC transporters which are inducible also supply Zn when the cell requires it (Dintilhac *et al.*, 1997; Lu *et al.*, 1997) (Figure 2-5). Bacteria can become Zn resistant due to efflux pumps such as p-type ATPase or proton antiporters (Nies, 1992a; Fagan and Saier, 1994; Srivastava *et al.*, 1999; Choudhury and Srivastava, 2001a), sequestration by Zn binding proteins, such as metallothioneins (MTs) (Bhagat and Srivastava, 1993; Morby *et al.*, 1993; Robinson *et al.*, 1998) or extracellular accumulation (Bhagat and Srivastava, 1993) (Figure 2-5).

Zn is effluxed out of cells by two systems, P-type efflux ATPases and RND driven transporters (Saier Jr *et al.*, 1994; Saier, 1994) (Figure 2-5). For example according to Beard *et al.* (1997) and Rensing *et al.* (1997a) the *zntA* p-type ATPases are responsible for efflux of Zn out of *E. coli* and the *ziaA* p-type ATPases are responsible for efflux of Zn out of cyanobacterium, *Synechocystis* (Thelwell *et al.*, 1998). These transport systems efflux Zn across the cytoplasmic membrane of bacteria, while RND transport, such as the cobalt/zinc/cadmium (*czc*) system can transport Zn across the cell wall of gram-negative bacteria, including the OM (Paulsen and Saier, 1997; Nies, 1999). The *czc* transport system has been identified as one of the best studied Zn resistant mechanisms in *Ralstonia eutropha,* a gram-negative soil bacterium (Mergeay *et al.*, 1985; Nies *et al.*, 1987) and confers

resistance to Cd, Zn and Co. It work as a cation/proton antiporter and consists of three structural genes including *czcC*, *czcB* and *czcA* (*czcABC*) (Nies and Silver, 1989; Nies, 1992a; Nies, 1995). All together these genes can transport Co, Zn and Cd across cytoplasmic membrane, periplasm and OM (Rensing *et al.*, 1997b) using proton motive force (Nies and Silver, 1995). Another gene cluster called *czr*, which confers resistance to Cd and Zn was also identified in *P. aeruginosa*, encoded by *czcCBA* genes which were very similar to those proteins in *R. eutrophus*, giving further evidence that the cation antiporter efflux system is involved in Zn and Cd resistance (Hassan *et al.*, 1999).

Bacteria continuously regulate the expression of Zn resistant genes so that they control the amount of Zn present in their cells, in order to have sufficient amounts for growth but to also avoid accumulating toxic levels. They do this by increasing the expression of resistant genes that export metals present in excess as well as down-regulating these genes when the concentration of metals is low, to prevent the starvation of Zn from cells (Jones *et al.*, 1997). Examples of some Zn specific proteins are *smtB*, *zntR*, *ziaR*, *zur*. In addition the *czc* gene cluster, consists of regulatory genes which are located in regions upstream and downstream of the *czcCBA* structural genes. These regulator regions contain the genes *czcD*, *czcR* and *czcS* with the latter two forming the two component regulatory system (van der Lelie *et al.*, 1997; Robinson *et al.*, 1998). *CzcR* and *czcD* may also form two component regulatory systems (Nies, 1992b; Nies and Silver, 1995) where *czcD*, part of the cation diffusion facilitator (CDF) family contains Zn transporters (Nies and Silver, 1995; Paulsen and Saier, 1997; Anton *et al.*, 1999).

Bacteria also use specific Zn binding proteins, such as MTs (Lazo *et al.*, 1998) and *znu* proteins (Patzer and Hantke, 1998) to regulate the passage of metal ions into and out of their cells. MT is a homeostatic stress induced protein that aims to protect bacterial cells from harmful components (Lazo *et al.*, 1998) and they can bind to a variety of metals including Cd, Zn, Cu and Ag (Brady, 1982). *Znu* proteins belong to the ABC protein family and are involved in the periplasmic transport of Zn in *E. coli* (Patzer and Hantke, 1998). Lastly cells also gain resistance to Zn due to extracellular accumulation, where studies showed that *P. stutzeri* RS34, which was isolated from industrially polluted soils, resisted Zn by accumulating it on its OM (Bhagat and Srivastava, 1993).

2.9   The link: antibiotic and metal resistance

Many studies have suggested that metal contamination in natural environments may be associated with the maintenance and proliferation of AR (Summers *et al.*, 1993; Alonso *et al.*, 2001). Mechanisms of AR can be similar in structure and function to mechanisms by which bacteria gain resistance to metals, such as Zn (Baker-Austin *et al.*, 2006). For example bacteria can gain resistance to both β-lactams and Zn by reducing the permeability of their membranes (Silver and Phung, 1996; Ruiz *et al.*, 2003), using efflux mechanisms (Levy, 2002; Nies, 2003) and modifying cellular targets (Barkay *et al.*, 2003; Roberts, 2005). Metal contamination can co-select for AR using a variety of mechanisms, including, co-resistance, cross resistance (Chapman, 2003), and co-regulation (Nakajima *et al.*, 1995; Baranova and Nikaido, 2002; Lee *et al.*, 2005).

Co-resistance occurs when metal resistance genes (MRGs) and ARGs are located on the same genetic elements such as on plasmids, transposons or integrons (Chapman, 2003). Multiple ARG cassettes are assembled within integrons (Davis *et al.*, 2005), which can exist within transposons, which means that the transfer of transposons will lead to the transfer of ARG cassettes within integrons (Wireman *et al.*, 1997; Bass *et al.*, 1999; Liebert *et al.*, 1999). Studies using transformation, plasmid curing and plasmid sequencing have reported this known physical linkage between ARGs and MRGs (Foster, 1983). Further, studies done by Summers *et al.* (1993) demonstrated a genetic link between mercury (Hg) and AR. Another study by Parkhill *et al.* (2001) revealed the presence of a plasmid (pHCM1) in *salmonella enterica* serovar Typhi CT18 that is resistant to the antibiotics, trimethoprim, sulphonamide, chloramphenicol, ampicillin and streptomycin and the metal, Hg (Figure 2-6).

Cross resistance occurs when the same target, pathway or common route of access into the cell, such as efflux pumps, are used by different compounds (Chapman, 2003). Therefore resistance to an antibiotic is accompanied by resistance to another compound, such as a metal (Chapman, 2003). Examples include MDR pumps in *Listeria monocytogenes* which can export both metal and antibiotics (Mata *et al.*, 2000), as well *tetL* proteins which can transfer tetracycline and Co (Cheng *et al.*, 1996) (Figure 2-6).
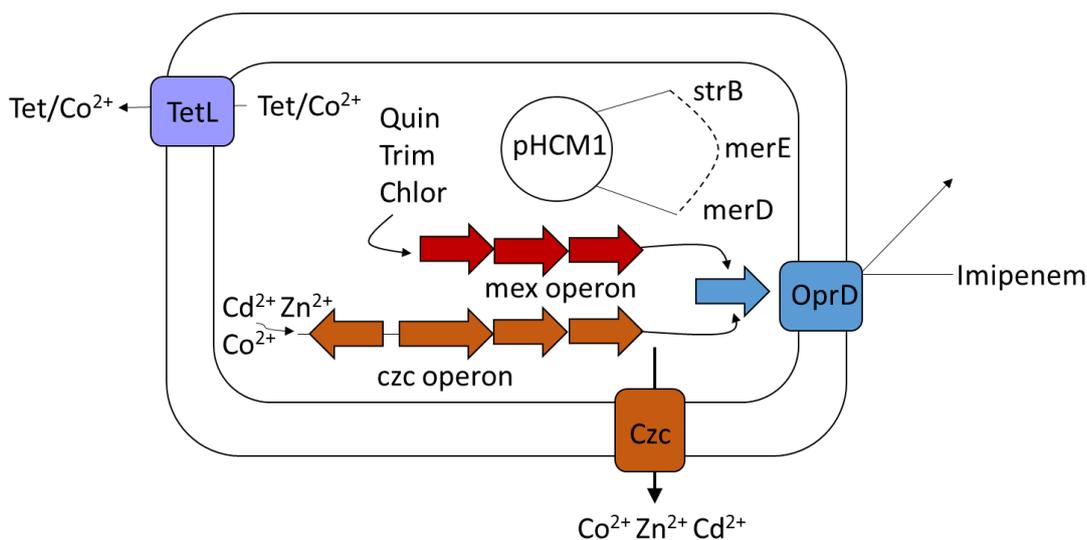
Chlor: chloramphenicol; pHCM1: Salmonella typhi CT18 resistance plasmid; Quin: quinolone; strB: gene encoding streptomycin-modifying enzyme; Tet: tetracycline; TetL: tetracycline efflux protein; Trim: trimethoprim.

Figure 2-6 Molecular mechanisms that are involved in metal and antibiotic co-selection in bacteria (Cheng et al., 1996; Perron et al., 2004; Lee et al., 2005; Baker-Austin et al., 2006)

Co-regulation occurs, when exposure to one toxic compound, can result in resistance to another compound, due to transcriptionally linked regulatory systems (Nakajima *et al.*, 1995; Baranova and Nikaido, 2002; Lee *et al.*, 2005). For example studies done by Lee *et al.* (2005) observed that excess Zn caused a mdtABC operon, which is a RND type efflux system to upregulate, conferring resistance to certain antibiotics. Another study done by Perron *et al.* (2004) also revealed that strains exposed to Zn were also resistant to other heavy metals and imipenem, a carbapenem antibiotic, due to the co-regulation of imipenem influx by Mex proteins and heavy metal efflux by *czc* proteins (Figure 2-6).

2.10   Zn and meropenem resistance: The Tyne basin and experimental bioreactors

Many studies have shown that the presence of heavy metals with and without the presence of antibiotic concentrations can exert a selective pressure on bacteria within natural environments, such as rivers, lakes, as well as in wastewater treatment systems and their effluents, which could contribute to the spread of AR (Summers *et al.*, 1993; Alonso *et al.*, 2001; Baker-Austin *et al.*, 2006; Jain *et al.*, 2009). Further, environments contaminated with high levels of metals such as Zn, Cr and Pb, can also contain high numbers of strains resistant to antibiotics (Rasmussen and Sorensen, 1998). Historic mining areas, such as the South Tyne catchment of the River Tyne basin, have high concentrations of heavy metals, especially Zn and Pb in their waters and underlying sediments (Macklin *et al.*, 1994; Macklin *et al.*, 1997). These high concentrations of heavy metals, can therefore maintain a selective pressure on bacterial communities for long periods of time, as they are not easily degraded, which can

also promote the co-selection of AR determinants due to a variety of mechanisms (Nakajima *et al.*, 1995; Baranova and Nikaido, 2002; Chapman, 2003; Lee *et al.*, 2005) described in section 2.9. Further, previous research has shown that there are relationships between heavy metals and AR bacteria and ARG abundances in natural environments, such as rivers and soils (Jain *et al.*, 2009; Berg *et al.*, 2010; Knapp *et al.*, 2011).

The study of highly metal contaminated natural aquatic environments, such as the Tyne basin can provide information on how the levels of contamination affect AR. This thesis, focuses predominately on the relationship between Zn levels and culturable Zn, meropenem and combined resistance. This relationship was firstly assessed in river water and sediments of sites, within two comparative catchments of the Tyne basin, the North and South Tyne, chosen due to their different baseline Zn concentrations, which to date has not been conducted and assessed (Chapter 3). Batch reactors were also used to further determine the effect of Zn on bacterial communities from sediments within the two basins, by assessing whether increased Zn amendments induced culturable meropenem resistance over time (Chapter 4). Previous experimental studies where co-selection was induced showed that there were relationships between increased metal amendments and increased AR (Berg *et al.*, 2010), however this has not been assessed using communities from sediments within the Tyne basin.

The interaction of bacteria from raw untreated sewage with bacteria in WWTPs could select for ARGs, as well as promote the transfer of these genes to other environmental bacteria (Kümmerer, 2004; Schluter *et al.*, 2007; Caplin *et al.*, 2008; Gullberg *et al.*, 2011; Zhang *et al.*, 2011), as described in section 2.3. Therefore wastewater systems and also their effluents could harbour potential dangerous and life threatening AR bacteria and ARGs. Zn levels may also be apparent in WWTPs, due to their use in industrial and manufacturing processes (Charif *et al.*, 2012). As concentrations of antibiotics and metals can both exert a selective pressure on bacteria within an ecosystem, the movement of ARGs among environmental bacteria could be promoted with such compounds (Alonso *et al.*, 2001; Stepanauskas *et al.*, 2005; Stepanauskas *et al.*, 2006; Wright *et al.*, 2006; Segura *et al.*, 2009). Therefore by using experimental bioreactors to treat wastewater, mimicking trickling filter beds, and amended with varying concentrations of Zn and meropenem (Chapter 5), the impact of these compounds on culturable organisms can be assessed and important information can be gained on whether meropenem resistance in cultured environmental strains can be selected for when organisms are under metal and-or antibiotic stress.

Chapter 3 Relationships between meropenem and metal resistance in the North and South basins of the River Tyne

3.1   Introduction

The emergence of AR bacteria is threatening and challenging the effectiveness of antibiotics, with ARGs present now in both natural and clinical settings (Baker-Austin *et al.*, 2006; Knapp *et al.*, 2011). There are many reasons why ARGs may be present in the environment. Antibiotics are believed to be prudently used for the treatment of infections in humans, however they are also extensively used in agricultural and veterinary applications, including for growth promotion, treatment of disease and improving feed efficiency (Sarmah *et al.*, 2006). Therefore, the use or misuse of antibiotics is widespread, and can select for and modify *in situ* AR in soil and aquatic environments.

Due to selective pressure in microbial communities from the presence of antibiotic concentrations, ARGs acquired by HGT or from mutations in pre-existing genes are being disseminated to other bacteria, sometimes pathogens (Alonso *et al.*, 2001; Boerlin and Reid-Smith, 2008), promoting the evolution and spread of resistance genes across nature (Thiele-Bruhn, 2003; Segura *et al.*, 2009). However, some ARGs originated from antibiotic producing organisms that are found in the environment and these are sometimes found in the same gene clusters as antibiotic biosynthesis pathway genes (Benveniste and Davies, 1973; Webb and Davies, 1993). In addition, the selection of ARGs can also occur without antibiotic selective pressure, for example, heavy metals or other chemicals can select for AR determinants and traits in natural environments (Foster, 1983; Alonso *et al.*, 2001; Stepanauskas *et al.*, 2006). Furthermore, such determinants may provide an ecological advantage to the bacteria for colonisation and survival (Alonso *et al.*, 2001). Therefore, it is clear that the environment is an important reservoir for ARGs and that it plays an important role in the selection of the ARGs, with or without the presence of antibiotics.

Metal contamination can provide a widespread and unmanageable selection pressure to both environmental and clinical bacteria that could potentially contribute to the spread of AR (Baker-Austin *et al.*, 2006). As heavy metals cannot be degraded and remain in the environment, they can maintain a selective pressure on bacterial communities for long periods, often due to co-presence of heavy MRGs (Stepanauskas *et al.*, 2006). Research has shown that heavy metal contaminated environments, such as those contaminated with Zn, Cr and Pb, can contain high numbers of AR strains and also higher plasmid abundances (Rasmussen and Sorenson, 1998). As MR and AR are often liked to similar plasmids,

increased mobilisation of genes under metal stress could also increase the mobilisation of ARGs among bacteria populations (Alonso *et al.*, 2001). Many reports have indicated that heavy metal contamination in natural environments can promote the development and proliferation of AR (Alonso *et al.*, 2001; Summers, 2002; Jain *et al.*, 2009). For example evidence from waste treatment systems show strong links between Zn and ambient AR levels where AR was higher when Zn levels were also high (Peltier *et al.*, 2010). Furthermore studies have been conducted where metal levels significantly correlated with specific ARGs in soils, including Zn (Knapp *et al.*, 2011).

This study aimed to assess the extent of meropenem and Zn resistance in sediments and water of two watersheds with very different baseline metal conditions; the North Tyne, which has very low metals and is comparatively pristine, and the South Tyne, which has very high heavy levels due to historic mining. The objectives were as follows:

a) To quantify total, soluble and colloidal heavy metal levels in the North and South Tyne.

b) Determine whether there is a difference in metal levels between the pore water within sediments and associated river water.

c) To determine whether there are differences in meropenem, Zn, and combined meropenem and Zn resistance (combined resistance) in microbial communities from the North and South Tyne.

d) To assess whether and how local Zn levels influence meropenem, Zn, and combined resistance in microbial communities within associated river sediments.

3.1.1 *The Tyne basin*

The Tyne catchment, located in the North East (NE) of the UK, covers an area of 2,933 kilometres square (km^2) that includes two geologically distinct sub-catchments, the North and South River Tyne catchments (Macklin *et al.*, 1994; EA, 2008). The Tyne basin is predominately carboniferous sandstone, limestone and shale (Klingeman *et al.*, 1998), some areas of which are rich in heavy metals. The North arm includes the Kielder Reservoir, which is a man-made lake built in the 1960s to support industrial growth of NE England (Archer, 2008; Water, 2010). In contrast, the South Tyne and its headwater tributaries rise from Cumbria, which lies in the North Pennines (Water, 2010). This area, now an 'Area of Outstanding Natural Beauty' due to its extensive elevated moorlands, once consisted of massive ore fields and was a highly productive Pb and Zn mining area in the 19th and early 20th centuries (Macklin *et al.*, 1994; Water, 2010). The South Tyne has a number of tributaries including the River Allen and River Nent (Water, 2010). The confluence of the North and

South Tyne river is just upstream of Hexham, to form the River Tyne, which continues towards Tynemouth and the North Sea (Water, 2010).

3.1.2 *The North Tyne basin*

The North Tyne catchment's flow is regulated from regular releases from Kielder Water reservoir, which is the largest artificial lake in Britain, covering an area of more than 10 km^2 with a catchment area of 240 km^2 and a capacity of nearly 200 million cubic meters (m^3) (Archer, 2008; Water, 2010). The North Tyne catchment has no history of mining or industry, and has very low or nearly absent Zn and Pb levels (Macklin *et al.*, 1994). The geology of the catchment consist of lower limestone and scremerston Group (EA, 2008). The operational catchment extends from just south of Bellingham, where the River Rede joins the North Tyne. Tributaries include Warks Burn, Crook Burn, Gunnerton Burn, Erring Burn and Swin Burn.

3.1.3 *The South Tyne basin*

Throughout the 19th century, areas of the South Tyne basin, more precisely Sellingstones, East Allen, West Allen and River Nent, were the most productive Zn and Pb mining areas of Britain (Macklin *et al.*, 1994; Macklin *et al.*, 1997). The area also produced substantial quantities of non-metallic minerals, such as fluorspar, witherite and barytes (Macklin *et al.*, 1997). Smelting, the dumping of mining residuals on land, and the release of fine-grained mining wastes into local streams was prominent, until legislation came into force in the latter part of the 19th century (Macklin *et al.*, 1994; Macklin *et al.*, 1997). These activities resulted in the South Tyne valleys becoming heavily polluted.

The Northern Pennine Orefield valleys were rich in mineral resources, which was as a result of mineralisation and other physical and chemical properties that are thought to have taken place at around 270 million years ago (Nuttall and Younger, 2002). Moreover, the development of sewage collection systems throughout the early years of the 'Industrial Revolution', also added to the pollution, locally discharging into the river, resulting in the river becoming comparatively toxic to aquatic organisms (Trust, 2012). Sedimentology profiles researched by Macklin (1994), showed that sediments laid down in the West Allen and River Nent had 100 times higher metal concentrations than, sediments, which had been deposited from the late Roman period, before any large-scale metal extraction began (Macklin *et al.*, 1994). Furthermore, metal concentrations in overbank sediments deposited in the late 19th century, during the mining period increased up-profile compared to concentrations that were taken in sediments after the 1900s, where they declined up-profile, which gives further evidence of the intensity of mining operations in the Tyne catchment (Macklin *et al.*, 1994).

The geology of the South Tyne catchment consists of Millstone Grit, Upper Limestone and Middle Limestone (EA, 2008).

3.1.4 *Contrasting in situ AR between the North and South Tyne Basins*

The Tyne basin includes two geologically distinct sub-catchments, the North Tyne, which is relatively pristine and contains very low concentrations of heavy metals and the South Tyne, which has very high levels of metals, due to its historic industrial and mining activities in the 19th and early 20th centuries (Macklin *et al.*, 1994; Water, 2010). Therefore high concentration of metals, especially Zn and Pb are present in the sediments of the South Tyne as well as its water. The North and South Tyne basins are ideal for examining how metals influence natural MR and AR in the environment due to the nearly absent metal concentrations and, high concentrations of heavy metals, such as Zn and Pb, incorporated into the sediments as well as the associated river water.

3.2 Materials and Methods

3.2.1 *Sampling sites*

Monthly samples were collected from five sites in the Tyne basin (Figure 3-1) to contrast metal levels and AR traits across the catchment. Two of the sites, Reaverhill (RH; NY90527323; Figure 3-2a) and Warks Burn (WB; NY86117651; Figure 3-2b), were located in the North Tyne catchment (Section 3.1.2), whereas the other sites were in the South Tyne catchment (Section 3.1.3). The two South Tyne sites within the historically mined areas, included the River Nent (RN) at Wellgill (NY77724421; Figure 3-3c) and the River West Allen (WA) in Ninebanks (NY78045400; Figure 3-3b). The third South Tyne site was at Featherstones (FS) (NY67186106; Figure 3-3a), which is 25 km downstream of the RN site.
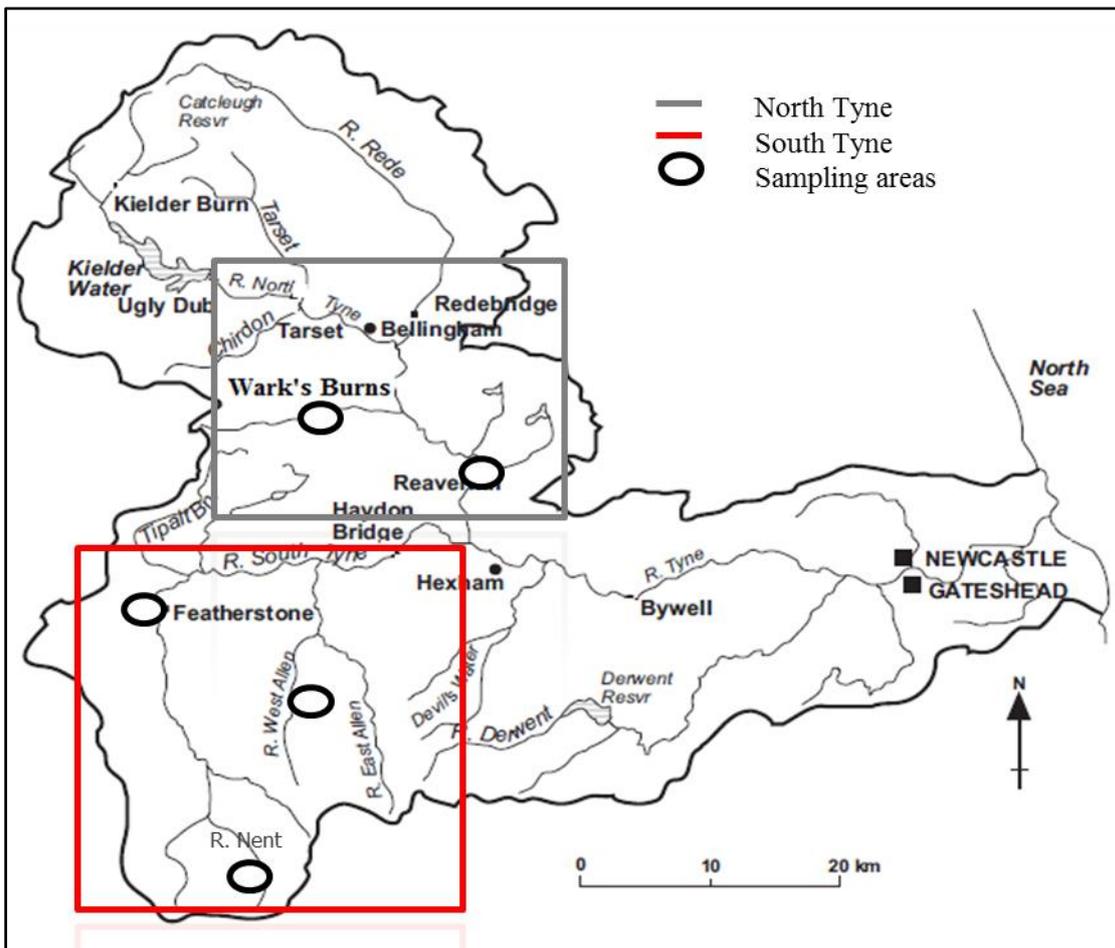
Figure 3-1 Map of the Tyne basin, UK outlining the North and South catchments and the sampling locations adapted from Archer (2008).

Figure 3-2 Photographs of sampling sites in the North Tyne basin; a) Reaverhill (RH); b) Warks Burn (WB).

Figure 3-3 Photographs of sampling sites in the South Tyne basin; a) Featherstones (FS); b) River West Allen (WA); c) River Nent (RN).

3.2.2 *Sample collection*

Duplicate sediment samples were collected from each of the five sampling sites (Section 3.2.1) every four weeks for six months (June – October, 2014). The samples were collected about 0.5-1.0 metres (m) from the river bank by using a plastic circular coring mould of 3-centimetres (cm) in depth with lids. The mould was used to also trap pore water around the sediment, such that water could be available for the analysis of colloidal metals. At each site, the moulds were pressed into the river bed and sterile plastic scoops were used below the mould to remove the collected sediment. Plastic scoops were then used to scoop the sediment from the mould into sterile 50-ml tubes (VWR, Leicestershire, UK) for microbiological analysis and also to sterile 250-ml Nalgene plastic bottles (VWR, Leicestershire, UK) for total, soluble and colloidal metal analysis. A single water sample also was collected in sterile 15-ml tubes (VWR, Leicestershire, UK) at each site for microbiological and metal analysis. Samples were kept in a cool box for transportation after which they were kept at 4°C until processing and analysis, either on the same or next day. In addition the pH, dissolved oxygen (mg/L DO), and temperature (°C) of river water at each site was measured using a digital two channel multi-metre (Hach- Lange, Salford, UK).

3.2.3 *Preparation of sediment and water samples*

Sediment samples for microbiological analysis were prepared the same day by filling sterile 15-ml tubes (VWR, Leicestershire, UK) to the 5-ml level with sediment from each site and adding the equivalent amount (5.00 ml) of prepared sodium pyrophosphate decahydrate ($Na_4P_2O_7 \cdot 10H_2O$; Sigma- Aldrich, St. Louis, MO, USA). Solutions of 2.00 g/L sodium pyrophosphate decahydrate were prepared in de-ionised water (Veolia Water Technologies, High Wycombe, UK) and sterilised by autoclaving for 15 minutes at 121°C (Rodwell Scientific Instruments, Essex, UK). These solutions were shaken overnight in an incubator (Infors HT, Basel, Switzerland), set at 112 revolutions per minute (rpm) and at a temperature of 20°C in order to release cells from the sediments into solution. Water samples were refrigerated at 4°C until needed the subsequent day for culturing.

3.2.4 *Quantifying meropenem and Zn resistance*

Samples were analysed for meropenem and Zn resistance using classical spread-plating techniques according to the spread plate (9215C) standard method (APHA, 2005). All preparation and culturing were done in a class II microbiological safety cabinet (Labcaire Systems Ltd., North Somerset, UK), where for each set of samples, agar plates were prepared using R2A agar (Sigma- Aldrich, St. Louis, MO, USA) amended with 40.0 mg/L

cycloheximide (to suppress mould growth; Sigma- Aldrich, St. Louis, MO, USA). The following agar amendments were then added as follows: no amendment (control), 2.00 mg/l meropenem, 65.0 mg/L Zn, and a combination of 2.00 mg/L meropenem and 65.0 mg/L Zn (preparations described in section 3.2.5). All amendments were added when the agar was at 55°C and mixed before the agar was poured into sterile 90-mm petri dishes (VWR, Leicestershire, UK).

3.2.5 *Selection and preparation of agar and amendments*

R2A (Sigma- Aldrich, St. Louis, MO, USA) agar was used as it is a lower nutrient media (often used for environmental isolations), which in combination with a lower incubation temperature and extended incubation times, enhances recovery and selection of environmental organisms (Reasoner and Geldreich, 1985). R2A was weighed out and autoclaved according to the manufacturer's instructions.

Concentrated stock solutions of 1.00 g/L meropenem (Flurochem Ltd., Hadfield, UK) were prepared by weighing out the appropriate amounts (Mettler-Toledo Ltd., Leicester, UK) and diluting in de-ionised water. In order to identify an appropriate meropenem concentration for isolation of presumptive "resistant" strains, trial experiments were conducted. Initially, plates were made with 0.25 mg/L meropenem based on levels typically used for clinical studies (Turner *et al.*, 1999). However, when this concentration was used for sediment samples, over 60.0% of culturable isolates were tolerant to meropenem at this level. Therefore, further trials ultimately led to the selection of 2.00 mg/L meropenem for subsequent work (Table 2-1), which was still not highly selective relative to clinical strains, but provided a range of differing responses among various sediment sites.

Table 3-1 Percentage (%) of bacteria in sediments from Warks Burn (WB) and River West Allen (WA) resistant to 0.25 mg/L and 2.00 mg/L meropenem. Standard errors (S.E.) indicated in brackets.

Sampling sites	Percentage of resistant colonies (%)	
	0.25 mg/L meropenem	2.00 mg/L meropenem
Warks Burn (WB)	85.0 (S.E. ± 1.02)	47.0 (S.E. ± 2.75)
River West Allen (WA)	64.0 (S.E. ± 0.24)	33.0 (S.E. ± 1.01)

Zn in the form of Zn sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Sigma- Aldrich, St. Louis, MO, USA) was used for preparing Zn-amended plates. Previous studies done by Nuttall and Younger (2002) on Zn levels in River Nent (RN) showed that *in situ* Zn concentrations in the river water were typically as $ZnSO_4$. Due to the potential for Zn tolerance in river microorganisms, especially in the South Tyne, $ZnSO_4$ was chosen as the Zn amendment. Stock solutions of 1.00 g-Zn/L were prepared by weighing out the appropriate amounts of $ZnSO_4$ (Mettler-Toledo Ltd., Leicester, UK) and diluting in de-ionised water. The concentration of $ZnSO_4$ needed to produce a known Zn concentration was calculated using Equation 3-1, given that the molecular weight of $ZnSO_4$ (Molecular weight) and atomic weight of Zn (Atomic weight) were known, as was the required Zn concentration ($Concentration_2$).

Equation 3-1 The equation used to determine the concentration of $ZnSO_4$ needed, when the molecular weight of $ZnSO_4$, the atomic weight of Zn and the required Zn concentration were known.

$$Concentration_1 = \frac{Concentration_2 \, (mg/L) \times Molecular \; weight \; (mg/L)}{Atomic \; weight \; (mg/L)}$$

Agar plates were amended with 65.0 mg/L Zn for selecting "Zn resistant" isolates (i.e., 1 mMol of Zn), which has been used previously for testing Zn resistance in environmental strains (Peltier *et al.*, 2010). A stock solution of 4.00 g/L cycloheximide (Sigma- Aldrich, St. Louis, MO, USA), a glutarimide eubacterial inhibitor, was also prepared and filter-sterilised (0.2-µm membrane filters; Pall Cooperation, MI, USA), and 40.0 mg/L was added to the plates to inhibit fungal growth. Cycloheximide specifically inhibits protein synthesis in eukaryotic cells, but not prokaryotic cells, therefore this amendment reduces fungi and mould growth and allows plates to last longer during incubation (Sigma-Aldrich, 2013). The volumes of meropenem, Zn and cycloheximide required for the agar preparation were calculated using Equation 3-2, as the initial and final concentrations, as well as the final volume required were known. All amendments were filter-sterilised before addition as noted above.

Equation 3-2 The equation used to determine the amounts of meropenem, Zn and cycloheximide required for the agar amendment, when the initial and final concentrations as well as the required volumes were known.

$$Volume_1 \times Concentration_1 = Volume_2 \times Concentration_2$$

3.2.6 *Inoculation of samples and colony analysis*

Sediment and water samples were serially diluted (10^0-10^2) in sterile phosphate buffered saline (PBS; Oxoid, Hampshire, UK; Figure 3-4) prior to plating. PBS was prepared according to manufacturer's instructions and autoclaved for 10 minutes at 110°C.
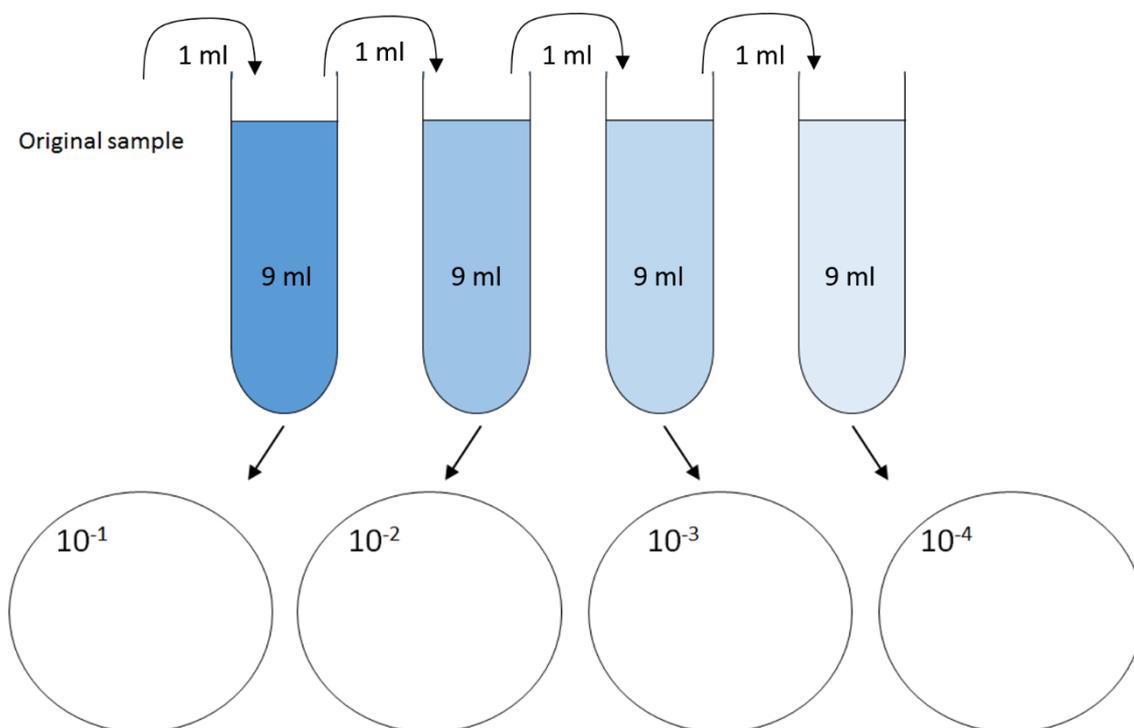
Figure 3-4 Diagram showing the process of serial dilution (APHA, 2005).

Each prepared plate was inoculated with 50-µl of each serially diluted sediment sample or 100-µl of water sample, and spread with a glass spreader, which was dipped in ethanol and flamed between plates. Each serial dilution was plated in triplicate, and were inverted and incubated for three days at 24 to 27°C (room temperature; section 3.2.5). After incubation, the plates were quickly examined prior to colony counting. Only plates with roughly 30-300 colony forming units (CFU) per plate were counted (in accordance with APHA (2005) Standard Method 9215A), using a colony counter (Weiss Technik, Gwent, UK). The number of CFU/ml were calculated using Equation 3-3, which depends on the dilution used and the volume inoculated. The percentage ratio of CFU/ml in amended petri dishes relative to un-amended control petri dishes was then calculated (Equation 3-4) to estimate the proportion culturable colonies that were resistant to the associated media.

Equation 3-3 The equation used to transform the number of colonies counted per petri dish to Colony Forming Units per ml (CFU/ml).

$$CFU/ml = \frac{Dilution}{(Volume\ innoculated\ (ml)\ \times\ Number\ of\ colonies)}$$

Equation 3-4 The equation used to calculate percentage ratio of CFU/ml in amended petri dishes relative to un-amended control petri dishes.

$$Resistant\ colonies\ (\%) = \frac{CFU/ml\ of\ amended\ petri\ dish}{CFU/ml\ of\ unamended\ (control) petri\ dish} \times 100$$

3.2.7 *Preparation of samples for metal analysis*

Prior to metals analysis, all glass vials (VWR, Leicestershire, UK) were washed in 0.20% Nitric acid (HNO_3) (68.0%, VWR, Leicestershire, UK), rinsed in de-ionised water and allowed to dry. They were then further dried for two minutes in a 550°C muffle furnace (S H Scientific, Blyth, UK) and, once cooled, the vials were pre-weighed. The sediment collected in the 250-ml Nalgene bottles was transferred to the "clean" glass vials and dried overnight in an oven (Weiss Technik, Gwent, UK) at 104°C. They were then re-weighed to determine the dry weight of the sediment present in each vial.

Samples for soluble metal analysis were prepared by filtering the river water collected with the sediment through 0.2-µm cellulose acetate membrane filters (VWR, Leicestershire, UK). Samples for colloidal metal analysis were prepared by further filtering the above through mixed cellulose hydrophilic 0.025-µm filters (Merck, NJ, United States). The river water collected from each site in 15-ml falcon tubes was also prepared for soluble and colloidal metal analysis.

3.2.8 *Metal analysis*

Sample acid digestion was performed on all dried sediments, according to the APHA (2005) Standard Method 3030 E.2 using 1N HNO_3 (65.0%, Suprapur®, Merck, NJ, United States), for 40 minutes at a temperature of 105°C. Acid digested samples were then filtered prior to analysis using 0.2-µm cellulose acetate membrane filters and diluted by at least a factor of 10. Soluble and colloidal metal analysis was measured by adding 0.50 ml 1N HNO_3 to 1-ml of the filtered samples and filling to a volume of 5-ml with de-ionised water. All prepared samples were analysed using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Vista MPX CCD Simultaneous ICP-OES, Varian, Australia), for metals including Cd, Co, Cu, Ni, Pb and Zn. Blanks with de-ionised water were prepared in the same

way as samples and standards were prepared using 1000 mg/L stock solutions (VWR, Leicestershire, UK).

3.2.9 *Data analysis*

All data was statistically analysed using Minitab (Version 17, Coventry, UK). Normality checks where performed to see if the data was normally distributed. Typically, the CFU and metal data were not normal, therefore Mann-Whitney non-parametric test were used to compare isolate and metal abundances between sites. This was conducted by assessing whether sample-pairs were significantly different to each other using 95.0% significance tests, where two samples were significantly different when p-values were < 0.050. Bi-variate correlation analysis also was performed to assess linear correlations and general trends among measured parameters. The Pearson's coefficient (value of r) signified the strength of the test, where the closer the coefficient to 1, the stronger the correlation, whereas p-values < 0.050 were significantly different.

3.3   Results and Discussion

3.3.1 *North and South Tyne river water characteristics*

Both biotic and abiotic stress can affect the growth and activity of microbes in the river water as well as in the sediment beds. Abiotic factors can include, the availability of light, temperature and pH, so the measurement of some of these factors were measured during each sampling month at each of the five sites within the North and South Tyne basins. Mean water temperatures ranged from 13.3 to 14.0°C across the six-month sampling campaign, with RN having the lowest and the two sites in the North Tyne (RH and WB) having the highest (Table 3-2).

Table 3-2 The water characteristics, including pH, temperature (°C) and oxygen saturation (% saturation) for each sampling site averaged over the six months of sampling. Ranges and standards errors (S.E.) are indicated below in brackets.

Sampling sites	pH	Temperature (°C)	Oxygen saturation (% saturation)
Reaverhill (RH)	7.16 (ranges: 6.21-7.82; S.E. ± 0.22)	14.0 (ranges: 11.8-16.3; S.E. ± 0.79)	91.0[1] (ranges: 84.0-94.0; S.E. ± 2.13)
Warks Burn (WB)	7.18 (ranges: 6.58-7.90; S.E. ± 0.17)	14.0 (ranges: 11.7-16.7; S.E. ± 0.82)	90.3[2] (ranges: 71.0-100; S.E. ± 5.84)
Featherstone (FS)	7.42 (ranges: 6.56-8.26; S.E. ± 0.28)	13.9 (ranges: 11.7-15.5; S.E. ± 0.70)	91.3[3] (ranges: 75.0-100; S.E. ± 4.96)
River West Allen (WA)	7.86 (ranges: 6.93-8.45; S.E. ± 0.22)	14.4 (ranges: 11.5-16.3; S.E. ± 0.83)	95.3[4] (ranges: 83.0-100; S.E. ± 3.68)
River Nent (RN)	7.62 (ranges: 7.03-8.18; S.E. ± 0.19)	13.3 (ranges: 10.4-15.6; S.E. ± 0.87)	92.3[5] (ranges: 85.0-95.0; S.E. ± 2.17)

[1]RH: 9.52 mg/L DO (± 0.13); [2]WB: 9.38 mg/L DO (.± 0.40); [3]FS: 9.80 mg/L DO (± 0.35); [4]WA: 9.99 mg/L DO (± 0.23); [5]RN: 9.64 mg/L DO (± 0.05)

Mean water pH values ranged from 7.16 to 7.86, with the highest pH recorded at WA and the lowest at the two North Tyne sites. Mean oxygen saturation rates (% saturation) varied from 90.3 to 92.3, with the highest saturation observed at WA and the lowest in WB (Table 3-2). The small error values for each value, showed that the pH did not vastly change over the six month period, thus it was mostly between the optimum pH of 6.00 to 8.00. Optimum DO in freshwater is usually between 8.00 and 15.0 mg/L DO (Pepper *et al.*, 1996), and as all the sites fall within this (9.38 to 9.99 mg/L DO), the quality of the water can be said to be quite high. Overall WA had the highest pH, temperature and saturation of oxygen, which is the highest sampled site in the River Tyne catchment.

3.3.2 *Total, soluble and colloidal metal levels in sediment and water samples*

Previous studies have shown the South Tyne has considerably elevated levels of heavy metals due to its past mining activity during the 19th century (Macklin *et al.*, 1994; Macklin *et al.*, 1997). Consistent with historic information, analyses here found mean total Zn levels were significantly higher (Mann-Whitney; p-values = 0.005) in WA and RN (3540 (S.E. $\pm$ 54.9) and 3560 (S.E. $\pm$ 226) mg-Zn/kg-dry sediment, respectively), both located in the South Tyne basin than in North Tyne basin sites, RH and WB (53.6 (S.E. $\pm$ 3.15) and 18.4 (S.E. $\pm$ 0.10) Zn/kg-dry sediment, respectively; Table 3-3). The intermediate site in the watershed, FS, had about half the total sediment Zn levels of WB and RH; i.e., 1550 (S.E. $\pm$ 210) mg-Zn/kg-dry sediment, which was significantly higher than total Zn levels in RH and WB (Mann-Whitney; p-value = 0.005).

The contrast in sediment metal levels between the North and South Tyne is profound, especially given their close proximity. Data show total Zn levels vary by over 200 times among the five sites, which provides a broad contrast of Zn conditions for comparison with *in situ* meropenem resistance. It should be noted that elevated metals levels in the South Tyne are suspected to be creating significant environmental (and maybe health) stress (Abel, 1996), which is consistent with anthropogenic processes often creating greater stress than parallel natural processes in metal cycling (Mitchell and Gu, 2010). For example previous studies on the WA catchment reported that the benthic fauna was severely impoverished due to the high levels of Zn (Abel, 1996). More recently the catchment was given a 'moderate' ecological status in 2009, due to the impacts of Zn levels on fish (EA, 2009).

Table 3-3 Measured total (mg/kg), soluble (mg/L) and colloidal (mg/L) levels of Zn in sediment and water samples from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN). Values indicated are averages over six months and standard errors (S.E.) are indicated in brackets (all values shown in Table A-1, Table A-2 and Table A-3).

Sampling sites	Zn levels in sediment			Zn levels in river water	
	Total	Soluble	Colloidal	Soluble	Colloidal
	(mg/kg dry sediment)	(<0.20 mg/L)	(<0.02 mg/L)	(<0.20 mg/L)	(<0.02 mg/L)
Reaverhill (RH)	53.6 (S.E. ± 3.15)	<0.01	<0.01	<0.01	<0.01
Warks Burn (WB)	18.4 (S.E. ± 0.10)	<0.01	<0.01	<0.01	<0.01
Featherstone (FS)	1550 (S.E. ± 210)	0.13 (S.E. ± 0.03)	0.10 (S.E. ± 0.03)	0.10 (S.E. ± 0.01)	0.07 (S.E. ± 0.01)
River West Allen (WA)	3540 (S.E. ± 54.9)	1.05 (S.E. ± 0.01)	0.77 (S.E. ± 0.02)	0.88 (S.E. ± 0.07)	0.63 (S.E. ± 0.08)
River Nent (RN)	3560 (S.E. ± 226)	3.89 (S.E. ± 0.85)	2.10 (S.E. ± 0.78)	1.22 (S.E. ± 0.22)	0.67 (S.E. ± 0.14)

The analysis of the pore water within the collected sediment samples showed that soluble and colloidal Zn levels were significantly lower than total Zn levels (Mann- Whitney; all p-values = 0.005), meaning that the majority of Zn was present in the sediment itself than in the aqueous phase (Table 3-3).

Heavy metals, such as Zn tend to bind with particles and can become incorporated into sediments as they pass through the water column (Manahan, 2010). However a certain amount remains in the water surrounding the sediment, which is often associated with inorganic anions or organic compounds, as metal complexes (Manahan, 2010). The presence of complexing agents, such as chelating agents, which can be found in many polluted waters, or natural forming humic substances often result in the solubilisation of metals from the sediment solid phase (Manahan, 2010). Therefore, sites with high total Zn levels often have high soluble Zn levels, and this was the case, where soluble Zn levels were significantly higher in FS, WA and RN (0.13; S.E. ± 0.03), 1.05; S.E. ± 0.01) and 3.89; S.E. ± 0.85) mg/L soluble Zn respectively) relative to levels in RH and WB (both below 0.01 mg/L Zn; Mann-Whitney; all p-values = 0.005; Table 3-3).

Colloidal Zn levels in the sediment pore water were significantly higher in FS, WA and RN (0.10; S.E. ± 0.03, 0.77; S.E. ± 0.02 and 2.10; S.E. ± 0.78 mg/L colloidal Zn respectively) relative to levels in RH and WB (both below 0.01 mg/L colloidal Zn; Mann-Whitney; all p-values = 0.005) which showed similar patterns as soluble Zn levels across the sites (Table 3-3). Furthermore colloidal levels were all significantly lower than total metal levels (Mann-Whitney; all p-values = 0.005; Table 3-3).

Soluble Zn levels in the river water were lower than soluble Zn levels in the sediment pore water; however, only Zn levels at RH and RN were significantly different between river and pore water levels (Mann-Whitney; both p-values = 0.005). This implies there are differences in Zn levels and possibly available Zn in the river water and the water around the sediments. Differences are likely due to elevated local solubilisation of Zn into the poorly mixed sediment pore water, which differs to the continual flushing of local sites by upstream water in the open river (Table 3-3). For both soluble and colloidal analysis of the river water, for sites in the North Tyne the levels were not detectable. However in WA and RN soluble Zn levels were detected with the highest being in RN, which were significantly higher than levels in RH, WB and FS (Mann- Whitney; all p-values = 0.005; Table 3-3). In addition in WA and RN colloidal Zn levels were also significantly higher than levels in RH (Mann-Whitney; WA: p-value = 0.005, RN: p-value = 0.013) and WB (Mann-Whitney; WA: p-values = 0.005, RN:

p-value = 0.013), while only WA colloidal Zn levels were significantly higher than those at FS (Mann-Whitney; p-values = 0.005; Table 3-3).

Cd, Co, Cu, Ni and Pb levels were also measured in sediment and water samples. The detected levels of these metals in the soluble and colloidal phases were always below detection limits. However, the concentrations of total metals from the digested sediment showed that there were high levels of Pb in FS, WA and RN (Table 3-4). This makes sense as previous mining activities in the South Tyne released other heavy metals, including Pb, as well as Zn. Combining data from all sites, strong positive significant correlations were found between total Zn levels and total Cd (Pearson's correlation = 0.973; p-value = 0.000; Table A-4) and Pb levels (Pearson's correlation = 0.951; p-value = 0.000;Table A-4). This suggests that these metals are present together at all sites and so sites with increased Zn also had increased Cd and Pb. In addition there were also correlations between total Zn and total Cu, however these were weaker than the latter (Pearson's correlation = 0.754; p-value = 0.012; Table A-4). Further there were positive significant correlations between total Pb levels and total Cd levels (Pearson's correlation = 0.864; p-value = 0.001; Table A-4). There were also correlations between total Pb and total Co (Pearson's correlation = 0.663; p-value = 0.037; Table A-4) and Cu levels (Pearson's correlation = 0.716; p-value = 0.020; Table A-4), however these were weaker than and not as strong as the latter.

Table 3-4 Measured total levels of Cd, (Co, Cu, Ni, Pb and Zn in mg/kg in sediment from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN). Values indicated are averages over six months and standard errors (S.E.) are indicated in brackets.

	Cadmium (Cd)	Cobalt (Co)	Copper (Cu)	Nickel (Ni)	Lead (Pb)	Zinc (Zn)
Reaverhill (RH)	0.67 (S.E. ± 0.29)	7.96 (S.E. ± 3.05)	3.22 (S.E. ± 0.72)	12.3 (S.E. ± 4.60)	9.95 (S.E. ± 2.16)	53.6 (S.E. ± 3.15)
Warks Burn (WB)	0.15 (S.E. ± 0.001)	1.93 (S.E. ± 0.001)	1.63 (S.E. ± 0.24)	3.60 (S.E. ± 0.49)	3.78 (S.E. ± 0.44)	18.4 (S.E. ± 0.10)
Featherstone (FS)	4.32 (S.E. ± 0.49)	9.08 (S.E. ± 0.17)	14.7 (S.E. ± 1.67)	15.4 (S.E. ± 0.10)	891 (S.E. ± 115)	1550 (S.E. ± 210)
River West Allen (WA)	13.6 (S.E. ± 0.06)	7.03 (S.E. ± 0.09)	14.4 (S.E. ± 0.29)	8.73 (S.E. ± 0.53)	1340 (S.E. ± 243)	3540 (S.E. ± 54.9)
River Nent (RN)	10.1 (S.E. ± 0.43)	11.5 (S.E. ± 1.53)	10.3 (S.E. ± 0.59)	11.9 (S.E. ± 0.95)	1880 (S.E. ± 345)	3560 (S.E. ± 226)

3.3.3 *Observed resistance in sediment samples among sampling sites using different selective media*

Microbial culturing of sediment samples on agar plates amended with Zn, meropenem and a combination of meropenem and Zn showed that there were significantly higher numbers of Zn resistant isolates in sediments from sites in the South Tyne (FS, WA and RN) compared to sites in the North Tyne (RH and WB) (Figure 3-5).
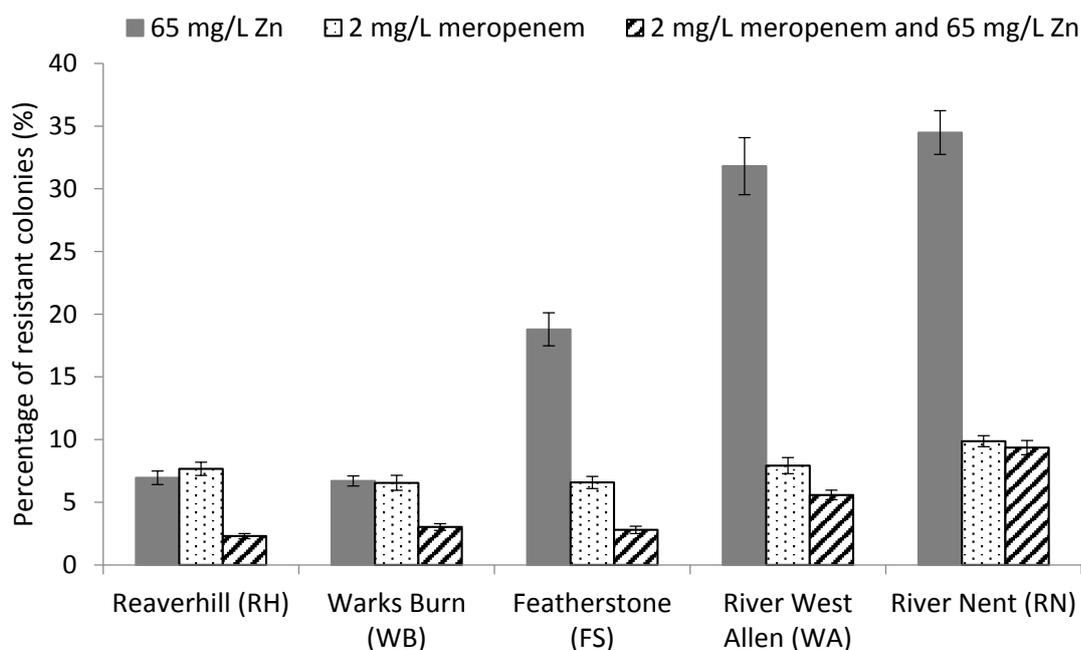
Figure 3-5 Percentage (%) of colonies in sediment resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN). Values indicated are averages over six months and standard error (S.E.) bars are shown (all values shown in Table A-5).

Levels of Zn resistance at FS, WA and RN were significantly higher relative to levels in RH (Mann-Whitney; FS: p-value = 0.013; WA: p-value = 0.008; RN: p-value = 0.005) and WB (Mann-Whitney; FS: p-value = 0.008; WA: p-value = 0.008; RN: p-value = 0.005). However, meropenem resistance did not significantly vary among the five sites (even between sites with low and high total Zn levels; Mann-Whitney; p > 0.050).

In contrast, when samples were cultured on medium containing both meropenem and Zn, it was found that "combined" resistance was statistically higher at RN relative to RH (Mann-Whitney; p-value = 0.008), WB (Mann-Whitney; p-value = 0.031) and FS (Mann-Whitney; p-value = 0.020), respectively (Figure 3-5). Combined resistance was also statistically higher at WA relative to RH (Mann-Whitney; p-value = 0.013), however levels were only weakly significant in WA relative to WB and FS (Mann-Whitney; p-values = 0.066 and 0.093,

respectively). Therefore, it is apparent that combined resistance is significantly higher in the higher Zn sediments in the South Tyne, which is most apparent in the sediments from WA and RN (Section 3.3.2; Table 3-3).

When characterising each site individually, no significant differences in culturable isolate abundances were observed between media amendments at the two North Tyne sites (RH and WB). Within the South Tyne catchment, Zn resistance was statistically higher than meropenem and combined resistance at FS (Mann-Whitney; p-values of 0.013 and 0.005, respectively). In addition, meropenem resistance was also significantly higher than combined resistance at FS (Mann-Whitney; p-value = 0.031; Figure 3-5).

Both WA and RN, which had considerably high levels of total and soluble Zn present in the sediment (>3540 mg/kg and >1.05 mg/L), had very high abundances of Zn resistant culturable organisms (Table 3-3). In WA, 31.8% of the CFU's were resistant to Zn, which were significantly higher than the meropenem and combined resistant colonies (Mann-Whitney; both p-values < 0.05). In RN, 34.5 % of isolates were Zn resistant, which was significantly higher than both meropenem resistance levels (Mann-Whitney; p-values = 0.008) and combined levels (Mann-Whitney; p-values = 0.005; Figure 3-5).

3.3.4 *Observed resistance in water samples among sampling sites using different selective media*

When assessing resistant culturable organisms among water samples, the highest levels of Zn resistance were apparent at the two South Tyne sites, WA and RN, which was similar to sediment observations (Section 3.3.3; Figure 3-5; Figure 3-6). Zn resistance was significantly higher in WA and RN in comparison to RH (Mann-Whitney; WA: p-value = 0.020, RN: p-value = 0.013) and WB (Mann-Whitney; WA: p-value = 0.045; RN: p-value = 0.045; Figure 3-6).
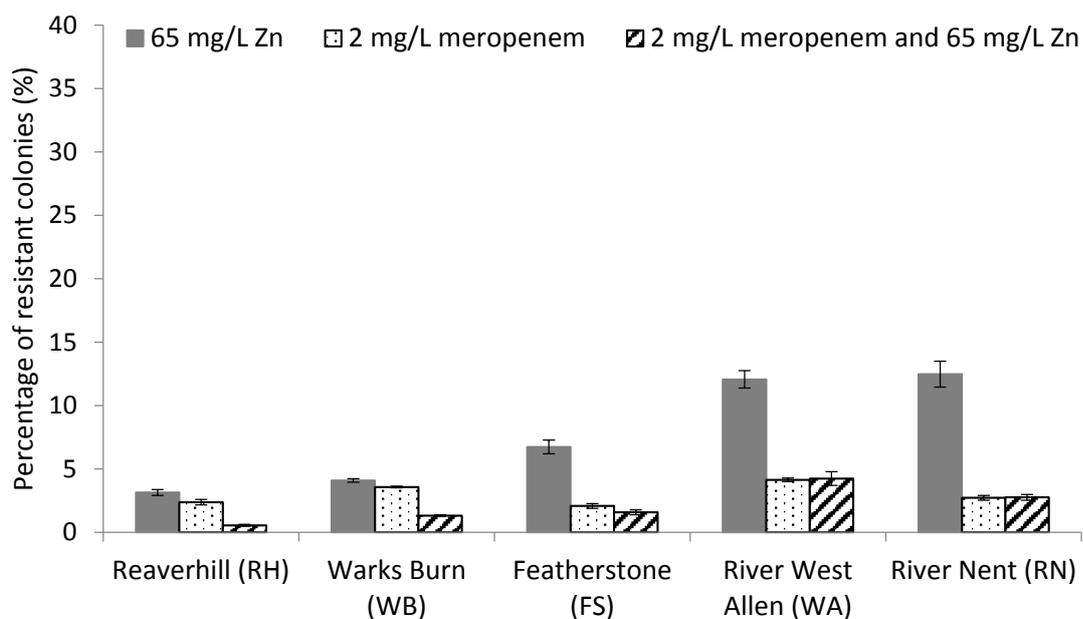
Figure 3-6 Percentage (%) of colonies in river water resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN). Values indicated are averages over six months and standard error (S.E.) bars are shown (all values shown in Table A-6).

Like the sediment samples, apparent meropenem resistance did not significantly vary among sites (Mann-Whitney; $p > 0.05$), whereas combined was significantly lower at RH relative to the FS, WA and RN sites (Mann-Whitney; p-values < 0.05; Figure 3-6).

When assessing different resistance types at RH, the percentage abundance of strains with combined Zn and meropenem resistance was broadly significantly lower, than strains with observed Zn resistance (Mann- Whitney; p-value = 0.020). The same was observed at WB, the other site along the North Tyne (Mann-Whitney; p-value = 0.045). All three sites on the South Tyne had significantly higher levels of Zn resistant organisms than meropenem resistant (Mann-Whitney; FS: p-value = 0.013; WA: p-value = 0.045; RN: p-value = 0.005) and combined resistant isolates (Mann-Whitney; FS: p-value = 0.005; WA: p-value = 0.031; RN: p-value = 0.008) Figure 3-6). FS, WA and RN also had the highest river water Zn levels, ranging from 0.09 to 1.22 mg-Zn/L, respectively (Table 3-3).

3.3.5 *Relationships between different resistance and Zn levels*

It is clear that the percentage of Zn and combined resistant strains were higher when Zn levels in sediment and water samples were higher. Combining data from all sites, positive significant correlation were found between relative percent Zn and combined resistant isolates, and total sediment Zn levels (p-value = 0.000 and 0.002, respectively), which was also seen when compared against soluble (p-value = 0.008 and 0.000, respectively) and

colloidal Zn levels (Pearson's correlation; p-value = 0.010 and 0.000, respectively; Figure 3-7 and Figure 3-8). However, no significant correlation was observed between the percent abundance of meropenem resistant isolates and total Zn levels, suggesting that meropenem resistance was not directly driven by local Zn levels.

The fact that a significant correlation was observed between relative combined resistance and total Zn levels implies Zn does conditionally influence apparent meropenem resistance, although this is only seen with plate media that contains extra Zn with meropenem. Similar correlations were seen between relative combined resistance and soluble and colloidal Zn levels, although correlations were stronger than for total Zn (Figure 3-8b and Figure 3-8c). This suggests that it was the soluble/colloidal fractions of Zn probably driving resistance (not total Zn level), which is with previous work that showed Zn is more bioavailable to microorganisms when present in soluble or colloidal particles (Manahan, 2010). Such colloids are suspended in water, either sediment pore water or the river water itself, usually as minerals, organic pollutants, algae and bacteria in the range from 0.001-1.00 µm (Manahan, 2010). A further study described in Chapter 4 confirms the relationship between soluble Zn levels and meropenem resistance using long-term laboratory incubations.

When looking at percent Zn resistance and Zn levels, correlations were actually better against total Zn, rather than soluble and colloidal Zn (Figure 3-7). A possible reason for this could be due to the non-linear trend between soluble and colloidal Zn and Zn resistance. Figure 3-7b and Figure 3-7c showed that Zn resistance became more constant as soluble and colloidal Zn increased. Therefore it is possible that the optimum concentration of Zn conferring resistance was reached, giving further evidence that soluble and colloidal Zn, is more bioavailable to organisms than total Zn.
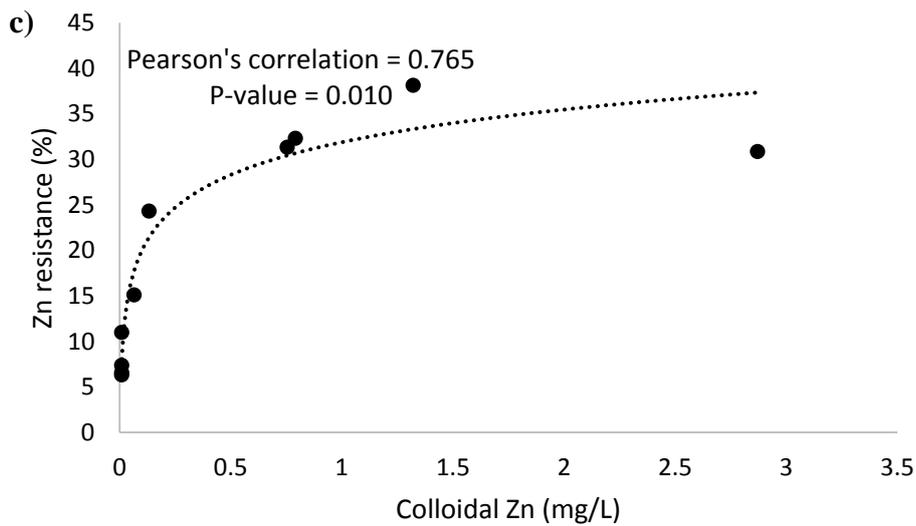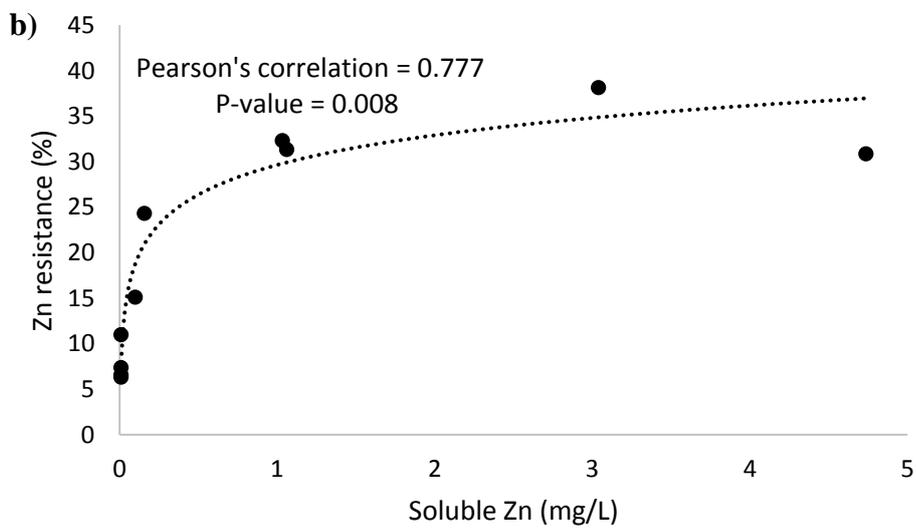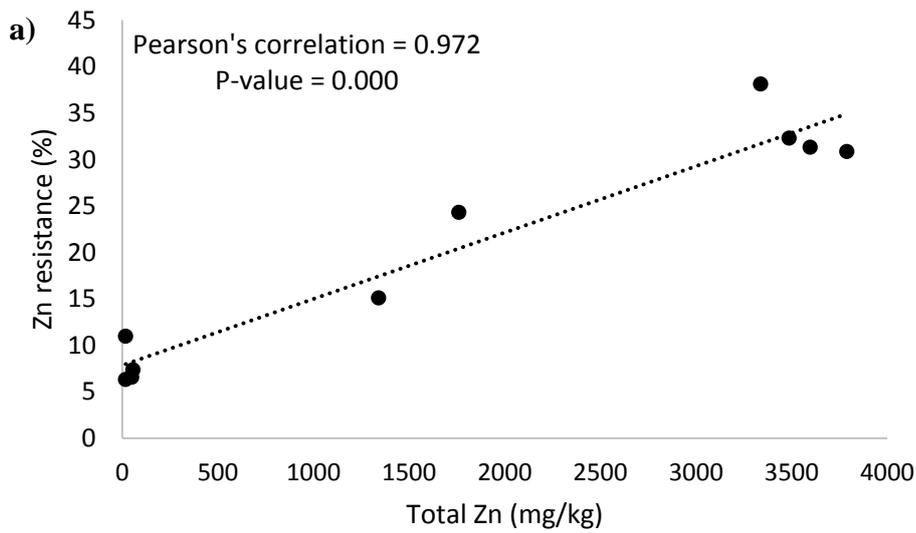
Figure 3-7 Pearson's correlations (r) between Zn resistance (%) and a) total (mg/kg), b) soluble (mg/L) and c) colloidal (mg/L) Zn.
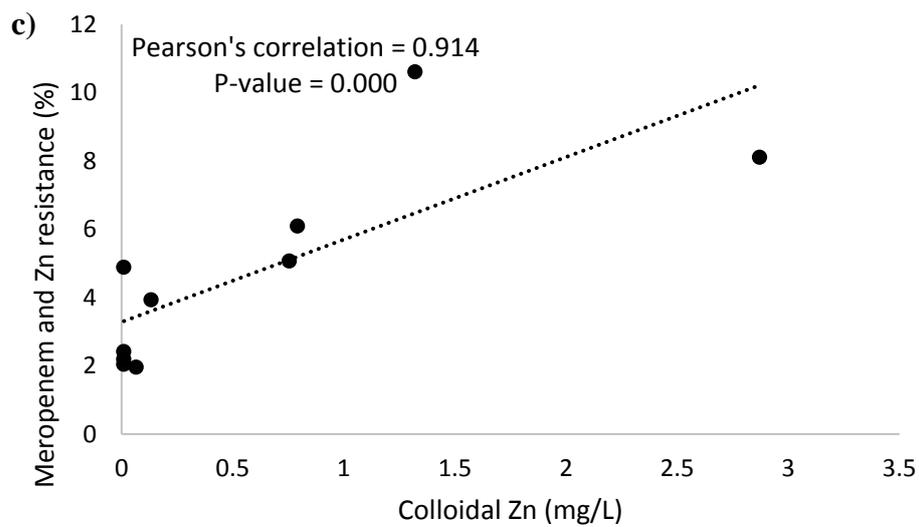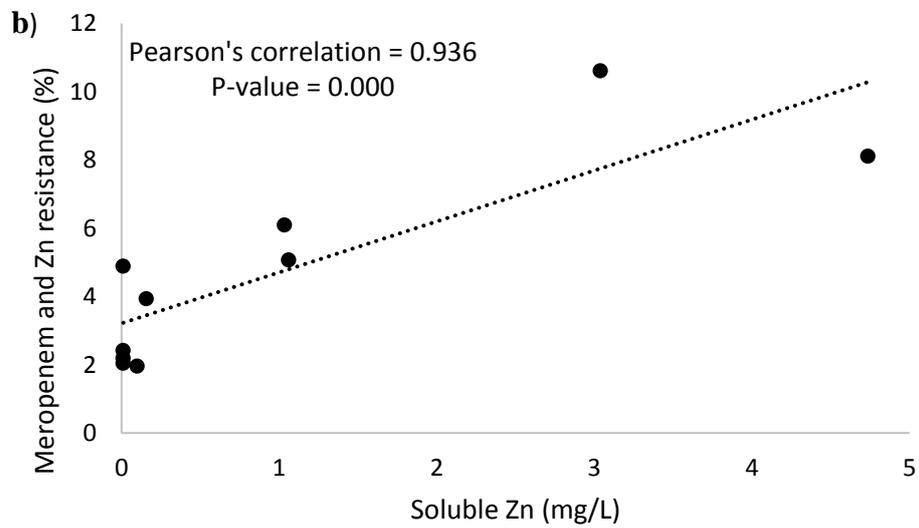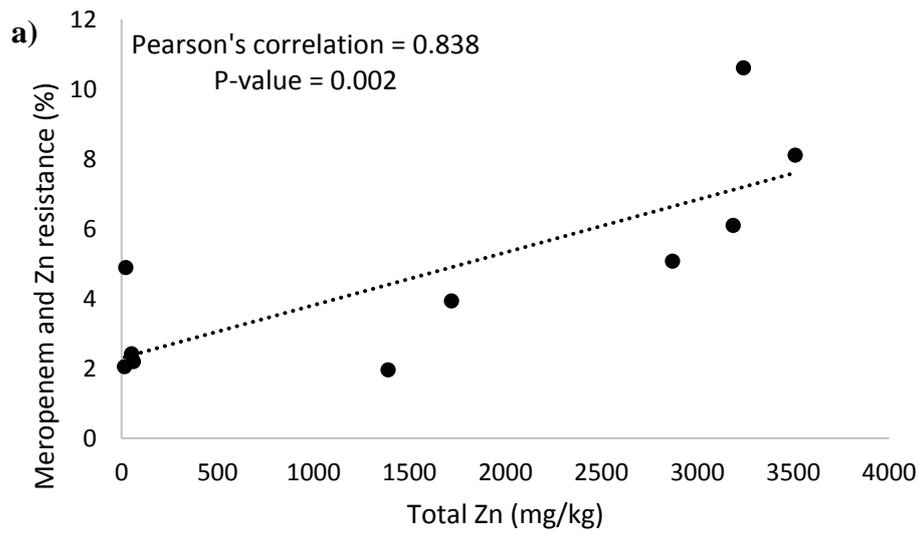
Figure 3-8 Pearson's correlations (r) between meropenem and Zn resistance (%) and a) total (mg/kg), b) soluble (mg/L) and c) colloidal (mg/L) Zn.

As Pb levels were also very high at the South Tyne sites, correlation analyses was also performed with total, soluble and colloidal Pb. This showed that relatively elevated total, soluble and colloidal Pb levels also correlated significantly with Zn resistance (p-values = 0.000, 0.001 and 0.026; Table A-7), and combined meropenem and Zn resistance (p-values = 0.002, 0.004 and 0.037; Table A-7). Further, there was also a significant correlation between total, soluble and colloidal Cd levels and, relative percent Zn (p- values = 0.000, 0.001 and 0.001; Table A-7), and combined resistant isolates (p-values = 0.011, 0.000 and 0.000; Table A-7). Therefore this suggests that the presence of Pb and Cd, as well as Zn in the sediments may also have an effect on the selection of combined meropenem resistant isolates. This observation could be explained due to strong positive correlations between total Zn and, Cd and Pb levels, as well as between total Pb and Cd levels (Section 3.3.2). Therefore these metals exist together and follow the same patterns and therefore could also contribute to the selection of combined Zn and meropenem resistant isolates. However further analysis, including the culturing of isolates on Pb and Cd amended agar plates is required to be certain.

3.3.6 *The effect of Zn levels on Zn resistance in river sediments and water*

Numerous Zn resistant colonies grew on R2A media containing 65.0 mg/L Zn, which were from sediment and water samples along the North and South Tyne (Section 3.3.3 and 3.3.4; Figure 3-5 and Figure 3-6). However, percent Zn resistant colonies were much greater at sites on the South Tyne (FS, WA and RN) where levels of Zn and other heavy metals were significantly higher. Furthermore, apparent Zn resistance in sediments, and total, soluble and colloidal Zn levels all positively correlated with each other (Figure 3-7; section 3.3.5), suggesting *in situ* Zn levels are important to Zn resistance.

As background, Zn is an essential nutrient for all living systems and plays many important roles in development, growth and differentiation of cells (Choudhury and Srivastava, 2001b) (Section 2.8). However, exposure to elevated Zn, such as that present in the South Tyne, can potentially have adverse and toxic effects on microorganisms, depending upon Zn speciation and other factors. Therefore, resident organisms have almost certainly adapted to such conditions through specific and non-specific mechanisms, ranging from simple physiochemical adaptations (e.g., bio-sorption) to the development of stringent efflux systems (Choudhury and Srivastava, 2001a).

When a cell is present in an environment with high heavy metal concentrations (especially ions; e.g., Zn^{2+}, Ni^{2+}, Co^{2+} or Mn^{2+}), metals initially accumulate in cells via transport by rapid, non-specific chemiosmotic membrane-integral proteins, such as CorA (Nies, 1999). For example the CorA transporter was responsible for the transfer of Zn in *Saccharomyces*

cerevisiae, a species of yeast and has since been reported present in archaea and many bacteria (Smith and Maguire, 1995; Smith *et al.*, 1998). As heavy metals cannot be degraded, many cells have evolved metal ion homoeostasis factors and MR determinants to reduce toxic intracellular metal accumulation (Nies, 1999). For example Zn specifically is effluxed from cells through, chemiosmotic systems, such as the CDF efflux protein and-or by inducible RND transporters, such as the *czc (cobalt, zinc and cadmium)* and *czr (cadmium and zinc)* defence systems (Mergeay *et al.*, 1985; Nies *et al.*, 1987; Hassan *et al.*, 1999; Nies, 1999). Such defence systems were seen in bacteria found in industrial polluted areas, with the first ever strain, *Ralstonia metallidurans* CH34, discovered in the 1970s in sediments around a Zn factory in Belgium (Mergeay *et al.*, 1978). In this case the highest resistance to Zn was seen in bacteria from South Tyne sediments, which had the highest Zn concentrations. Therefore, it is very likely that organisms in the South Tyne (FS, WA and RN) continually monitor metal stress and vary the expression of MR defence genes acquired through the *czc* and *czr* systems allowing them to survive in high Zn environments by exporting metals present in excess, as well as down-regulating the expression of these genes when metal levels are low to, prevent starvation of Zn within cells (Choudhury and Srivastava, 2001b).

3.3.7 *The relationship between high Zn levels and combined meropenem and Zn resistance*

Many studies have suggested that there is link between metal presence and AR (Alonso *et al.*, 2001; Peltier *et al.*, 2010; Knapp *et al.*, 2011). In this case the presence of Zn increased combined meropenem and Zn resistance across samples. Despite there not being an apparent significant increase in meropenem resistance in areas of high Zn, the fact that combined meropenem and Zn resistance was higher than meropenem alone in plated colonies suggests that the Zn must indirectly influence meropenem resistance.

Similar findings have been reported in other studies; for example, sediments analysed along the Almendares River near Havana, Cuba, which also had high metal contamination, showed that elevated Cu, Pb, Co and Zn correlated with certain ARGs (Graham *et al.*, 2011). Another study, such as that reported by Stepanauskas *et al.* (2006) showed that AR resistant bacteria were higher in metal-amended microcosms, which reflected the indirect selection for AR taking place in natural environments contaminated with heavy metals. Other river studies also found that bacterial tolerances to metals and antibiotics were highest at river sites with the highest metal contamination (Wright *et al.*, 2006). Wright *et al.* (2006) also revealed that antibiotic tolerances were highest in bacteria from sediment, where metal levels were also higher. This is consistent with the findings from this study, where resistance to meropenem

and Zn were higher in bacteria from sediments compared to water (Sections 3.3.3 and 3.3.4). Further Zn levels were also higher at all sites in sediment, suggesting that bacteria in sediments may have more bacteria with both metal and meropenem resistant traits.

One option for this pattern observed may be the co-presence of explicit AR and MR resistant determinants within the cells (Alonso *et al.*, 2001; Martinez, 2009a), but there are other options. For example, data here suggest that a subtype of Zn resistant bacteria may confer defence to meropenem. Such a phenomena has been reported in studies using *P. aeruginosa*, as these organisms thrive in environments polluted with organic matter and are often highly resistant to xenobiotics, including heavy metals, antimicrobial agents and solvents (Wang *et al.*, 1997). In this case, conferred resistance resulted from a combination of altered OM permeability and multiple RND efflux pumps, which are upregulated in many gram-negative bacteria under metal stress (Nikaido, 1994; Saier Jr *et al.*, 1994). RND efflux systems can control the removal of metal ions from the cell, but also of antibiotics, and other biochemical systems such as Mex pumps, can allow isolates to display simultaneous metal and antibiotic resistance in the presence of elevated metals (Ramos- Aires *et al.*, 1999; Ziha-Zarifi *et al.*, 1999). Furthermore, the *czc* and the *czrCBA* systems (mentioned earlier), which are both RND type efflux pumps, confer Zn resistance in *R. metallidurans* (Mergeay *et al.*, 1985; Nies *et al.*, 1987) and *P. aeruginosa* (Hassan *et al.*, 1999), respectively. Therefore, the organisms selected on the combined meropenem and Zn media could be using similar types of efflux systems that provide cross resistance.

The field data reported here can be compared to a study done by Perron *et al.* (2004), where a clinically isolated *P. aeruginosa* was exposed to Zn, which led to the selection of strains that were simultaneously resistant to both Zn and the carbapenem antibiotic, imipenem, used to treat gram-positive bacteria (Bedenic *et al.*, 2014). The organisms showing combined meropenem and Zn resistance could be using the same mechanisms as those identified in Perron *et al.* (2004), but this cross resistance is only apparent in isolates from plates with both Zn and meropenem. The mechanism behind this cross resistance (Perron *et al.*, 2004) revealed that a single amino acid in the two-component sensor protein *czcS* (a structural gene from the *czcCBA* efflux pump) that regulated heavy metal efflux pump expression, had changed resulting in a co-regulation between carbapenem influx and heavy metal efflux. Further analysis showed that Zn induced both the *czcCBA* efflux pump operon and the transcription of the two component regulator genes *czcR* and *czcS*, creating a positive auto regulation loop. Therefore, the presence of Zn, could allow cells to overexpress heavy metal RND pumps, which include Mex pumps. The OprD porin, which facilitates in the diffusion of basic amino

acids and carbapenem antibiotics, was also expressed in Zn induced strains in heavy MR mutants.

Therefore, due to the co-regulation between the simultaneous overexpression of an efflux pump and down regulation of a porin pathway, intracellular accumulation of toxins is reduced. Thus, the environmental exposure of cells to Zn and other heavy metals, such as those organisms present in the sediment of sites in the South Tyne, could cause an external stress signal, resulting in a decrease in the expression of the OprD expression and increased expression of the multidrug efflux pumps. This confers resistance to carbapenem antibiotics, such as imipenem and meropenem, but it only would be apparent when Zn was also present, which is evident in Figure 3-5. Although this may not be the only mechanism contributing to combined meropenem and Zn resistance, it is highly possible that increased combined resistance in sites in the South Tyne was due to the expression of multidrug efflux pumps due to the presence of Zn, which implies the observed resistance to meropenem may disappear in cells after they are removed from the environment with elevated metals. This differs significantly from carbapenem resistance apparent in organisms that carry specific resistance genes like *bla-*NDM-1 that directly target the carbapenem and are not metal dependent (Yong *et al.*, 2009).

3.4   Conclusions

This study assessed Zn, meropenem, and combined meropenem and Zn resistance in sediment and water in the North and South basins of the River Tyne. Six months sampling in the watershed showed significantly higher abundances of Zn resistant organisms in sediments at sites FS, WA and RN in the South Tyne. Furthermore, WA and RN had greater numbers of culturable Zn resistant isolates relative to meropenem alone and combined meropenem and Zn resistant organisms, showing that baseline Zn resistance was dominant in these sites than the other types of resistance investigated. Significantly higher total, soluble and colloidal Zn levels were apparent at sites FS, WA, RN in the South Tyne, suggesting elevated Zn resistance and Zn levels are related at these sites.

Meropenem resistance did not differ among sites, including the low Zn sites in the North Tyne. However, higher combined meropenem and Zn resistance was evident in sediments from RN, a high Zn site in the South Tyne. Further, combined meropenem and Zn resistance correlated with total and soluble Zn concentrations across all sites, the latter displaying stronger correlations. This implies soluble Zn is more bioavailable and stress-inducing than total Zn, which appears to increase Zn resistance and, in turn, confers higher meropenem resistance due to Zn facilitated cross resistance. When looking at resistance in the river water

Zn resistance was also higher in the South Tyne, precisely WA and RN, and combined meropenem and Zn resistance was higher in FS, WA and RN. Meropenem resistance followed the same pattern as the sediment samples.

Chapter 4 Evolution of meropenem resistance in batch reactors seeded with different river sediments and levels of soluble zinc

4.1   Introduction

Many reports have suggested heavy metal contamination in natural environments can promote the development and dissemination of AR (Alonso *et al.*, 2001; Summers, 2002; Jain *et al.*, 2009), often being associated with high numbers of AR strains and high plasmid abundances relative to background conditions (Rasmussen and Sorenson, 1998). Pressure by elevated metal levels can promote increased levels of MR genes on gene vectors (i.e., plasmids), which allows MR to spread across bacterial communities (Alonso *et al.*, 2001; Martinez, 2009a). Given MR and AR genes are often found on similar plasmids, increased MR mobilisation also increases the mobilisation of ARGs in environmental bacterial communities as well as in human and other animal pathogens (Alonso et al., 2001; Martinez, 2009a; Martinez, 2009b). Heavy metals can, therefore, promote the selection of AR determinants in the environment and co-resistance of ARGs, which can be accelerated through HGT. This can allow the development and maintenance of AR without direct exposure to antibiotics (Alonso *et al.*, 2001; Jain *et al.*, 2009; Martinez, 2009a).

Evidence from previous work suggest there is a link between Zn contamination and AR. For example, Peltier *et al.* (2010) showed in waste treatment systems that systems with elevated Zn levels also display higher AR, while other work showed positive significant correlations between Zn levels and ARGs in soils (Knapp *et al.*, 2011). Furthermore, a link between Zn levels and meropenem was shown and assessed in the previous chapter (Chapter 3), where Zn, meropenem and combined Zn and meropenem resistance (combined resistance) was significantly higher in river sediments that contained high levels of Zn. Experimental studies where co-selection was induced, demonstrated that soil microbes from Cu amended-fields were more resistant to Cu and clinically important antibiotics, than those not exposed (Berg *et al.*, 2010).

This chapter focuses on the co-selection of AR and MR in bacterial communities in batch reactors that were seeded with different River Tyne sediments and amended with different levels of extraneous Zn. Therefore, this work aimed to assess whether sediment source (i.e., the North versus the South Tyne) or amended Zn was most responsible for observed levels of meropenem resistance over time. The specific objectives were as follows:

    a) Assess the link between Zn amendment, sediment source, and observed meropenem resistance over time in mixed, bench-scale batch reactors differentially

seeded with sediments from the North and South Tyne and amended with 2.00 mg/l and 100 mg/l of Zn, respectively (2 x 2 test matrix; in triplicate)

b) Test whether increased Zn levels, both soluble and total, correlate with observed meropenem resistance in sediment slurries

c) Contrast meropenem and Zn resistance in different reactors by using molecular biological and classical microbiological methods.

4.1.1 *The North and South Tyne basins*

The North and South Tyne river systems (described in detail in sections 3.1.1-3.1.4) in NE England are two geologically distinct sub-catchments, which are part of the larger Tyne catchment that spans an area of 2,933 km^2 (Macklin *et al.*, 1994; EA, 2008). The North Tyne catchment has no history of mining or industry and has broadly low heavy metal levels in its water and sediments, whereas the South Tyne catchment has much higher levels of Zn and Pb (especially) due to extensive historic mining over hundreds of years (Macklin *et al.*, 1994; Macklin *et al.*, 1997). Contrasting these two sites is ideal for examining how metals from mining, which have accumulated in sediments, might influence *in situ* MR and AR and also resistance development in seeded batch reactors.

4.1.2 *Combining molecular and classical microbiological methods*

In the previous chapter, resistance to Zn, meropenem and a combination of both was assessed using classical microbiological methods, which included culturing isolates from sediment and water samples. In this chapter, both microbiological and molecular methods were used to assess how microbial communities varied within seeded reactors based on extended exposure to different levels of Zn (2.00 mg/L and 100 mg/L) and seeded from different original sediments (North and South Tyne). Relative abundances of isolates resistant to 2.00 mg/L meropenem were quantified over time, which was contrasted with specific presumptive ARGs associated with meropenem resistance in other settings.

The first step to any molecular biological work is the extraction of DNA from environmental samples. Since the first published protocol 1980 (Torsvik, 1980), there have been many new methods developed that use a combination of chemical lysis, physical lysis and heat to extract DNA; methods being chosen depending on sample characteristics and the quality of DNA required. Nowadays, extraction kits, which consist of all reagents, tubes and protocols, combining physical and chemical lysis and DNA purification are typically used. However, DNA detection and interpretation require further methods.

The next step is to amplify specific nucleic acids, using a rapid and simple method known as polymerase chain reaction (PCR) (Mullis *et al.*, 1986). PCR is performed in three stages, including denaturation, annealing and extension. Denaturing of the extracted DNA takes place between temperatures of 93 and 95°C for several minutes, where the double-stranded DNA is denatured and two strands of complementary sequence are yielded. The annealing step then takes place, where temperatures are lowered to between 40 and 60°C and specific primers are allowed to bind to target sites on each complementary strand. Strand extension occurs using the enzyme DNA polymerase and is performed at 72°C, and is where the DNA fragments annealed to primers, are elongated and new complimentary strands are synthesised. The three-step cycle is then sequentially repeated, which results in many replicate copies of the original template DNA being created.

A fluorescent DNA binding dye is typically used in constructing the amplified product, which can then be detected by using gel electrophoresis and can be used in other culture independent techniques, such as community fingerprinting (Muyzer, 1999). One genetic fingerprinting technique is denaturing gradient gel electrophoresis (DGGE), where the overall microbial community structure can be assessed at a genetic level (Muyzer, 1999). DGGE works by separating double stranded DNA fragments of the same length, and using difference in bond strengths between Adenine and Thymine (AT) (contains two hydrogen bonds) and Guanine and Cytosine (GC) (contains three hydrogen bonds), to give distinct banding patterns that characterise genetic differences within the environmental sample community. These can then be interpreted using similarity statistical methods, such as Bray-Curtis (Muyzer *et al.*, 1993; Muyzer, 1999).

Furthermore, quantitative PCR (qPCR) can be used to quantity the abundance of specific genes present in environmental samples. This process is similar to PCR, however the amplified product can be detected and measured during the reaction progress, without the use of gel electrophoresis. In a qPCR reaction, a fluorescent DNA binding dye, such as SYBR Green is included, where the measured fluorescence reflects the amount of amplified DNA. The copy number of DNA is determined by using serial dilutions of a known template (a target with known DNA concentration) to generate a standard curve. In this chapter, DGGE was used to examine microbial community differences at a genetic level in different batch sediment-slurry reactors over time, whereas qPCR was used to assess the abundance of selected genes associated with ESBL resistance, *bla*-CTX-M, and carbapenem resistance, *bla*-NDM-1, which might be influenced by Zn and sediment source.

4.2 Materials and methods

4.2.1 *Sampling sites and collection*

Samples were collected from two contrasting sites in the Tyne basin, Warks Burn (WB; NY86117651; Figure 3-2b) in the North Tyne and River West Allen (WA; NY78045400; Figure 3-3b) in the historically mined area of the South Tyne basin (Figure 3-1). Duplicate sediment samples were collected in sterile 500-ml Nalgene plastic bottles (VWR, Leicestershire, UK) from each sampling site about 0.5–1 m from the river bank using plastic scoops. Nalgene plastic bottles were sterilised by autoclaving at 121°C for 15 minutes (Rodwell Scientific Instruments, Essex, UK), while the scoop was sterilised with 70.0% ethanol before each use. Samples were kept in a cool box for transportation after which they were kept at 4°C until needed the next day.

4.2.2 *Batch reactor set-up*

Four bench-scale reactors in replicates of three (n = 12) were established using 1-L Erlenmeyer flasks with cotton-stopper lids. R2A broth (Lab M Limited, Lancashire, UK) diluted to 20.0% with de-ionised water was the liquid growth medium. Typically, 500-ml of the medium was prepared in each flask, according to the manual's instructions, and pre-sterilised in the autoclave for 15 minutes at 121°C before sediment addition. The sediment collected from WB and WA was weighed out (Mettler-Toledo Ltd., Leicester, UK) and 400 g of sediment from each site was passed through a 2-mm sieve (Endecotts Ltd, London, UK) to remove any large stones and collected on a clean tray. Both the sieve and tray had been sterilised by spraying and wiping down with 70.0% ethanol. The sieved sediment from each site was then transferred into six labelled, sterile pre-weighed 50-ml falcon tubes (VWR, Leicestershire, UK).

All 12 tubes (two sites times six fractions) were then re-weighed to determine the wet weight of sediment in each tube and, 50.0 mg of wet sediment was then added to the appropriate reactor followed by 2.00 mg/L (low Zn) or 100 mg/L (high Zn) of Zn, prepared as described in section 3.2.5 (Table 4-1).

Table 4-1 Amendments of Zn added to each batch reactor.

Batch reactor	Zn amendments (mg/L)
Low Zn Warks Burn (WB)	2.00
High Zn Warks Burn (WB)	100
Low Zn West Allen (WA)	2.00
High Zn West Allen (WA)	100

A low Zn amendment of 2.00 mg/L was chosen, based on a study by Peltier *et al.* (2010), where 5.00 mg/L of Zn was used to assess the influence of Zn in activated sludge bioreactors. Another study also found that Zn had toxic effects on organisms in bench-scale activated sludge reactors at 10.0 mg/L (Cabrero *et al.*, 1998). Therefore a low value of Zn was necessary, to assess the influence of Zn on typical values rather than toxic levels and to ensure organisms did not select for resistance. The high Zn amendment for batch reactors was calculated by using the ratio of typical Zn concentrations in WA (12000 mg/kg) and WB (233 mg/kg) relative to the low Zn amendment (Equation 4-1) (Macklin *et al.*, 1994; Gozzard *et al.*, 2011).

Equation 4-1 The equation used to determine the concentration of Zn (mg/L) required for high Zn amended batch reactors using the Zn concentration required for the low amended batch reactors and typical total Zn (mg/kg dry sediment) levels for WA and WB.

$$\frac{High\ Zn\ amendment\ (mg/L)}{Low\ Zn\ amendment\ (mg/L)} = \frac{Total\ WA\ Zn\ concentration\ (mg/kg\ sediment)}{Total\ WB\ Zn\ concentration\ (mg/kg\ sediment)}$$

After all amendments were added, the level of liquid was marked on each batch reactor and noted for subsequent work and they were continuously mixed on a shaking incubator (Infors HT, Basel, Switzerland), set up to a speed of 121 rpm and a temperature of 20°C (Figure 4-1).

Figure 4-1 Photograph showing some of the batch reactors.

4.2.3 Sampling procedure and regime

Samples were taken from each reactor every 28 days for a total duration of 309 days, on a fill-and-draw basis, using a 10-ml Gilson pipette and sterilised cut tips. All reactors were removed from the shaking incubator and allowed to settle for 10 minutes before samples were taken for immediate cultural analysis, where 2.00 ml was added to the equivalent amount (2.00 ml) of prepared sodium pyrophosphate decahydrate ($Na_4P_2O_7 \cdot 10H_2O$; Sigma- Aldrich, St. Louis, MO, USA) in sterile 15-ml tubes (VWR, Leicestershire, UK). Samples of 4.00 ml were also taken for molecular and metal analysis and stored at -20°C until processed and analysed. Each reactor was amended with the equivalent amount of de-ionised water to replace the sample taken.

4.2.4 Quantifying relative meropenem resistance

Samples were analysed for meropenem resistance using classical spread-plating techniques (Section 3.2.4) according to the spread plate (9215C) standard method (APHA, 2005). All preparation and culturing were done in a class II microbiological safety cabinet (Labcaire Systems Ltd., North Somerset, UK), where for each set of samples, agar plates were prepared using R2A agar and amended with 40.0 mg/L cycloheximide (to suppress mould growth; Sigma- Aldrich, St. Louis, MO, USA) (Section 3.2.5) and the following agar amendments: no amendment (control) and 2.00 mg/L meropenem. Each agar plate was inoculated with 50-µl of each, serially diluted sample and incubated for three days at 24-27°C, chosen according to Reasoner and Geldreich (1985), where lower incubation temperatures and extended

incubation times, enhanced recovery and selection of environmental organisms (Section 3.2.5). Agar plates with roughly 30-300 colony forming units (CFU) per plate were counted (in accordance with APHA (2005) standard method 9215A) and the number of CFU/ml were calculated using Equation 3-3, followed by the calculation of the percentage ratio of CFU/ml in amended petri dishes relative to un-amended control petri dishes (Equation 3-4; section 3.2.6).

4.2.5 *Preparation and analyses of samples for metal analysis*

Prior to metal analysis, all glass vials (VWR, Leicestershire, UK) were prepared as described in section 3.2.7. The samples stored for metal analysis from each reactor were thawed and centrifuged at a speed of 13,000 rpm (Sartorius, Surrey, UK). The pellet was then re-suspended in 1-ml of de-ionised water and transferred to the "clean" glass vials, dried overnight at 104°C and re-weighed to determine the dry weight of solids present in each vial (Section 3.2.7).

The method of acid digestion using 1N HNO_3 (65.0%, Suprapur®, Merck, NJ, United States), according to the APHA (2005) standard method (3030 E.2) was performed on the dried sediment samples (Section 3.2.8). All acid digested samples were then filtered prior to analysis using 0.2-µm cellulose acetate membrane filters (VWR, Leicestershire, UK) and diluted by at least factor of ten for quantification. Soluble metal analysis was performed by filtering the remaining supernatant through 0.2-µm cellulose acetate filters and 0.50 ml of 1N HNO_3 was added to 1-ml of the filtered samples and filled to a volume of 5-ml with de-ionised water (Section 3.2.8). All prepared samples were analysed using an ICP-OES (Vista MPX CCD Simultaneous ICP-OES, Varian, Australia), for Cd, Co, Cu, Ni, Pb and Zn levels (Section 3.2.8).

4.2.6 *Extraction of DNA from samples*

Samples from each of the 12 batch reactors for days 0, 112, 204 and 309 were thawed and mixed using a vortex to allow for the samples to become homogenised. The DNA from each of these samples was extracted by using BIO 101 FastDNA Spin Kits (for soil; Q-Biogene, MP Biomedicals, Cambridge, UK). All tubes were labelled appropriately and 250 µl of each sample was added to separate Lysing Matrix E Tubes (provided in the DNA extraction kit), followed by 978 µl of sodium phosphate buffer and 122 µl MT buffer. The tubes were then secured in a Ribolyser (Fastprep ® Instrument or Hybaid Ribolyser) and processed for 30 seconds at a speed of 6.5. The rest of the process followed was according to the manufacturer's instructions. All solutions used, except the ethanol were provided in the kit, as

well as spin column tubes and collection tubes. Once completed the extracted DNA was kept at -20°C.

4.2.7 *Polymerase Chain Reaction (PCR) protocol*

PCR was used to amplify target DNA from all extracted DNA samples, in order to assess the prevalence of eubacterial (as 16S rDNA) genes present in each sample using DGGE. Megamix Blue (Microzone Ltd, UK) was used for the PCR reaction, which contains recombinant Taq polymerase that is required for the extension step in PCR, 220 μM deoxyribonucleoside triphosphates (dNTPs: where N is A, T, C, and G); and blue agarose loading dye in a 2.75 mM $MgCl_2$ buffer (required for the subsequent analysis of PCR products by agarose gel electrophoresis). Reaction volumes totalled 50.0 μl and, were made up of 47.0 μl of MegaMix Blue, 1.00 μl of DNA extracted from the sample and a primer mix of 1.00 μl forward primer and 1.00 μl of reverse primer, both diluted 10 times (Table 4-2). All preparations were done in a class II microbiological safety cabinet (Envair, Lancashire, UK), and positive and negative controls were included, where positive controls contained 1.00 μl of DNA extract, that contained the correct PCR fragment and negative controls contained 1.00 μl of nuclease free water, in place of the sample DNA. The PCR reactions were run with the appropriate primers (required to examine the products by DGGE) and thermo-cycler programme (Table 4-2) using a CFX96 Real-time System (Bio-Rad, Hertfordshire, UK).

Table 4-2 Primer sets used for PCR analysis of all samples, followed by their sequences and appropriate reaction conditions.

Primer	Batch	Sequence	Initial denaturation	Denaturation	Annealing	Extension	Number of cycles[1]
2/3-GC clamped (Muyzer *et al.*, 1993)	VR	5' - ATTACCGCGGCTGCTGG - 3'	95°C for 1 minute	95°C for 30 seconds	65°C for 1 minute and reduce by 1°C every 2nd cycle	72°C for 1 minute	24 cycles followed by a further 15 at annealing temperature of 53°C
	VF-GC	5' – CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG - 3'					

[1]Number of cycles for denaturation, annealing and extension steps.

4.2.8 *Agarose Gel Electrophoresis*

Products of the PCR reaction on the samples were examined firstly by agarose gel electrophoresis, in order to assess whether the correct sized gene had been amplified and for any reaction contamination. A 1.50% agarose gel was used, which was prepared by adding 1.50 g of agarose (pre-weighed) to 100 ml of 1x TAE buffer (2 M Tris-Acetate, 0.05 M EDTA, pH 8.3, Eppendorf Scientific Inc., New York, USA), and then heating the solution to melt the agarose. 20.0 µl of Nancy 520 DNA strain (Sigma- Aldrich, St. Louis, MO, USA) was added and the mixture was poured into a casting tray, containing combs for well formation and allowed to set for 30 minutes. Once set, the combs were removed and the tray was placed into the electrophoresis tank. The tank was then filled up to the level with 1x TAE solution and 7.00 µl of PCR product was added to each well. A reference PCR marker (PCR marker solution in loading buffer, P9577, Sigma- Aldrich, St. Louis, MO, USA; 8 fragment sizes, 50–2,000 base pairs) was used at either end of the gel, where 5.00 µl was added. Electrophoresis was run for 45 minutes at 100 volts (v) and the stained DNA was visualised by UV illumination (Bio-Rad Fluor-S ® MultiImager, Bio-Rad, Hertfordshire, UK).

4.2.9 *Denaturing Gradient Gel Electrophoresis (DGGE)*

PCR amplified products were first visualised with agarose gel electrophoresis (Sections 4.2.8) and then were examined using DGGE to assess the overarching diversity of bacterial communities and also assess dominant DNA bands from the samples. A 10.0% polyacrylamide gel (w/v) was prepared with a denaturing gradient of 30.0-60.0% (Table 4-3), which suited the primers and allowed for maximum band separation.

Table 4-3 Solutions used to prepare a 10.0% polyacrylamide gel denaturing gradient of 30.0-60.0%.

Solutions	Upper denaturing gradient solution 30.0%	Lower denaturing gradient solution 60.0%
40.0% Bis/Acrylamide	25.0 ml	25.0 ml
50x TAE buffer	2.00 ml	2.00 ml
Formamide (de-ionised)	12.0 ml	24.0 ml
Urea (molecular grade)	12.6 g	25.2 g
dH$_2$O (made up to)	100 ml	100 ml

The PCR amplified products containing 2/3 GC clamped primers (230 base pairs; section 4.2.7) were prepared in a 96-well plate by adding 11.0 µl of each PCR product to 11.0 µl of loading buffer (Thermo Scientific, Leicestershire, UK), followed by carefully loading the prepared gel with each 22.0 µl. A reference bacterial marker, designed by Fiona Read (School of Civil Engineering and Geosciences, Newcastle University) from cloned organisms, that contained 11 strong bands and maintained the same DNA concentrations, was run three or four times on each gel, at either end of the gel and in the centre. By running this marker and using the appropriate software (Section 4.2.12), gels can be corrected in case of any unevenness and lanes from more than one gel can be compared. The gel was run using a BioRad system and a Power Pac 3000 at 60°C using electrophoresis for 900v hours (200v per). Once complete the gel was removed from the gradient gel sandwich, and it was stained with a solution containing 200 ml of 1x TAE buffer and 20.0 µl of SYBR Green I (Sigma-Aldrich, St. Louis, MO, USA; diluted 1/10000 in 1 × TAE) in a staining tray covered with aluminium foil for 30 minutes. The stained gel was then viewed using a UV transilluminator, with the program 'Quantity One' (Bio-Rad Fluor-S MultiImager, Bio-Rad, Hertfordshire, UK) and photos of the gel were taken and stored.

4.2.10 *Quantitative polymerase chain reaction (qPCR)*

The abundance of eubacteria (as 16S rDNA), *bla*-CTX-M and *bla*-NDM-1 genes were analysed using qPCR for DNA samples from all reactors from days 0, 112, 204 and 309. All preparations were done in a class II microbiological safety cabinet (Envair, Lancashire, UK), and the Qiagility pipetting robot (Qiagen, Manchester, UK) was used to prepare 96-well plates (Bio-Rad, Hertfordshire, UK), all with triplicate samples, standards and non-template controls (NTCs). Total reaction volumes of 8.00 µl, were made up of 5.00 µl of SsoFast EvaGreen (which contained SYBR Green I, which binds non-specifically to double-stranded DNA; Bio-Rad, Hertfordshire, UK), 2.00 µl of nuclease free water and 0.50 µl of forward primer and 0.50 µl of reverse primer, both diluted 10 times. The appropriate volumes of SsoFast, primers and nuclease free water were calculated (depending on the number of samples) and were placed in the robot. In addition, the DNA extracts, diluted appropriately (x10, x50 or x100) were placed in the robot, where 2.00 µl of DNA was added to each well of the plate. For negative controls, 2.00 µl of nuclease free water was added instead of DNA.

Standards of seven different dilutions were used per plate (10^{+8}-10^{+2}) and per primer set to produce a standard curve, in order to quantify the relative abundance of target genes. Standards for 16S and *bla*-NDM-1 gene qPCR reactions were prepared from growing clones containing the necessary fragments and extracting and quantifying the concentrations of the plasmids (Section 4.2.11). Standards for *bla*-CTX-M qPCR reactions were prepared from DNA extracts containing the relevant gene (Section 4.2.11). Once prepared the reactions were run using a CFX96 Real time System (Bio-Rad, Hertfordshire, UK) with appropriate programs, determined for each set of primers (Table 4-4).

Table 4-4 Primer sets used for qPCR analysis of all samples followed by their sequences and appropriate reaction conditions.

Primer	Batch	Sequence	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles[1]
16S (Yu *et al.*, 2005; Huber *et al.*, 2007)	BAC338F	ACTCCTACGGGAGGCAGC	98°C for 3 minutes	98°C for 5 seconds	60°C for 10 seconds		40
	BAC1046R	CGACARCCATGCANCACCT					
bla-CTX-M (Birkett *et al.*, 2007)	CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	95°C for 1.3 minutes	95°C for 5 seconds	58°C for 10 seconds	72°C for 10 seconds	40
	CTX-M-R	ATCACKCGGRTCGCCNGGRAT					
bla-NDM-1 (Naas *et al.*, 2011)	NDM-1-F	ATTAGCCGCTGCATTGAT	95°C for 5 minutes	95°C for 15 seconds	60°C for 1 minute		50
	NDM-1-R	CATGTCGAGATAGGAAGTG					

[1]No.of cycles for denaturation, annealing and extension steps

The results from each qPCR reaction were then analysed using absolute quantification, where CT values of test samples were compared to the standard curve (produced from using relevant standards) and the gene abundance of the test samples (Starting Quantity; SQ) were determined. The gene abundances (copy number) per DNA extract were determined using Equation 4-2, and the value from this was used to calculate the gene abundance (copy number) per ml of sample (Equation 4-3). Further to this, the average and log gene copy-numbers per sample were determined.

Equation 4-2 Calculation used to determine the gene abundance per DNA extract, using the Starting Quantity (SQ), the dilution factor by which all samples were diluted by (10) and the final volume of extracted DNA (50 µl).

$$Gene\ abundance\ of\ extract = (SQ \times A) \times B$$

Where:

SQ = Starting quantity

A = Dilution factor

B = Volume of DNA (µl)

Equation 4-3 Calculation used to determine the gene abundance per ml of sample, using the gene abundance per DNA extracted (calculated using Equation 4-2) and the volume of sample used for the DNA extraction procedure (250 µl).

$$Gene\ abundance\ (ml) = \frac{gene\ abundance\ of\ extract}{A} \times 1000$$

Where:

A = Volume of sample used for DNA extraction procedure (µl)

4.2.11 *Preparation of qPCR standards*

In order to determine the abundance of genes for any set of primers using qPCR, standards of a known concentration were prepared. For 16S and *bla*-NDM-1 analysis *E .coli* plasmids with the appropriate gene fragments, were used. These were obtained from cloning DNA with the gene fragments into a 3956 base pair plasmid (pCR 4-TOPO), using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions. The plasmids were cultured in 50.0 ml of Luria-Bertani (LB) Broth (Oxoid Limited, Basingstoke, UK) amended with 50.0 mg/L ampicillin (Sigma-Aldrich, St. Louis, MO, USA) at 150 rpm and 37°C for 24 hours. To ensure the cultures were pure, they were streaked on LB agar plates with 50.0 mg/L ampicillin and incubated at 37°C for 24 hours. Both the LB broth and agar were prepared according to the manufacturer's

instructions, and a stock solution of 50.0 g/L ampicillin was prepared with de-ionised water and filter-sterilised (0.2-µm membrane filters; Pall Cooperation, MI, USA). All culturing and streaking were done in a class II microbiological safety cabinet.

After growth, the plasmids were extracted using the Roche High Pure Plasmid Isolation kit (Roche Diagnostics Limited, West Sussex, UK) and PCRs were carried out as described in section 4.2.7, but using different reaction conditions. The reaction conditions used for the plasmids containing 16S fragments were 95°C for 4 minutes, followed by 36 cycles of 95°C for 1 minute, 60°C for 45 seconds and a final 1 minute at 72°C for extension. The reaction conditions used for the plasmids with *bla*-NDM-1 fragments were 95°C for 3 minutes, followed by 31 cycles of 95°C for 30 seconds, 60°C for 1 minute and a final 1 minute at 72°C for extension.

For *bla*-CTX-M quantification, a PCR product was used, due to problems faced when cloning DNA using the TOPO TA Cloning Kit for Sequencing, as above. To produce standards of a known concentration using PCR, *E. coli* with the appropriate insert was cultured in 50.0 ml of LB broth at 150 rpm and 37°C for 24 hours and also streaked on LB agar plates and incubated at 37°C for 24 hours, to ensure purity. After growth, the DNA was extracted using a BIO 101 FastDNA Spin Kit and a PCR was also carried out (Section 4.2.7) with the appropriate primers (Table 4-4) and reaction conditions of 95°C for 3 minutes, followed by 31 cycles of 95°C for 1 minute, 58°C for 1 minute and a final 1 minute at 72°C for extension. All PCR products were examined by agarose gel electrophoresis, using a 1.50% agarose gel, reference PCR markers and negative and positive controls that were run for 45 minutes at 100v and visualised by UV illumination (Section 4.2.8). The *bla*-CTX-M PCR products were then cleaned-up using a QIAquick PCR Purification Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

The DNA concentration of the *bla*-CTX-M PCR product, as well as the original plasmids extracted for 16S and *bla*-NDM-1 were quantified using a Nano Drop ND-1000 Spectrophotometer (Thermo Scientific, Leicestershire, UK). The copy number per µl of sample was determined using Equation 4-3 and the volume of DNA needed to produce a 10^{+9} standard was determined using Equation 4-4.

Equation 4-4 Calculation of copy number per μl of sample DNA, using 6.023 × 1023 (molecules/mol), the concentration of DNA quantified (ng/μl) and the molecular weight (MW) of the fragment (g/mol) using Daltons (DA), the average weight of base pairs in DNA (660 Da × number of base pairs).

$$Copy\ number\ per\ \mu l\ of\ sample\ DNA$$
$$= \frac{6.023 \times 10^{23}\ (molecules/mol) \times Concentration\ of\ DNA\ (ng/\mu l)}{MW\ of\ fragment\ (g/mol)}$$

4.2.12 *Data analysis*

The CFU, metal and qPCR data were statistically analysed using Minitab (Version 17, Coventry, UK). Normality checks where performed to see if the data was normally distributed. Where data was normal, including the CFU and qPCR data, analysis of variance (ANOVA) sample tests were used, using Tukey's pairwise comparisons to assess differences between batch reactors and time-points. Where data was not normal, which included the Zn data, Mann-Whitney non-parametric test were used to compare metal abundances between batch reactors. All tests were conducted by assessing whether sample-pairs were significantly different to each other using 95.0% significance tests, where two samples were significantly different when p-values were < 0.050. Bi-variate correlation analysis also was performed to assess linear correlations and general trends among measured parameters. The Pearson's coefficient (value of r) signified the strength of the test, where the closer the coefficient to 1, the stronger the correlation, whereas p-values < 0.050 were significantly different.

DGGE images produced from the illumination of the gels (Section 4.2.9), were analysed using BioNumerics software (Applied Maths, Austin, USA). The software aligns sample lanes from a number of DGGE gels based on the marker lanes, producing a normalised image with band height quantification values. The values were then exported into Primer v6 software (Plymouth Routines In Multivariate Ecological Research version 6: PRIMER-E Ltd., Ivybridge, UK) to allow for diversity statistics, non-metric multi-dimensional scaling (MDS) analysis and analysis of similarity (ANOSIM) statistical tests (Clarke and Gorley, 2006). For non-metric MDS analysis, the band height quantification values were used to generate presence-absence data, which was used to produce Bray-Curtis similarities (Equation 4-5) and cluster analysis to produce MDS ordination plots.

Equation 4-5 Calculation of Bray-Curtis similarity values using presence-absence data generated from the band-height quantification values calculated from the BioNumerics software.

$Bray - Curtis\ similarity$

$$= \frac{2 \times Number\ of\ shared\ taxa\ (bands)}{Number\ of\ taxa\ (bands)\ in\ A + Number\ of\ taxa\ (bands)\ in\ B}$$

Ordination plots showed a visual representation of the similarities between reactors, time-points and Zn amendments. One-way ANOSIM were used to assess the differences between all samples taken from each reactor and, two-way crossed ANOSIMS, to assess the differences between samples taken on specific days versus sediment source and Zn amendments (Clarke, 1993).

4.3 Results and Discussion

4.3.1 *Total and soluble metal levels in batch reactors*

The total and soluble levels were measured in the batch reactors before Zn was added and throughout the study, from day 14 to 309 (all data shown in Table B-1 and Table B-2). Reactors with high Zn amendments and seeded with WB sediment had significantly higher total Zn levels of 1580 mg-Zn/kg dry sediment (S.E. ± 35.9) than reactors dosed with low Zn, where total Zn levels were 319 mg-Zn/kg dry sediment (S.E. ± 55.3; Mann-Whitney; p-value = 0.000; Figure 4-2). These levels were consistent with the reactor amendments, where one would expect high Zn to be present in reactors where 100 mg/L of Zn (high Zn) was dosed. However the total level of Zn prior to amendment was not significantly different to levels measured in low (Mann-Whitney; p-value = 0.117) and high (Mann-Whitney; p-value = 0.246) Zn-amended reactors seeded with sediment from WB (Figure 4-2).

Total Zn levels in reactors dosed with low Zn and seeded with WA sediment were significantly higher (12400 mg-Zn/kg dry sediment; S.E. ± 524) compared to levels measured in reactors dosed with low Zn and seeded with WB sediment (319 mg-Zn/kg dry sediment; S.E. ± 55.3; Mann-Whitney; p-value = 0.000; Figure 4-2).
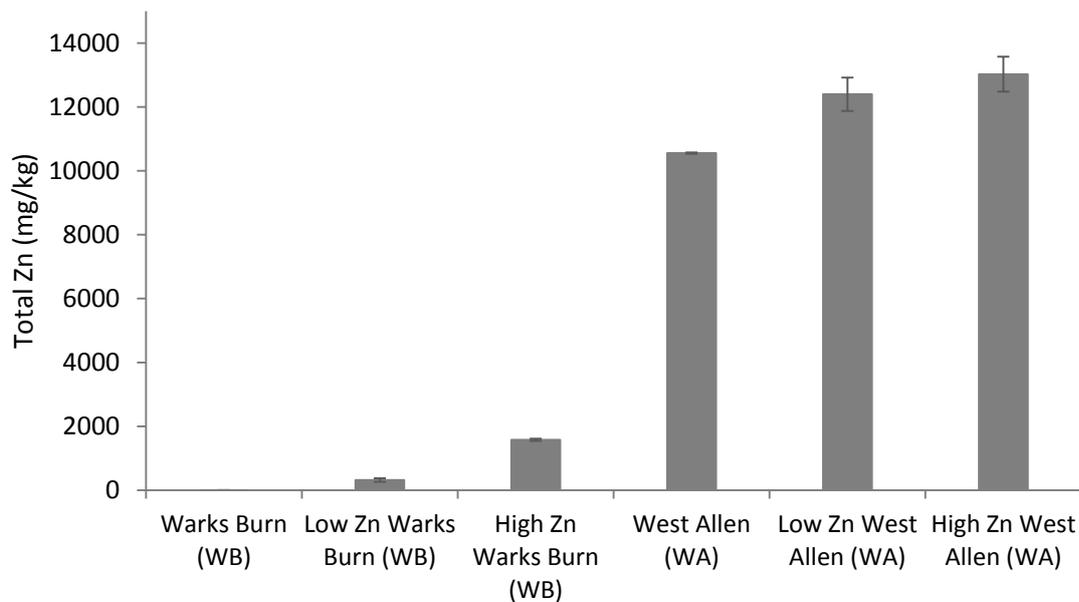
Figure 4-2 Measured total Zn levels (mg/kg) in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn. Values indicated for each condition are averages of triplicate reactors and also averaged data of all 309 sampling points. Standard error (S.E.) bars are shown.

Similarly there were also higher levels of Zn present in reactors amended with high Zn and seeded with sediments from WA (13000 mg-Zn/kg dry sediment; S.E. ± 547) compared to those measured in reactors with low Zn and WB sediment (319 mg-Zn/kg dry sediment; S.E. ± 55.3; Mann-Whitney; p-value = 0.000; Figure 4-2). Therefore, there is wide contrast of Zn conditions, which was intended in the experiment.

Overall, Zn levels are consistent with historic information from the river, which reflect levels approaching those in the South Tyne (Macklin *et al.*, 1994; Macklin *et al.*, 1997) as well as with the previous study (Chapter 3; section 3.3.5). Despite the different Zn amendments, in reactors seeded with WA sediment, there were no significant differences between the low and high Zn batch reactors seeded with WA sediments (Mann-Whitney; p-value = 0.658); however, there were significantly lower levels of Zn measured in WA sediment of 10600 mg-Zn/kg dry sediment (S.E. ± 21.1) compared to those measured in reactors seeded with the same sediment and amended with low (12400 mg-Zn/kg dry sediment; S.E. ± 524; Mann-Whitney; p-value = 0.000) and high (13000 mg-Zn/kg dry sediment; S.E. ± 547; Mann-Whitney; p-value = 0.000) Zn (Figure 4-2). It must also be noted that levels of total Zn measured prior to amendment were considerably higher than those concentrations measured in sediment from WB and WA in Chapter 3. Despite the collection of sediment from the same area, fluvial processes and weather conditions may have caused erosion or transportation of

sediment, resulting in heterogeneous sediment samples, with higher Zn content, being collected.

The analysis of the soluble Zn levels from each reactor over time showed that reactors dosed with high Zn and seeded with WB sediment had significantly higher soluble Zn levels than reactors dosed with low Zn (Mann-Whitney; p-value = 0.001), where the measured average level over 309 days was 1.29 mg/L soluble Zn (S.E. ± 0.34; Figure 4-3). In addition soluble Zn levels were also higher in reactors dosed with low Zn and seeded with WA sediment (0.77 mg/L; S.E. ± 0.29), compared with those dosed with low Zn and seeded with WB sediment (0.11 mg/L Zn; S.E. ± 0.02; Mann-Whitney; p-value = 0.006; Figure 4-3).
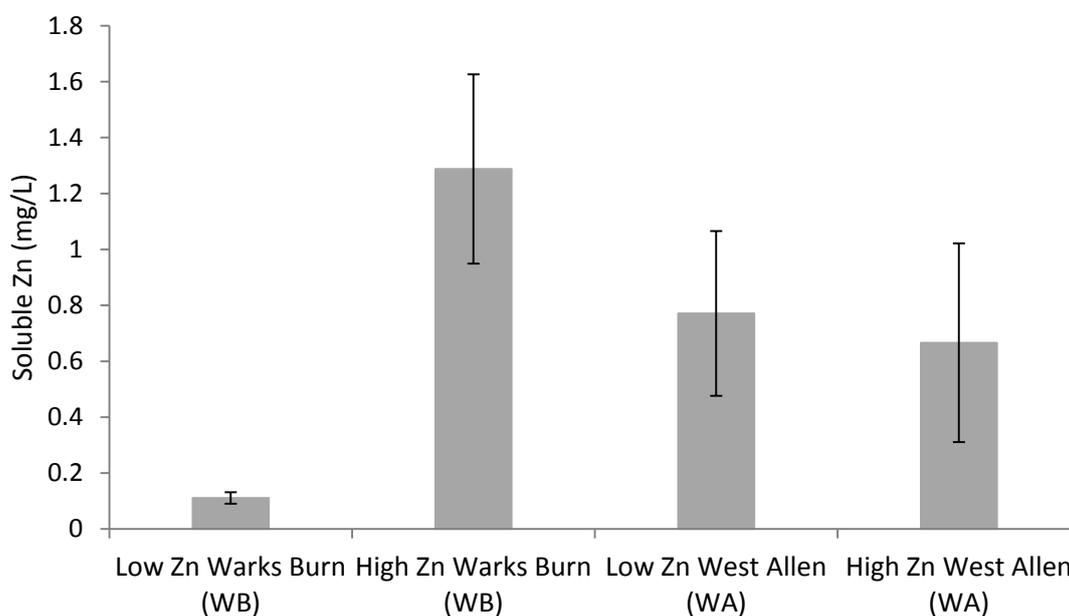
Figure 4-3 Measured soluble Zn levels (mg/L) in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn. Values indicated for each condition are averages of triplicate reactors and also averaged data of all 309 sampling points. Standard error (S.E.) bars are shown.

Soluble Zn levels were higher in reactors amended with high Zn and seeded with WA sediment (0.67 mg/L soluble Zn; S.E. ± 0.36) compared to those measured in reactors with low Zn and WB sediment (0.11 mg/L Zn; S.E. ± 0.02; Mann-Whitney; p-value = 0.004; Figure 4-3). Interestingly soluble Zn levels were higher in reactors with high Zn and WB sediment compared to reactors with high Zn and seeded with WA sediment, however these were only significant at a 0.10% level (Mann-Whitney; p-value = 0.093; Figure 4-3). In reactors seeded with WA sediment, no significant differences were observed between the low and high Zn amended reactors (Mann-Whitney; p-value = 0.859) and, soluble Zn levels were actually lower in reactors containing the highest Zn amendment (0.67 mg/L soluble Zn; S.E. ± 0.36) compared to the levels measured in low Zn amended reactors (0.77 mg/L; S.E. ± 0.29;

Figure 4-3). There were also differences between total and soluble Zn measured in all reactors, with total Zn levels significantly higher than soluble Zn levels (Mann-Whitney; p-value = 0.000; Figure 4-3). In summary, a significant contrast in Zn conditions was developed across the 12 reactors, which was the intent of the experimental design.

4.3.2 *Observed relative meropenem resistance in batch reactors*

In reactors dosed with low Zn and WB sediment, relative meropenem resistance levels progressively increased with time, although percent levels did not substantially increase after 169 days. The highest percent resistance was observed at day 309 (13.7%; S.E. ± 2.52; Figure 4-4).
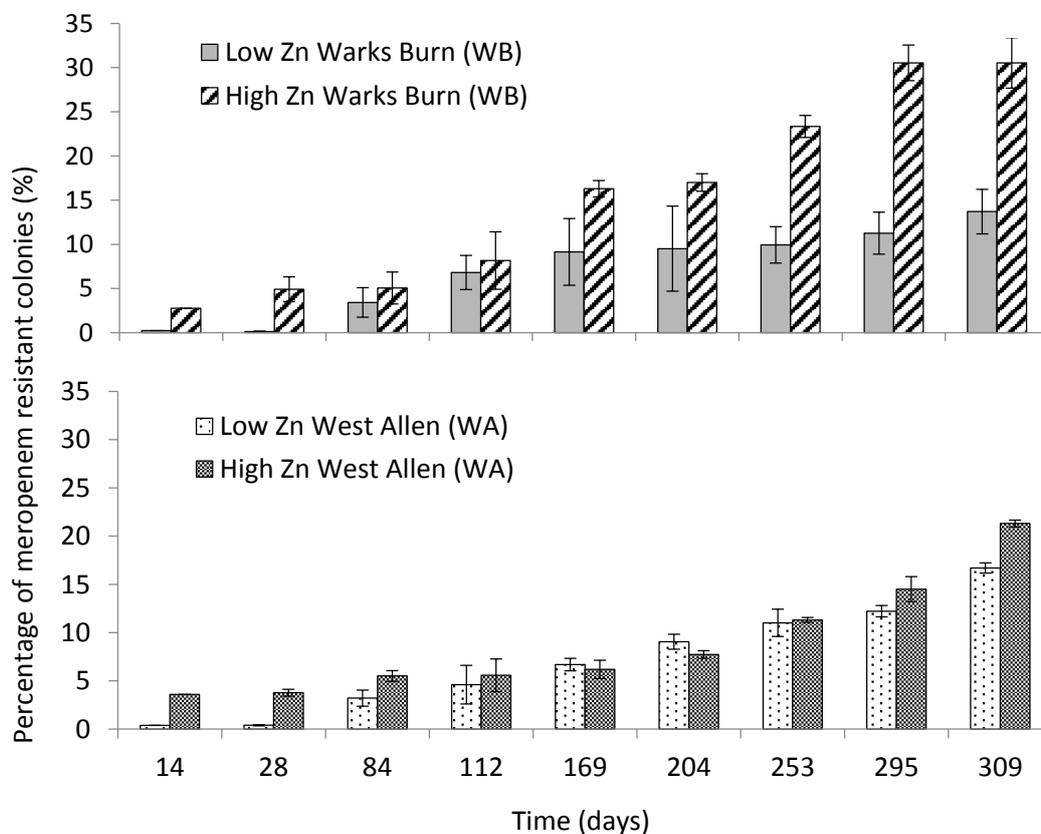
Figure 4-4 Percentage (%) of colonies resistant to 2.00 mg/L meropenem over a time period of 309 days, in reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn. Values indicated for each time point are averages of triplicate reactors (all data shown in Table B-3) and standard error (S.E.) bars are shown).

In reactors dosed with high Zn and WB sediment, meropenem resistance also increased with time, with the highest resistance observed at days 295 (30.5%; S.E. ± 2.01) and 309 (30.5%; S.E. ± 2.85; Figure 4-4). Resistance on days 253, 295 and 309 in reactors with high Zn and WB sediment were significantly higher than that observed on days 14, 28 and 84 (ANOVA;

Tukey's comparison; p-values < 0.05; Table B-4; Figure 4-4). In addition, resistance on days 295 and 309 were also significantly higher than resistance observed on day 112 (ANOVA; Tukey's comparison; p-values < 0.05; Table B-4; Figure 4-4) in reactors with high Zn and WB sediment. Therefore apparent percent meropenem resistance increased with time in reactors amended with high levels of Zn and seeded with WB sediment.

Resistance in reactors seeded with high Zn and WB sediment also were statistically higher on days 309 (30.5%; S.E. ± 2.85; ANOVA- Tukey's comparison; p-value = 0.033) and 295 (30.5%; S.E. ± 2.01; ANOVA- Tukey's comparison; p-value = 0.005) compared to resistance observed in reactors dosed with low Zn and WB sediment on the same days (13.7%; S.E. ± 2.52 and 11.3%; S.E. ± 2.37, respectively; Figure 4-4). This suggests that the level of Zn amended in reactors on those days did have an effect on apparent meropenem resistance, with higher levels of Zn resulting in higher percent meropenem resistant isolates.

In reactors dosed with low Zn and WA sediment, meropenem resistance increased from day 14 to day 309, with significantly higher meropenem resistance observed on day 309 (16.7%; S.E. ± 0.52) compared to that observed on day 14 (ANOVA- Tukey's comparison; p-value = 0.048) and 28 (ANOVA- Tukey's comparison; p-value = 0.049; Figure 4-4). The trend was similar in reactors dosed with high Zn and WA sediment, where meropenem resistance increased with time, with the highest on day 309 (21.3%; S.E. ± 0.35). Resistance at this time-point was significantly higher than resistance observed on days 14 (ANOVA- Tukey's comparison; p-value = 0.017), and 28 (ANOVA- Tukey's comparison; p-value = 0.019; Figure 4-4). Thus reactors amended with high levels of Zn and seeded with WA sediment, also showed similar patterns to resistance observed in reactors seeded with WB sediment, where apparent meropenem resistance increased with time.

Resistance in reactors dosed with high Zn and seeded with WA sediment were also statistically higher on day 295 (14.5%; S.E. ± 1.30; ANOVA; Tukey's comparison; p-value = 0.044), than in reactors with low Zn on that day (12.2%; S.E. ± 0.59; Figure 4-4), further suggesting that the level of Zn has an effect on the level of resistance seen.

4.3.3 *Relationships between meropenem resistance and Zn levels*

The culturing data show that relative percent meropenem resistance increased in all reactors over time. In addition the reactors dosed with the higher Zn levels had increased levels of relative meropenem resistance than those with lower Zn levels. When combining data from all reactors, there was no significant correlation between percent meropenem resistant isolates

and total Zn levels (Pearson's correlation = -0.294; p-value = 0.354; Figure 4-5a) which suggests that total Zn did not directly influence the apparent level of meropenem resistance.
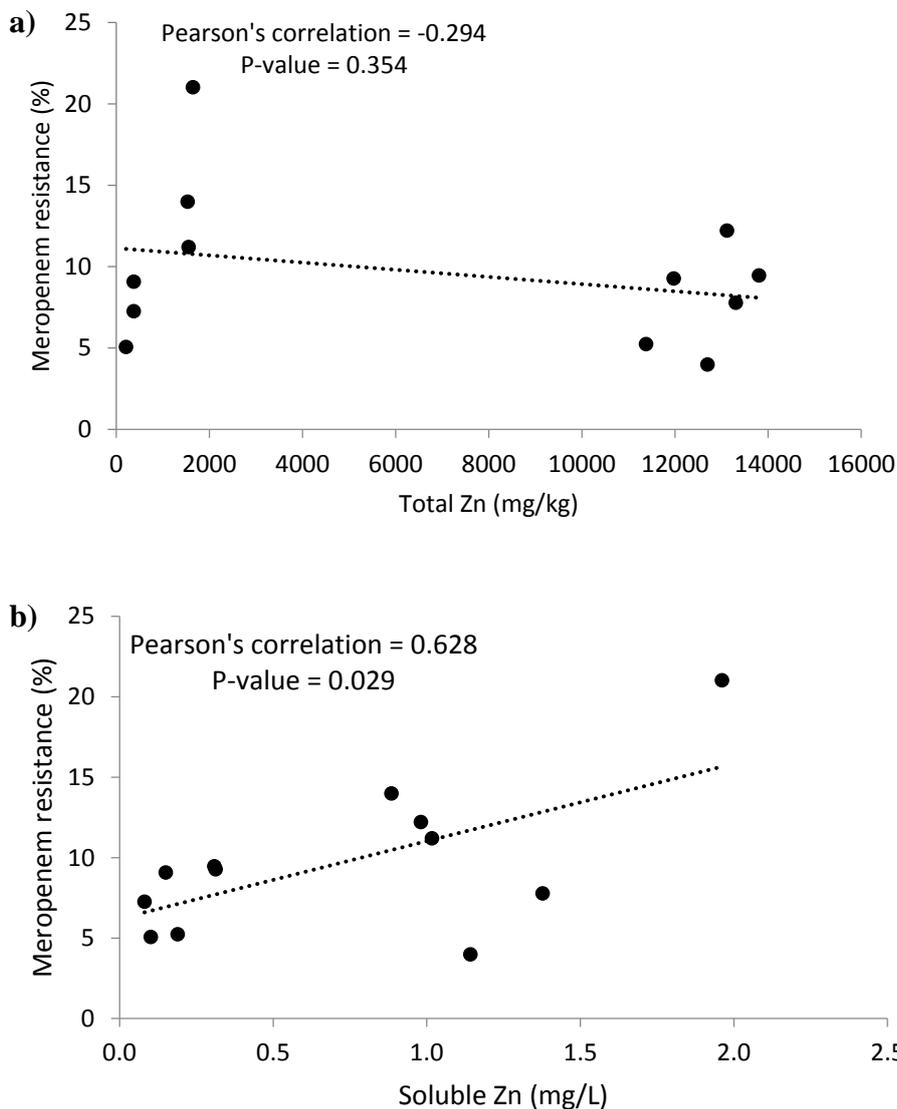
Figure 4-5 Pearson's correlations (r) between meropenem resistance and (a) total Zn (mg/kg) and, (b) soluble Zn (mg/L) in reactors seeded with sediment from Warks Burn (WB) and West Allen (WA) and amended with low (2.00 mg/L) and high (100 mg/L) Zn. Total and soluble Zn data used are averages from Zn measured over all time-points for each reactor.

However, when looking at the measured soluble Zn levels in all reactors, a positive significant correlation was demonstrated between percent meropenem resistant isolates and total soluble Zn levels (Pearson's correlation = 0.628; p-value = 0.029; Figure 4-5b). This suggests that the soluble fraction of Zn may be driving meropenem resistance, rather than total Zn levels in the sediment or the reactor seed sediment. Previous studies (described in Chapter 3) showed a similar correlation between soluble and colloidal Zn levels and combined meropenem and Zn resistance (combined resistance) in actual sediments from parallel river sediment sites (Section 3.3.5).

When combining data from the reactors seeded with WB sediment only, correlation analysis showed that the relationship between relative meropenem resistant isolates and soluble Zn is most apparent in reactors seeded with WB sediment, where highly significant, positive correlations were observed (Pearson's correlation = 0.953; p-value = 0.003; Figure 4-6).
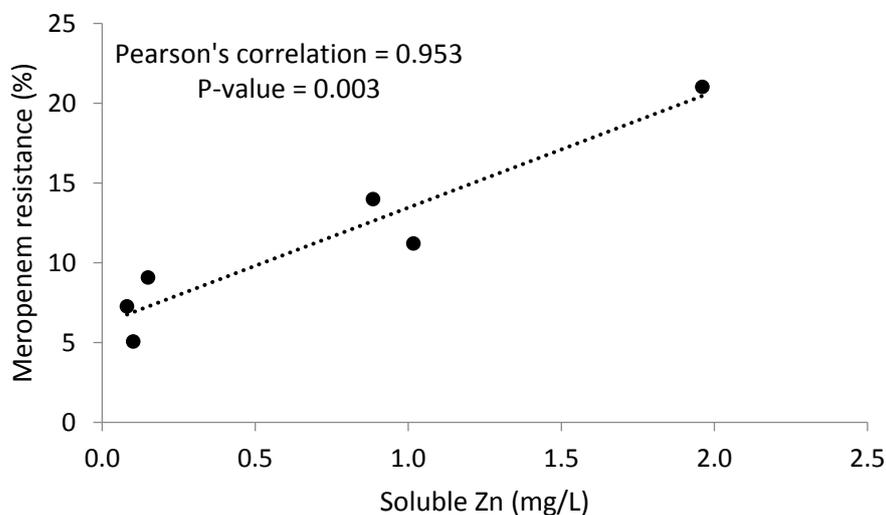
Figure 4-6 Pearson's correlation (r) between meropenem resistance and soluble Zn (mg/L) in reactors seeded with sediment from Warks Burn (WB). Soluble Zn data used are averages from Zn measured over all time-points.

However, when combining data from the reactors seeded with WA sediment only, correlations between meropenem resistant isolates and soluble Zn were not statistically correlated (Pearson's correlation = -0.052; p-value = 0.922). Average measured soluble Zn levels in these reactors seeded with WA sediment, showed that indeed reactors amended with high levels of Zn had lower soluble Zn levels (0.67 mg/L; S.E. ± 0.36) than reactors amended with low levels of Zn (0.77 mg/L; S.E. ± 0.29). The concentrations and bioavailability of heavy metals in sediments and water can influenced by environmental factors, including the pH and the level of oxygen (Seiler and Berendonk, 2012). Therefore this relationship suggests that high native total Zn levels present in these sediments (10600 mg/kg-Zn) as well as such environmental factors may be influencing the amount of available Zn present in the reactors and to microbial communities.

4.3.4 *The effect of batch reactor seed source on meropenem resistance*

Reactors amended with high soluble Zn and seeded with WB sediment generally had higher resultant meropenem resistance than those seeded with WA sediment, where meropenem resistance observed on day 295 in reactors with high Zn and WB sediment was significantly higher, at 30.5% compared to resistance observed in high Zn and WA reactors, at 14.5%

(Section 4.3.2; Figure 4-4). However, resistance levels in reactors amended with low levels of Zn and respective sediments were not that different.

This trend suggested that resistance was higher in reactors seeded with WB a relatively pristine and low metal sediment when further soluble Zn was added. This can be compared to a similar study by Knapp *et al.* (2011) where it was firstly demonstrated that relatively low total metal levels correlated with ARG abundances in soils. The study further suggested that low metal levels may also co-select for AR. The level of resistance seen in these reactors, could be due to the amount of available Zn present in the environment of each reactor, rather than the total metal concentrations in the seed. Average soluble Zn levels in reactors with WB sediment and amended with high Zn were higher (1.29 mg/L) than the soluble Zn in reactors from WA sediment and high Zn (0.66 mg/L), and therefore more Zn was available to organisms in these reactors. As mentioned above the solubility of heavy metal cations, such as Zn are influenced by many factors, such as pH, oxygen level, and pollutants. For example the presence of organic matter can act as a 'sink' for metals and may result in the reactor environment becoming anaerobic due to high decomposition. This can influence the oxygen level and the redox potential, which in turn reduces the solubility of Zn (Schulz-Zunkel and Krueger, 2009; Seiler and Berendonk, 2012). In contrast, low pH values can increase the solubility of Zn.

Furthermore, polluted sediments contain high anions levels, which can also influence Zn availability, by binding with soluble Zn. (Schulz-Zunkel and Krueger, 2009). As sediments from WA contain high native total Zn levels, due to previous industrial activities, it is possible that they contain other contaminants, which may be influencing the available Zn, which in turn impacts the level of meropenem resistance. Further analysis, including anion analysis, DO and pH measurements are needed to estimate the impact of these factors on Zn availability.

The fact that resistance did not differ between sediment seed in reactors where low levels of Zn were added, suggests that the type of sediment used did not influence the relative percent meropenem resistance in isolates. Therefore the level of native Zn in sediments had less of an impact on meropenem resistance, than the addition of soluble Zn to the reactors.

4.3.5 *The effect of batch reactor Zn amendment on meropenem resistance*

In reactors dosed with high levels of 100 mg/L Zn and seeded with WB sediment, there was an increase in meropenem resistance over time (Section 4.3.2; Figure 4-4). Similarly the same pattern was seen, where reactors seeded with high Zn and WA sediment, had significantly

higher levels of meropenem resistance over time than reactors amended with low Zn (Section 4.3.2; Figure 4-4). Therefore, this suggests that the increased level of Zn (100 mg/L) was likely responsible for the increased resistance. However, increased resistance was only statistically higher on days 253, 295 and 309, in both WB and WA sediments (Appendix B3; Table B4 and Table B5).

One reason for this increased resistance observed on those days could be due to a change in the microbial community. The survival of microorganisms depends on intrinsic factors, such as the availability of food and extrinsic factors such as temperature and pH (Chung *et al.*, 2006). As nutrients were not replenished in reactors after sample collection every month, the community was probably under starvation and nutrient stress. Under such stressed conditions, microorganisms use signal systems to sense environmental stresses that in turn control the expression of genes involved in cellular defence mechanisms (Kennelly and Potts, 1996).

When looking at the number of isolates present in each reactor, over all the time-points, there was a decline in bacteria, of an average two orders of magnitude (Figure 4-7).
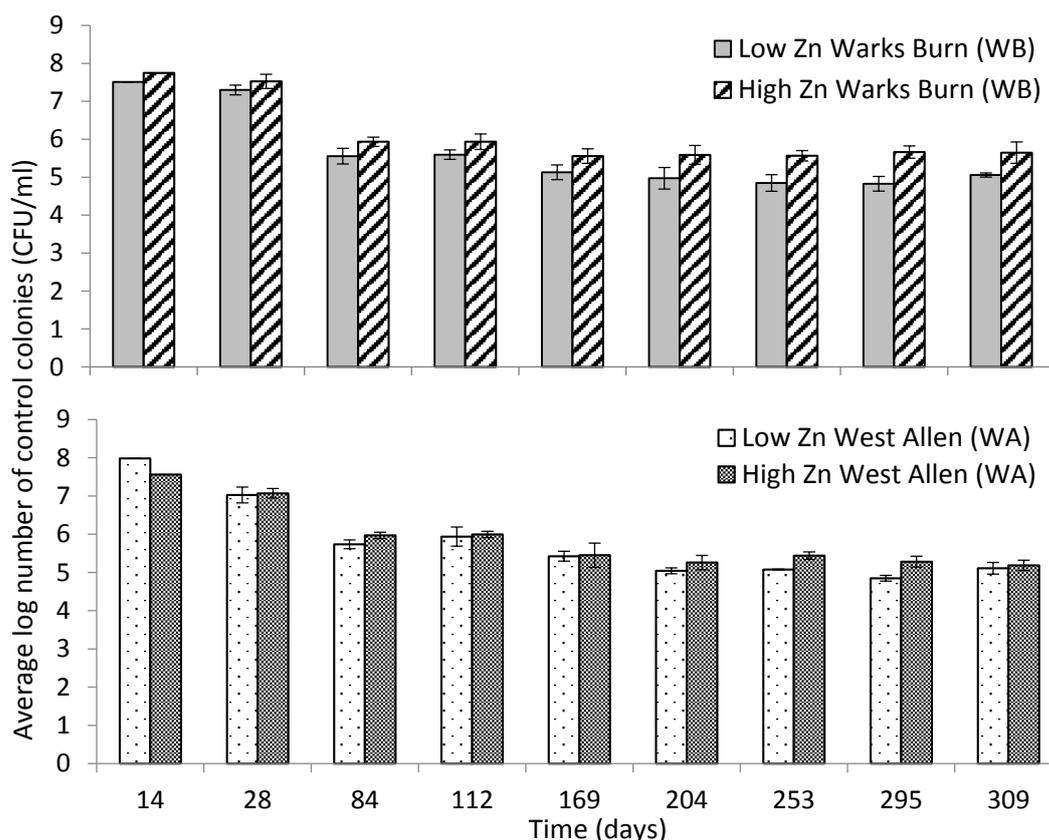
Figure 4-7 Logarithmic number of colonies present on non-amended agar plates (control colonies) over a time period of 309 days, in reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn. Values indicated for each time point are averages of triplicate reactors and standard error (S.E.) bars are shown.

This decline could be due to increased competition for food and nutritional resources, which is the main focus of microbial competition, and many mechanisms, including the production of antibiotics, motility and co-ordinated behaviour are used by bacteria to acquire these resources (Hibbing *et al.*, 2010).

As well as nutritional stress, the community within these sediments were also under high metal stress, due to the high level of soluble Zn, therefore only bacteria capable of tolerating these high levels would survive. Such bacteria employ cell defence mechanisms, such as metal ion homeostasis factors and MR determinants to reduce and regulate intracellular metal accumulation in their cells (Nies, 1999). Zn specifically can be removed from cells using the CDF and RND efflux pumps (Nies *et al.*, 1987; Hassan *et al.*, 1999; Nies, 1999; Mergeay *et al.*, 2003).

The results from this work can be compared to the river sediment studies described in Chapter 3, where bacteria within sediments with high levels of Zn showed increased resistance to combined meropenem and Zn. The results can also be compared to similar studies looking at the link between high metal levels and increased MR and AR. For example experimental studies done in Cu amended soils, found that there were increased numbers of Cu resistant bacteria and an associated increase of antibiotic resistant bacteria relative to non-amended control soils (Berg *et al.*, 2005). This suggested that indirect selection for antibiotic resistance occurred during the field experiment (Berg *et al.*, 2005). A further study demonstrated that soil microbes from a Cu contaminated site were more resistant to Cu and clinically important antibiotics, including vancomycin and tetracycline than the associated control site (Berg *et al.*, 2010).

Some possible reasons for this resistance, observed in Chapter 3 included, cross resistance and the influence of co-regulation mechanisms with the bacterial community. In this case, the presence of high Zn resulted in the community showing increased resistance to meropenem over time, despite the source of the sediment. Therefore it is possible that the bacteria both under metal and nutrient stress developed resistance due to the up-regulation of a combination of altered OM and RND efflux pumps (Nikaido, 1994; Saier Jr *et al.*, 1994). This data can also be compared to a study by Perron *et al.* (2004) (described previously in Chapter 3), where it was revealed that the presence of a two component sensor protein *czcS*, responsible for the regulation of a heavy metal efflux pump, had resulted in a co-regulation between carbapenem influx and heavy metal efflux.

Generic stress as well as abiotic factors, including temperature, pH, nutrient availability, oxygen and moisture content and, biotic factors, including commensal and mutualistic

relationships among the microorganisms can also contribute to increased rates of HGT, which can result in the transfer of ARGs to other organisms (Aminov, 2011). For example, work by Ilves *et al.* (2001) showed that the mobility of transposons were affected by starvation. Stress factors such as the induction of the SOS response or the deprivation of certain enzymes can also lead to further HGT of DNA (Melechen and Go, 1980). Further the SOS response also controls the recombination of resistance gene cassettes such as integrons which are common and abundant in environmental organisms (Stokes *et al.*, 2001; Holmes *et al.*, 2003; Guerin *et al.*, 2009; Gillings, 2014). These integrons tend to be linked to mobile elements, such as transposons (Mazel, 2006) and can enhance the potential exchange of resistant cassettes (Guerin *et al.*, 2009). Furthermore the evolution of these integrons is further driven by their exposure to selective agents, such as heavy metals, where metals such as Zn can co-select for ARGs, as these resistance genes are carried on mobile elements that also carry MR genes (Baker-Austin *et al.*, 2006; Wright *et al.*, 2008; Rosewarne *et al.*, 2010; Seiler and Berendonk, 2012; Gillings, 2013). Therefore it is possible that such gene cassettes contained within integrons are present in some of the bacteria within these reactors, which can be a vital component of bacterial adaptation; for example in polluted marine sediments, cassettes can encode a variety of functions responsible for the catabolism of industrial waste (Koenig *et al.*, 2009).

Although these may not be the only mechanisms contributing to meropenem resistance, it is possible that increased resistance in the batch reactors amended with high Zn was due to the expression of efflux pumps associated with Zn removal and increased HGT, as a result of increased microbial stress. Therefore a key piece of future work would be to quantify known efflux pump genes over all time-points and in all reactors. The results from this study support the trends seen in Chapter 3, where it is clear that increased Zn levels in both studies increased the abundance of meropenem resistant organisms.

4.3.6 *Diversity of microbial communities within batch reactors*

Despite the same general sediments being assessed *in situ* in Chapter 3 work, it is very probable that dominant organisms in the reactors are different than those seen in the environment, due to the significant different habitat conditions. To assess how the communities might have changed in the reactors relative to the source, samples from day 0, 112, 204 and 309 of each batch reactor were analysed using DGGE.

The visualising of the DGGE gels showed that reactors seeded with WB sediment and low and high Zn all have some similar dominant bands (Figure 4-8). When looking at each reactor specifically, there were distinct differences in bands observed on day 0 compared to days 112,

204 and 309, at which there are more bands (Figure 4-8a). In reactors amended with high Zn there are also differences in bands observed at day 0 compared to days 112, 204 and 309 (Figure 4-8b). In reactors seeded with WA sediment there were also some dominant bands that were present throughout the study (Figure 4-9). However there were differences in bands observed over time, such as bands observed in reactors amended with low Zn, although bands were more closely related at days 204 and 309 (Figure 4-9a). In reactors amended with high levels of Zn, bands were distinctly different between day 0 and days 112, 204 and 309 (Figure 4-9b). Therefore this suggests that apparent dominant organisms may have changed throughout the experiment, compared to what they were at the start (day 0).
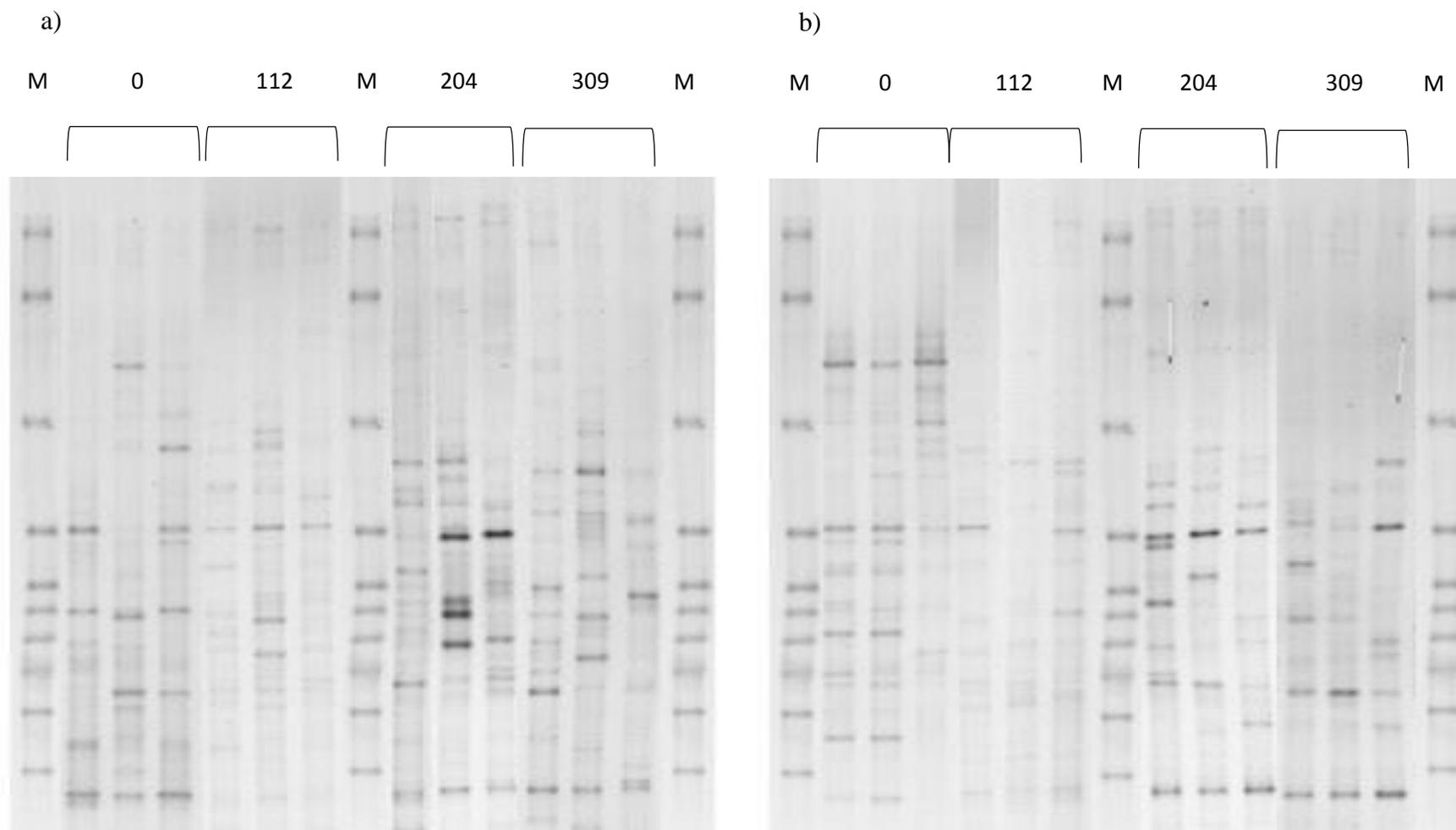
Figure 4-8 DGGE gels of 16S rDNA products from batch reactors seeded with WB sediment and amended with, a) low Zn and b) high Zn for days 0, 112, 204 and 309. Markers (M) on either side and in the centre.
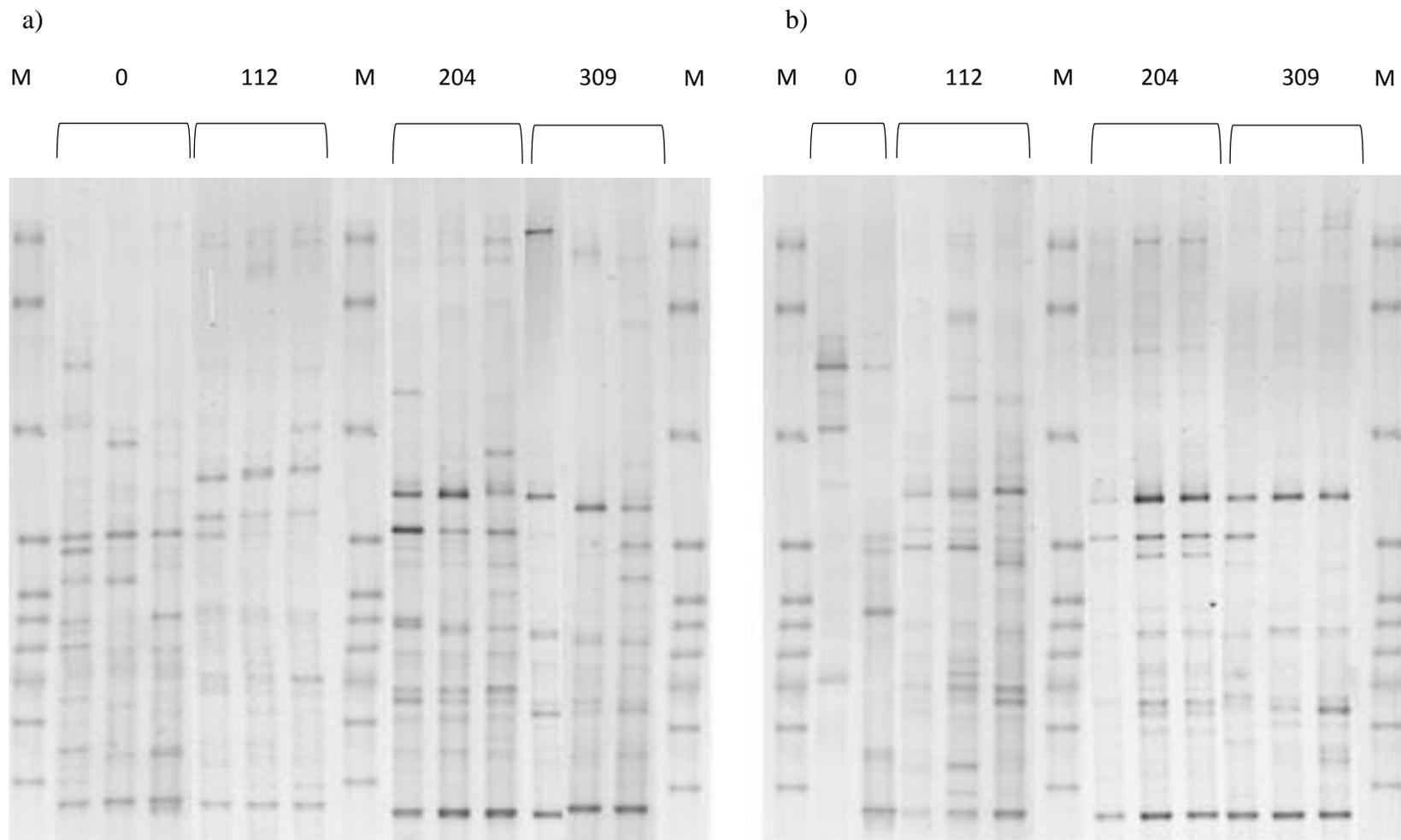
Figure 4-9 DGGE gels of 16S rDNA products from batch reactors seeded with WA sediment and amended with, a) low Zn and b) high Zn for days 0, 112, 204 and 309. Markers (M) on either side and in the centre.

The analysis of the presence and absence data, obtained from the visual representation of each sample and using BioNumerics and Bray-Curtis similarity tests was expressed using MDS ordination plots (Figure 4-10). In order to express how well the data is represented by the statistical analysis, a goodness of fit statistic was used, known as a stress value. For MDS ordination plots, the stress value is based on the differences between the actual distances and their predicted values. Here the stress value of 0.24 on the MDS ordination plot, suggests that the spatial representation of the data was good and not random (values close to 0 = excellent, 0.1 = good, <0.3 = poor).

Two-way crossed ANOSIM tests comparing the time points (0, 112, 204 and 309) and the sampling sites (WB and WA), showed that the apparent diversity in reactors seeded with sediment from WB on days 112, 204 and 309 were significantly different to diversity on day 0 (ANOSIM; all p-values = 0.010; Figure 4-10). Furthermore, apparent community characteristics on day 309 significantly differed to those seen on day 112 (ANOSIM; p-value = 0.010; Figure 4-10). In reactors seeded with sediment from WA, community characteristics significantly differed between each time point (ANOSIM; all p-values = 0.010; Figure 4-10). This shows that there were increasing differences in community species composition as time proceeded, especially in reactors seeded with WA sediment.

When comparing the time points and Zn amendments (low and high), reactors dosed with low Zn (2.00 mg/L) had significantly different apparent community characteristics at day 0 compared to days 112, 204 and 309 (ANOSIM; all p-values = 0.010; Figure 4-10). In addition, characteristics of samples from day 309 were significantly different that samples taken on days 112 and 204 (ANOSIM; p-values = 0.010 and 0.040 respectively; Figure 4-10), therefore apparent communities changed over time throughout the experiment. Finally, community conditions in reactors dosed with high Zn (100 mg/L) significantly differed between each time point (ANOSIM; all p-values = 0.010; Figure 4-10), which implies the Zn dose influenced communities in the reactors, especially in reactors dosed with high Zn.
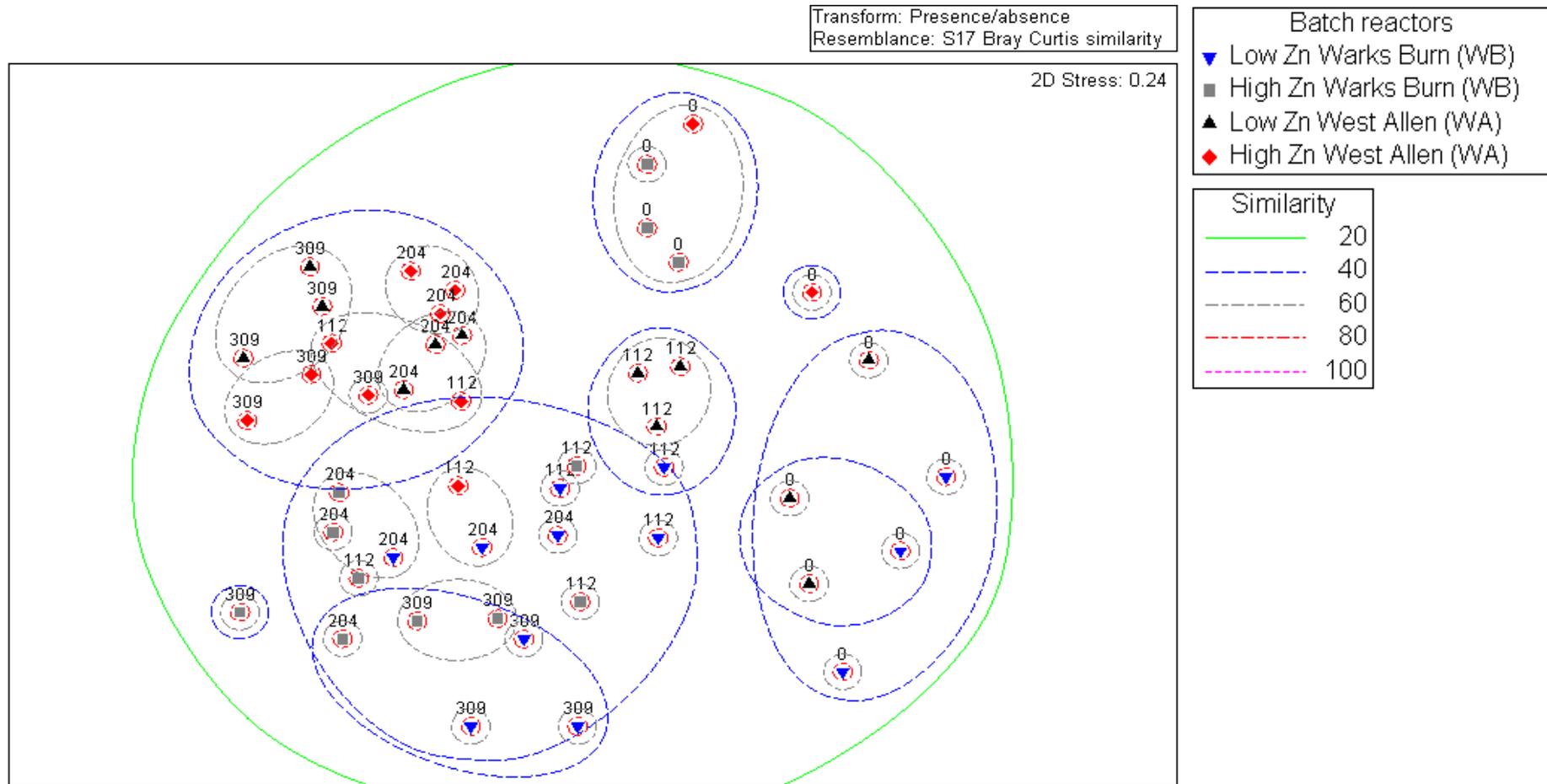
Figure 4-10 Multi-dimensional Scaling analysis ordination plot, with overlay of cluster analysis. Contours of 20, 40, 60 and 80 showed similarities between samples taken on days 0, 112, 204 and 309 from batch reactors seeded with Warks Burn (WB) sediment and dosed with low Zn (blue) and high Zn (grey) and, batch reactors seeded with West Allen (WA) sediment and dosed with low Zn (black) and high Zn (red).

When comparing the time points and the sampling sites plus their Zn amendments, one-way ANOSIM tests showed that there were trends between all samples, although differences were only apparent at a 90.0% confidence level (ANOSIM; all p-values = 0.100; Figure 4-10). However there were no differences in apparent diversity at day 0, between reactors seeded with WB and WA sediments and amended with low Zn (ANOSIM; p-value = 0.500) and reactors seeded with WA sediment and amended with low and high Zn (ANOSIM; p-value = 0.300; Figure 4-10). In addition there were no differences between reactors seeded with WA sediment and amended with low Zn on day 0 and those seeded with WB sediment and amended with low Zn on day 112 (ANOSIM; p-values = 0.800; Figure 4-10).

It is reasonable to assume that the microbial community at day 0 was roughly similar to the community present in the original river sediments, whereas community conditions changed by day 309, although data imply changes in community conditions differed among treatments. There may be many reasons for this that need further evaluating using different studies, however one reason could be due to competition between the species for resources, which tend to reduce biodiversity in most ecosystems (Hibbing *et al.*, 2010). It is possible that the communities within the batch reactors increased in response to richer media conditions in the 20.0% R2A media (i.e., richer than natural sediments), but apparent diversity decreased as resources became sparser as the experiment proceeded and starving ensued. Interestingly, community conditions were still surprisingly similar on day 309 which implies microbial stress and starvation that prevailed in all reactors might have been more important than environmental treatment in the resultant microbial communities. However, this speculation needs more work to distinguish between treatment specific and generic starvation effects.

4.3.7 *Abundance of genes in batch reactors*

To determine whether there was any relationship between resistance levels and selected genes abundances, *bla*-CTX-M and *bla*-NDM-1 were quantified using qPCR, to assess the abundance of selected genes associated with ESBL resistance (*bla*-CTX-M) and MBL/carbapenem resistance (*bla*-NDM-1) (Figure 4-11 and Figure 4-12). Figure 4-11 shows that there was no obvious trend between variation of relative *bla*-CTX-M gene levels and batch reactor conditions. Further there was no trend between gene levels and time points (0, 112, 204 and 309; Figure 4-11).
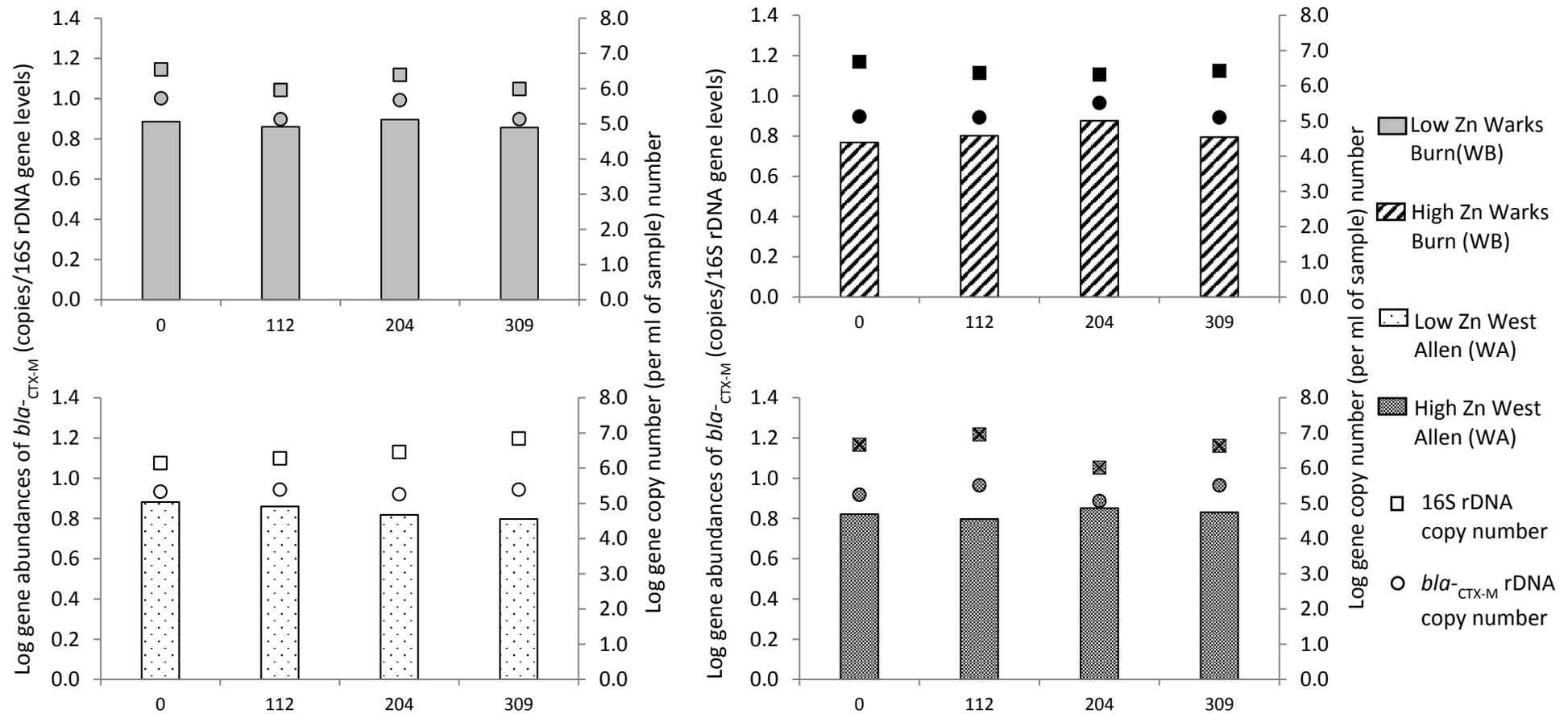
Figure 4-11 Relative abundances of bla-CTX-M genes and absolute bla-CTX-M and 16S genes in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and dosed with low (2.00 mg/L) and high (100 mg/L) Zn on days 0, 112, 204 and 309. Relative abundances were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances. Values indicated for each time point are averages of triplicate reactors (all relative abundance data shown in Table B-5).
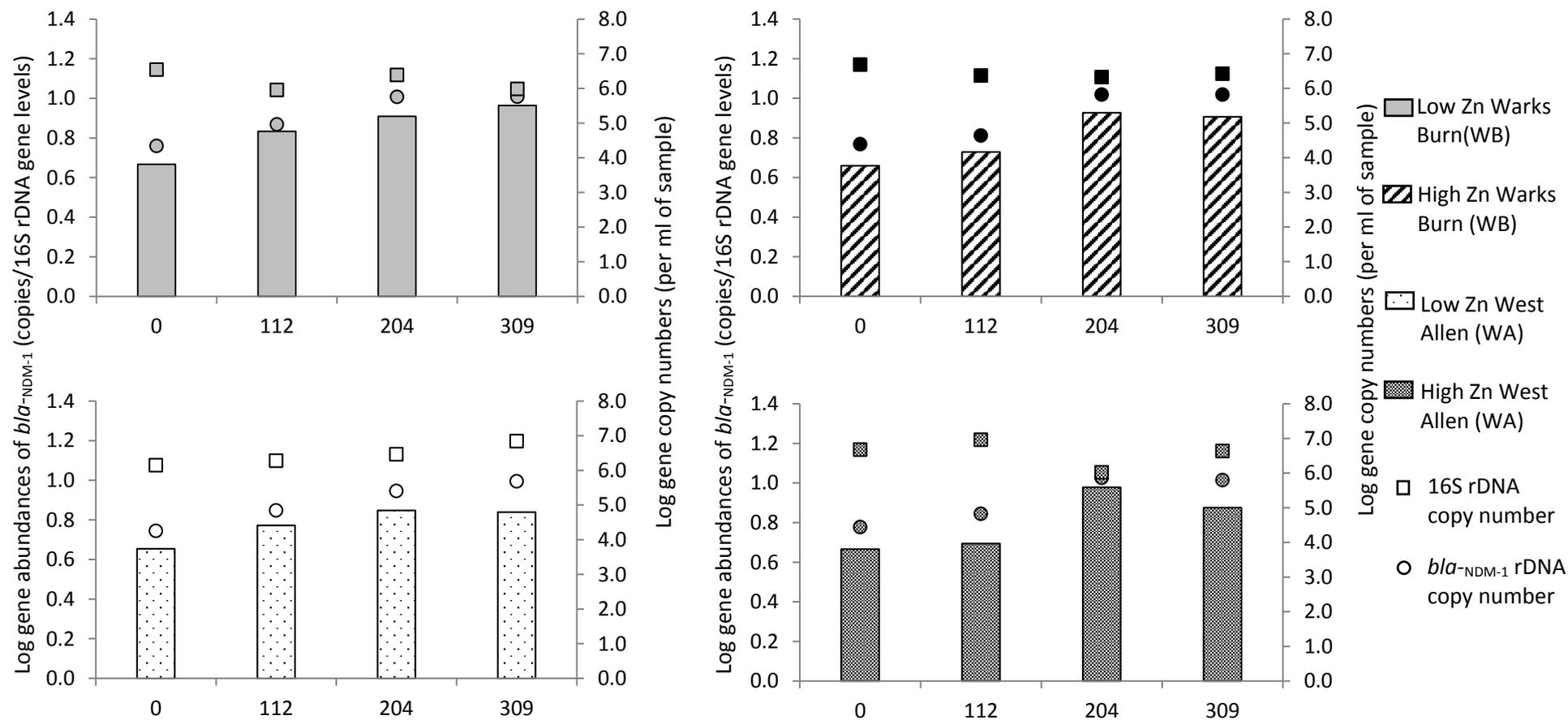
Figure 4-12 Relative abundances of bla-NDM-1 genes and absolute bla-NDM-1 and 16S genes in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and dosed with low (2.00 mg/L) and high (100 mg/L) Zn on days 0, 112, 204 and 309. Relative abundances were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances. Values indicated for each time point are averages of triplicate reactors (all relative abundance data shown in Table B-6).

When looking at relative gene abundances of *bla*-NDM-1, significantly higher relative *bla*-NDM-1 gene levels were detected on day 112 in reactors dosed with low Zn and seeded with WB sediment, compared to levels in reactors seeded with WA sediment and dosed with low (ANOVA; Tukey's comparison; p-value = 0.038) and high (ANOVA; Tukey's comparison; p-value = 0.000; Figure 4-12) Zn. There were also higher *bla*-NDM-1 gene levels on day 112 in reactors with low Zn and WB sediment compared to reactors with the same seed but high Zn (ANOVA; Tukey's comparison; p-value = 0.002; Figure 4-12). In addition there were also higher gene levels in reactors seeded with low Zn and WA sediment on day 112, compared to those seeded with the same sediment but dosed with high Zn (ANOVA; Tukey's comparison; p-value = 0.001; Figure 4-12).

When looking at variations between gene levels and time-points for each reactor, reactors with low Zn and WB sediment had significantly higher *bla*-NDM-1 gene levels on days 112, 204 and 309 compared to those on day 0 (ANOVA; Tukey's comparison; p-value = 0.046, 0.006 and 0.002 respectively; Figure 4-12). Reactors seeded with the same sediment but high Zn, also had significantly higher gene levels on days 204 and 309 compared to those on day 0 (ANOVA; Tukey's comparison; p-value = 0.002 and 0.004 respectively) and 112 (ANOVA; Tukey's comparison; p-value = 0.014 and 0.025 respectively; Figure 4-12). Reactors seeded with WA sediment only had increased gene levels in reactors dosed with high Zn on days 204 compared to levels on day 0 and 112 (ANOVA; Tukey's comparison; p-value = 0.014 and 0.011 respectively; Figure 4-12).

These results suggest that relative *bla*-NDM-1 gene levels did increase over time, especially in those reactors seeded with WB sediment. When looking at absolute 16S levels, gene levels slightly decreased over time in all reactors except reactors seeded with WA and amended with low Zn levels. Interestingly when comparing the number of isolates in each reactor (Figure 4-7) and absolute 16S gene levels (Figure 4-11 and Figure 4-12), the number of isolates dropped more rapidly between days 204 and 309 than the number of absolute 16S genes. A possible reason for this, could be that the 16S qPCR reaction is detecting all cells in the sample, including the dead cells or the DNA of some of them (Chaiyanan *et al.*, 2001; Nogva *et al.*, 2003; Pathak *et al.*, 2012). As mentioned in section 4.3.5, nutrients were not replenished in reactors after sample collection every month, putting the microbial community under starvation and nutrient stress. Therefore some organisms would not be able to survive, hence the lower number of total isolates, but higher number of absolute 16S gene levels, due to the persistence of DNA after cell death.

In contrast *bla*-NDM-1 gene levels increased at a faster rate than 16S genes decreased. This suggests that the increase in relative *bla*-NDM-1 gene abundances might have resulted from the presence of Zn, but also due to environmental stress, such as starvation and nutrient stress.

However, it must be noted that that the abundance of genes detected may not be *bla*-NDM-1 genes but similar carbapenem or *bla*-NDM-1 like genes. This is due to the fact that the absolute abundance of *bla*-NDM-1 genes was considerably higher than expected. For all reactions, all efficiency amplification values were between 90.0 -105%, and the r^2 of the linear standard curve was > 0.980. Further all replicates were consistent. Despite this indication that the qPCR reactions were sufficiently optimised, there may be several reasons for this higher abundance.

Firstly the primer set chosen, was based on a primer set used to detect positive isolates directly from clinical samples (Naas *et al.*, 2011). Hence pure isolates were tested rather than environmental ones, as in this case, and the primer set may have detected closely related genes, rather than the rare *bla*-NDM-1. Therefore the primer design and specificity needs to be considered for future analysis. Secondly it is possible, that the mixture used in the qPCR reaction, in this case Ssofast, was inhibiting the reaction. Therefore different reaction mixtures need to be tested to compare the results with this study. A further reason that could have had an effect on all qPCR reactions, was that the DNA isolation procedure and-or the kit used to extract the DNA from the samples, did not produce high quality total DNA. The sequencing of PCR amplicons could verify whether the *bla*-NDM-1 gene levels detected in the batch reactors were targeting carbapenemase associated sequences or similar carbapenemase like sequences.

4.4   Conclusions

In this study, batch reactors were set up and seeded with different sediments, taken from a metal free environment in the North Tyne (WB) and a high Zn contaminated environment in the South Tyne (WA). The batch reactors were then amended with low (2.00 mg/L) and high (100 mg/L) levels of Zn and assessed microbiologically and molecularly for 309 days. The culturing results showed that the relative percent of meropenem resistant isolates increased over time in all reactors. Further, there were significantly higher meropenem resistant organisms in reactors amended with high levels of Zn compared to those amended with low levels of Zn. Therefore this suggests that the amendment of high levels of Zn in reactors seeded with either sediment, selected for higher meropenem resistance levels.

When looking at the diversity of species at four time-points throughout the study for each batch reactor, there was a clear shift in the apparent diversity between days 0 and 309, indicating that the species richness changed over time. Samples from reactors seeded with the same sediment tend to cluster together, while there were also differences between reactor amendments, indicating the amendment of reactors with low or high Zn also had an effect on the overall diversity. Therefore it is possible that the increased meropenem resistance seen towards the end of the study was due to a group of different microbial species making up the overall environment, which were able to withstand and tolerate the level of meropenem present in the agar plates.

The increased abundance of $bla\text{-}_{NDM\text{-}1}$ gene levels in all reactors over time, suggested that the apparent community in the reactors had changed, possibly due to the presence of soluble Zn but also due to environmental stress. However it is possible, that the gene abundances detected were similar carbapenem genes and not pure $bla\text{-}_{NDM\text{-}1}$. This data can be related to the DGGE fingerprinting data, as well as the culturing data, suggesting that over 309 days, there were shifts in the apparent communities present in the reactors, which could have resulted to the increased relative percent meropenem resistant isolates observed, as well as the increases in carbapenem $bla\text{-}_{NDM\text{-}1}$ gene abundances.

Chapter 5   Impact of meropenem and zinc levels on resistance development in biofilms in rotating tubular reactors

5.1   Introduction

The presence of potentially toxic compounds, such as xenobiotics, heavy metals and organic solvents, can select for AR determinants in natural environments (Foster, 1983; Alonso *et al.*, 2001; Stepanauskas *et al.*, 2006). This was shown and assessed in the previous chapters, where a clear link was seen between high Zn levels in river sediments, and elevated meropenem and combined Zn and meropenem resistance (combined resistance) in sediment bacterial isolates (Chapter 3). Further, batch reactors seeded with river sediments amended with varied concentrations of Zn showed that meropenem resistance increased over time in reactors with highest Zn concentrations, regardless of sediment source (Chapter 4).

Another environmental system where elevated Zn in wastes might be apparent is in WWTPs, where effluents are eventually returned to the environment and may ultimately have an impact on human health (Wellington *et al.*, 2013) (Section 2.1). It is also known that the transfer and emergence of ARGs can take place in compartments with high bacterial densities, such as in biofilms (Murray, 1997), which exist in WWTPs. Biofilms may be present in pipes within treatment systems or in certain treatment processes, such as aerobic or anaerobic treatment units, making them potential hotspots for HGT (Kümmerer, 2004).

Many studies have shown that WWTPs do contain abundant ARGs, for example β-lactam and aminoglycoside resistance genes were identified in activated sludge in a WWTP in Germany (Tennstedt *et al.*, 2005). Furthermore, ESBL resistance genes have also been identified in sewage sludge in Portugal (Henriques *et al.*, 2006) and, ESBL producing-bacteria have been found in all treatment stages of a hospital sewage treatment works in Brazil (Prado *et al.*, 2008). In addition other compounds, such as metals, precisely Zn will also enter WWTPs. Zn is used in many industrial and manufacturing processes, such as paint production, smelting and in fertilisers and pesticides. It is also used as a pigment, such as in plastics, wallpaper and printing inks (Charif *et al.*, 2012).

As concentrations of antibiotics and metals can both exert a selective pressure on bacteria within an ecosystem, the movement of ARGs among environmental bacteria could be promoted with such compounds (Alonso *et al.*, 2001; Stepanauskas *et al.*, 2005; Stepanauskas *et al.*, 2006; Wright *et al.*, 2006; Segura *et al.*, 2009). Hence the study and impact of heavy metals, such as Zn on AR in communities within WWTPs, such as those present in biofilms is important and should be assessed. Therefore, this study aimed to measure and assess changes

in meropenem and combined resistance in microbial biofilm communities and effluents of rotating tubular reactors, treating domestic sewage. These rotating tubular reactors mimicked trickling filter beds and were amended with different levels of meropenem and Zn. The study was done in accordance to 'Organisation for Economic Co-operation and Development' (OECD) guidelines (OECD, 2015b) using the 'Activated Sludge Respiration Inhibition Test (ASRIT; Test No. 209)' (OECD, 2010) and the 'Simulation Test for Aerobic Sewage Treatment using biofilms (303B)' (OECD, 2001); however, some methods were modified. The objectives of the study were as follows:

a) To determine the concentrations of meropenem and Zn that influenced microbial communities within WWTPs.

b) To assess whether varied levels of Zn had an effect on relative Zn, meropenem and combined resistance in microbial communities in wastewater biofilms and effluents.

c) To investigate differences in relative Zn, meropenem and combined resistance, and measured Zn levels between three different sampling points of each rotating tubular reactor.

d) To determine whether elevated Zn and meropenem levels selected for MBL associated genes.

5.1.1 *Organisation for Economic Co-operation and Development (OECD)*

The OECD is an intergovernmental organisation which promotes policies with the aim of improving the economic and social welfare of people globally and has established a number of guidance protocols related to chemical safety and biosafety (OECD, 2015a). As background, the OECD, was established in 1961 and consisted of 18 European members and the United states of America (USA) and Canada, and now consists of 34 members ranging from North and South America to Europe and Asia- Pacific (OECD, 2015a). Among other roles, it provides a common forum to work together in order to solve problems and share experiences (OECD, 2015a), including the development of 'a collection of internationally agreed test methods used by government, industry and independent laboratories' (OECD, 2015b). This allows test methods for chemical safety and chemical preparation, such as good laboratory practice (GLP), to be harmonised within member countries, with the aim of collecting high quality and reliable data and include industrial chemicals, such as antibiotics and pesticides (OECD, 2015b).

Throughout this study, 'OECD guidelines for the testing of chemicals', consisting of 150 internationally agreed methods, were used to assess the effects of chemicals on the

environment and human health (OECD, 2015b). Specifically, the 'Activated Sludge Respiration Inhibition Test (ASRIT; Test No. 209)' (OECD, 2010) was initially employed for assessing effects of Zn and meropenem on biotic systems to determine the appropriate concentrations for use in the main study. Secondly, the 'Simulation Test for Aerobic Sewage Treatment using biofilms (303B)' (OECD, 2001) was used to assess the degradation and accumulation of Zn, meropenem and combined resistance in rotating tubular reactors.

5.1.2 *Activated Sludge Respiration Inhibition Test (ASRIT; 209)*

This OECD test guideline assesses the effects of a specific substance or chemical on microorganisms present in activated sludge of WWTPs (OECD, 2010). This is done by measuring how much oxygen is consumed by such microorganisms in the presence of a substance or combination of substances after three hours of exposure (OECD, 2010). For this study, various combinations of Zn and meropenem were assessed and total inhibition of oxygen uptake was quantified. Preliminary and definitive ASRITS tests were conducted and, based on results, appropriate concentrations and combinations of meropenem and Zn were chosen and used in the main study, using method 303B.

5.1.3 *Aerobic Sewage Treatment Simulation Test: Biofilms (303B)*

This OECD test guideline assesses the behaviour of a chemical or substance to wastewater treatment methods with biofilms, such as percolating or trickling filters, rotating biological contactors and fluidised beds (OECD, 2001). The test is performed by adding chemicals or substances within synthetic and-or domestic sewage to slowly rotating and inclined tubular reactors (OECD, 2001). Biofilms form on the internal walls of the reactor tubes after acclimation. Effluent is then collected and analysed for dissolved organic carbon (DOC) and the concentration of the substance is measured to determine "effects". The difference in DOC between reactors amended with substances and control reactors is presumed to be caused by added substance(s). The fate of substance from influent to effluent also can reflect effects (OECD, 2001).

In this study this test was modified, where the same test apparatus was used and the same conditions were followed. However, the concentration of the chemical (i.e., antibiotic itself) was not measured due to its known chemical instability (Mendez *et al.*, 2006; Cielecka-Piontek *et al.*, 2008). Instead samples were collected from two different parts of the biofilm, as well as the effluent to assess changes in meropenem, Zn and combined resistance levels across amended and control reactors.

5.2 Materials and Methods

5.2.1 *Inoculum of rotating tubular reactors*

Wastewater was collected once a week from the overflow channel of the primary sedimentation tank at the Totnes WWTP (Figure 5-1), which predominantly treats domestic sewage.
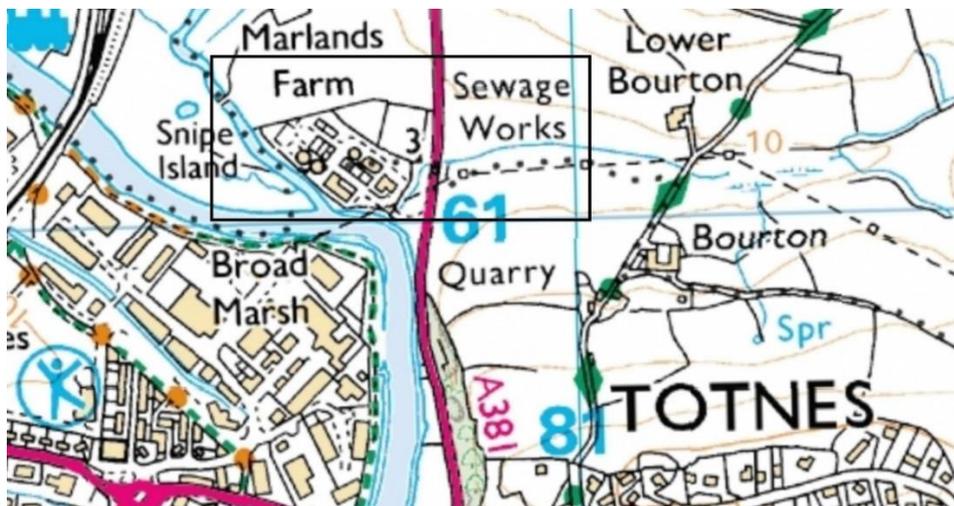
Figure 5-1 Location of Totnes sewage treatment works in Totnes, Devon, UK (Bing, 2015).

The collected wastewater was stored at 4°C, until use, where it was added together with a 100-fold concentrated stock of synthetic sewage to a common influent tank and used as the inoculum to the rotating tubular reactors (Table 5-1). Each prepared stock of synthetic sewage was autoclaved at a temperature of 121°C for 15 minutes (Touchclave R, LTE Scientific, Oldham, UK) and stored at 4°C and the pH was measured and adjusted to 7.5 ± 0.5. A volume of 10.0 ml was added to every L of fresh wastewater inoculum to achieve DOC concentration of 100 mg/L.

Table 5-1 Amounts of chemicals used for the preparation of 1.00 L of 100 fold concentrated stock of synthetic sewage (OECD, 2001).

Chemical	Amounts added (g)
Peptone	16.0
Meat extract (or comparable vegetable extract)	11.0
Urea	3.00
Sodium chloride (NaCl)	0.70
Calcium chloride dehydrate (CaCl$_2 \cdot$ 2H$_2$O)	0.40
Magnesium sulphate heptahydrate (MgSO$_4 \cdot$7H$_2$O)	0.20
Anhydrous dipotassium hydrogen phosphate (KH$_2$PO$_4$)*	2.80

* KH$_2$PO$_4$ added after the previous chemicals are dissolved in 800 ml of warm de-ionised water, and then filled to a 1.00 L level.

5.2.2 *Activated Sludge Respiration Inhibition Test (ASRIT)*

In order to determine the suitable levels of meropenem and Zn to be added to the rotating tubular reactors, an ASRIT was conducted in accordance to OECD Test Guideline 209 (OECD, 2010). The aim of the test was to measure inhibition of respiration caused by meropenem, Zn and a series of combinations of meropenem and Zn to activated sludge systems.

a. Preparation and procedure of ASRIT

A total of 20.0 ml activated sludge from tanks at Totnes WWTP (Figure 5-1) was collected two days prior the test, allowed to settle, and half the total volume of liquid was decanted to obtain a sludge solids concentration of 3.00 g/L. The solids were fed with 50.0 ml synthetic sewage feed/ L of activated sludge and the pH was measured and adjusted to 7.0 ± 1.0. The activated sludge was then continuously aerated at 2.00 L/minute and kept at ambient room temperature of 20 ± 2°C until use. The sludge solids concentration (total filterable solids) of the activated sludge was determined by filtering a known amount of sample on pre-weighed filter papers and drying in an oven at 105°C, for one hour according to standard methods

(APHA, 2005) and using Equation 5-1. The volume of activated sludge required for a set volume was then calculated using Equation 5-2.

Equation 5-1 The equation used to determine the sludge solids concentration.

$$Sludge\ solids\ concentration\ (mg/L) = A \times B$$

Where:

A = Weight of dry solids (mg)

B = Sample volume (ml)

Equation 5-2 The equation used to determine the volume of activated sludge required for each 150 ml flask when a sludge solids concentration of 1500 mg/L (1.50 g/L) is required.

$$Volume\ required = \frac{Volume\ required\ \times sludge\ solids\ required\ (mg/L)}{Sludge\ solids\ concentration\ (mg/L)}$$

For each ASRIT a total of 36 glass test vessels, each of a total capacity of 250-ml were used (OECD, 2010). These were prepared in sets of six, with intervals of 15 minutes between each set, where each set included one blank control vessel containing the prepared activated sludge at a sludge solids concentration of 1.50 g/L, (determined using Equation 5-1 and Equation 5-2), 5.00 ml of synthetic sewage feed (prepared as described in Table 5-1) and de-ionised water to give a total volume of 150 ml (Figure 5-2).

Figure 5-2 Photo of the set-up of aerated glass test vessels used for the definitive ASRIT study, with the appropriate meropenem and Zn amendments, activated sludge at a sludge solids concentration of 1.50 g/L, 5.00 ml of synthetic sewage feed and, de-ionised water to give a total volume of 150 ml.

The remaining five vessels contained activated sludge and synthetic sewage at the same concentrations, together with targeted concentrations of meropenem and Zn, filled to a total volume of 150 ml with de-ionised water. A set of 6 vessels were also inoculated with a reference inhibitory substance, 3,5-dichlorophenol (3,5-DCP), as it is a known inhibitor of respiration. Furthermore, the pH of each flask was measured and then aerated via glass sinters, for a three-hour exposure period. Compressed air from the laboratory supply was used at 0.50 L/min, to maintain a DO concentration at 60.0% saturation and all flasks were kept at an ambient temperate of $20 \pm 2°C$. After the three-hour exposure period, the pH and temperature of each vessel was measured and the respiration rate of each set of six vessels was measured using a Strathkelvin Strathtox respirometer (Strathkelvin Instruments, Scotland, UK), which measures oxygen uptake rate of six samples simultaneously.

The respirometer consisted of a six-place stirrer unit, with 20-ml nominal volume glass sample tubes. Six microcathode oxygen electrodes, mounted in electrode holders, fitted into the tubes to measure oxygen concentrations of the test solutions. The electrodes were connected to an interface unit, which in turn, were connected to a computer that then determined respiration rates for the six samples and, calculated the 50.0% effect concentration value (EC_{50}), based on amendment concentrations logged in the computer. The EC_{50} is the concentration that has a 50.0% effect on organisms within the activated sludge based on the exposure substance. This value provides an approximation of the toxicity of each amendment to activated sludge; i.e., aerobic wastewater bacteria. The percentage inhibition (I_T) of total oxygen consumption at each concentration was then calculated using Equation 5-3.

Equation 5-3 The equation used to calculate the percentage inhibition (I_T) of total oxygen consumption.

$$I_T = \left[1 - \frac{R_T}{R_{TB}} \right] \times 100\%$$

Where:

I_T = percentage inhibition (%)

R_T = respiration rate of test vessel (mg O_2/L/h)

R_{TB} = mean respiration rate of control flasks (mg O_2/L/h)

b. Experimental design

Two different ASRITs were set-up. The first was a preliminary study that employed concentrations of 0.00, 0.20, 2.00 and 20.0 mg/L of meropenem and 0.00, 2.00, 10.0, 30.0 and 100 mg/L of Zn, and included different combinations of these concentrations (Table C-1). The

first three sets of test vessels consisted of vessels where de-ionised water replaced the activated sludge in order to determine if meropenem or Zn alone showed any respiration activity. The second study, the definitive study, included test combinations of 2.00 mg/L meropenem plus 0.00, 2.00, 20.0 or 100 mg/L Zn, as well as 2.00 mg/L of meropenem and 2.00 mg/L of Zn alone (Table 5-2). These concentrations were chosen based on the preliminary ASRIT study (Table C-1) and performed in quintuplicate.

Table 5-2 Concentrations of meropenem and Zn used in the definitive ASRIT study, determined from the preliminary ASRIT.

Test vessel number	Test substance	Nominal concentrations (mg/L)		Solids concentration (g/L)
1		0.00		1.50
2		1.00		1.50
3	3,5-dichlorophenol	3.20		1.50
4		10.0		1.50
5		32.0		1.50
6		32.0		0.00
7	Meropenem	0.00		1.50
8- 12		2.00		1.50
13	Zn	0.00		1.50
14- 18		2.00		1.50
		Meropenem	Zn	
19	Meropenem and Zn	0.00	0.00	1.50
20-24		2.00	2.00	1.50
		Meropenem	Zn	
25	Meropenem and Zn	0.00	0.00	1.50
26-30		2.00	20.0	1.50
		Meropenem	Zn	
31	Meropenem and Zn	0.00	0.00	1.50
32-36		2.00	100	1.50

For both the preliminary and definitive ASRITs, stock solutions of 3,5-DCP, meropenem and Zn (as zinc sulfate heptahydrate; $ZnSO_4 \cdot 7H_2O$) (Sigma- Aldrich, St. Louis, MO, USA) were prepared by weighing out the appropriate amounts (Sartorius, Surrey, UK) and diluting in de-ionised water. The concentration of $ZnSO_4$ needed to produce defined Zn concentrations in the test vessels was calculated using Equation 3-1 and the volumes of 3,5-DCP, meropenem and Zn needed by each vessel was calculated using Equation 3-2 (Section 3.2.5), as the initial and final concentrations, as well as the final volume required were known.

5.2.3 *Rotating Tubular reactor set-up*

An apparatus containing six aerobic rotating tubular reactors was assembled at a constant room temperature of $22 \pm 2°C$ in accordance with OECD Test Guideline 303B (OECD, 2001). The apparatus consisted of a bank of acrylic tubes, each 30.5-cm long and 5-cm internal diameter, supported on rubber-rimmed wheels contained within a metal supporting frame (Figure 5-3).

Figure 5-3 Photo of the six rotating tubular reactors, inclined at an angle of approximately one degree to the horizontal. Starting from the left the reactor amendments were as follows: Control (R1; black label), 2.00 meropenem (R2, red label), 2.00 Zn (R3; green label), 2.00 mg/L meropenem and 2.00 mg/L Zn (R4; yellow label), 2.00 mg/L meropenem and 20.0 mg/L Zn (R5; blue label), 2.00 mg/L meropenem and 100 mg/L Zn (R6; yellow/green label).

Each tube had an outside lip, approximately 0.5-cm deep, to retain it on the wheels and a 0.5-cm deep internal lip at the upper (feed) end to retain the liquid. The internal surface of each tubular reactor was roughened with coarse wire. The rubber-tyred wheels were rotated using a motor at 18 ± 2 rpm. Lubricant was also applied to the tubes initially and periodically through the test to ensure proper functioning and to prolong the life of the rolling tubes. The tubes

were inclined at an angle of approximately one degree to the horizontal to achieve consistent slow residence time of 125 ± 12.5 seconds. Chosen rates, rotation speeds and times were used as they provide 80.0% DOC removals and higher, according to previous studies (OECD, 2001). The feed medium was contained in a 100-L storage vessel (influent tank), which was retained at $4 \pm 2°C$ to minimize biological activity before entering each tube. The storage vessel had an outlet that was connected by silicone rubber tubing (Thermo Fisher Scientific, Waltham, MA, USA) via a peristaltic pump (Watson Marlow, Cornwall UK), adjusted to deliver 125 ± 12.5 ml/h of the feed medium at the top end of each inclined tube. Effluent was allowed to drip from the lower end of each inclined tube to be collected in a separate storage vessel per tube. The rubber tubings in the peristaltic pump were changed every week and the influent vessel was replaced weekly with fresh waste and synthetic sewage and also wiped down with 1.00% Virkon (VWR, Leicestershire, UK) to remove any microbial growth.

5.2.4 *Experimental design and sampling procedure*

A mixture of domestic primary settled sewage and synthetic sewage feed (Section 5.2.1) was pumped through each rotating tubular reactor and samples were taken from the influent tank and each of the six effluent tanks twice per week for DOC (Carbon Analyser IL500, Hach Lange, Salford, UK) and pH analysis. Samples were filtered through Whatman (No. 4) filter papers (Thermo Fisher Scientific, Waltham, MA, USA) before analysis. The measured DOC values were used to determine the percentage removal of DOC from each reactor using Equation 5-4.

Equation 5-4 The equation used to determine the percentage (%) removal of DOC.

$$D_B(\%) \ = 100(\frac{1 - E_o}{C_m})$$

Where:

E_o = measured DOC of the reactor effluent at time t (mg/L)

C_m = DOC of the influent at time t (mg/L)

After 21 days, when surface biofilms had reached optimal thickness, determined once DOC removals of 80.0% were consistent in each rotating tubular reactor, targeted concentrations of meropenem and Zn were introduced to the reactor feeds, except the control reactor that was provided only with waste and synthetic sewage feed (Figure 5-3; Table 5-3).

Table 5-3 The concentration of meropenem and Zn added to each reactor after 21 days of biofilm growth.

Rotating Tube	Rotating Tube condition	Nominal Amendments (mg/L)	
		Meropenem	Zn
R1	Control	0.00	0.00
R2	Meropenem	2.00	0.00
R3	Zn	0.00	2.00
R4	Meropenem and Zn	2.00	2.00
R5	Meropenem and Zn	2.00	20.0
R6	Meropenem and Zn	2.00	100

Nominal amendments for each rotating tubular reactor were determined based on results from the definitive ASRIT study (Section 5.2.2). Syringe pumps (Harvard Apparatus, MA, USA), using syringes (Hamilton, GR, Switzerland) connected to the top end of each tubular reactor by PTFE tubing (Harvard Apparatus, MA, USA), were employed to dose each tubular reactor with the appropriate amendment at a constant rate of 125 ml/hr. One syringe was used per concentration of amendment and fresh stock solutions of 1.00 g/L meropenem and 1.00, 10.0 and 50.0 g/L Zn were prepared (Section 3.2.5) twice weekly and dosed into each tubular reactor for 29 days. During the dosing period, samples for microbiological and metal analysis were taken once a week from the influent and effluent of each reactor. Samples were also taken from biofilms at the top (upper) and bottom (lower) ends of each tube, by using separate and sterilised plastic spatulas, to scrape off 2 cm of biofilm. DOC and pH analysis were continued during the dosing period.

5.2.5 *Quantifying meropenem and Zn resistance*

Samples were collected and analysed for meropenem and Zn resistant bacteria using classical spread-plating techniques (Section 3.2.4) according to the spread plate (9215C) standard method (APHA, 2005). All media preparation and culturing were done in a class II microbiological safety cabinet (Thermo Fisher Scientific, Waltham, MA, USA) where for each set of samples, agar plates were prepared using R2A agar and amended as follows: no amendment (control), 2.00 mg/l meropenem, 65.0 mg/L Zn, and a combination of 2.00 mg/L

meropenem and 65.0 mg/L Zn (as described in sections 3.2.4 and 3.2.5). Each prepared agar plate was inoculated with 50.0 µl of each, serially diluted sample, and incubated for 4 days at $20 \pm 2°C$ (a day longer than previous studies described in Chapter 3 and Chapter 4, due to the lower incubation temperatures of 20 ± 2 °C; section 3.2.5). Petri dishes with roughly 30-300 colony forming units (CFU) per plate were counted (in accordance with APHA (2005) standard method 9215A) and the number of CFU/ml were calculated using Equation 3-3, followed by the calculation of the percentage ratio of CFU/ml in amended petri dishes relative to un-amended control petri dishes (Equation 3-4; section 3.2.6).

5.2.6   *Preparation and analysis of samples for metal analysis*

Prior to analysis, all glass vials (VWR, Leicestershire, UK) were prepared as described in section 3.2.7. Biofilm samples from the upper and lower ends of each tubular reactor were transferred to the "clean" glass vials of known weight, which had been dried overnight at 104°C, and the vials were re-weighed to determine the dry weight of solids present in each biofilm sample. Samples were acid digested using nitric acid (1N HNO_3; 65.0%, Suprapur®, Merck, NJ, United States), according to the APHA (2005) standard method (3030 E.2), prior to metal analysis (Section 3.2.8). All acid-digested samples were then filtered prior to analysis using 0.2-µm cellulose acetate membrane filters (VWR, Leicestershire, UK) and diluted by at least factor of ten. Samples taken from the influent tank and each of the six effluent tanks were also filtered through 0.2-µm cellulose acetate membrane filters and, 0.5 ml 1N HNO_3 was added to 1-ml of each of the filtered samples and filled to a volume of 5-ml with de-ionised water. All prepared samples were analysed for metals including Cd, Co, Cu, Ni, Pb and Zn (Section 3.2.8) using an ICP-OES (Vista MPX CCD Simultaneous ICP-OES, Varian, Australia).

5.2.7   *Extraction of DNA from samples*

Samples for DNA extraction were collected weekly for 28 days during the dosing period, including samples from the influent tank; from the six effluent tanks; and from the upper and lower biofilms on each reactor. DNA extractions were performed using BIO 101 FastDNA Spin Kits (Q-Biogene, MP Biomedicals, UK) as described in section 4.2.6.

5.2.8   *Quantitative polymerase chain reactions (qPCR)*

The abundance of eubacterial (as 16S rDNA), integron-1 *(IntI1)* and *bla-*NDM-1 genes were analysed using qPCR for all DNA samples, as described in section 4.2.10. As mentioned in section 4.3.5, integrons are common and abundant in environmental organisms (Stokes *et al.*,

2001; Holmes *et al.*, 2003; Guerin *et al.*, 2009; Gillings, 2014). Generally integrons are genetic elements that incorporate small mobile elements called gene cassettes into a recipient genome, via site-specific recombination (Wright *et al.*, 2008). *Intl1* genes were assessed as previous studies have reported that they play a role in the dissemination of AR gene cassettes in clinical bacteria (Stokes *et al.*, 2001) and, in a broad range of clinical and environment gram-positive and gram-negative bacteria (Nandi *et al.*, 2004; Stokes *et al.*, 2006; Boucher *et al.*, 2007).

Standards of 7 different dilutions were used per plate (10^{+8} - 10^{+2}) and per primer set to produce a standard curve, in order to quantify the relative abundance of target genes. Standards for 16S rDNA and *bla*-NDM-1 gene qPCR reactions were prepared from growing clones containing the necessary fragments, and extracting and quantifying concentrations of the plasmids (Section 4.2.110). Standards for *IntI1* qPCR reactions were prepared from DNA extracts containing the relevant gene (Section 5.2.9). Once prepared the reactions were run using appropriate programs, determined for each set of primers (Table 5-4). The results from each qPCR reaction were analysed using absolute quantification, where CT values of test samples were compared to the standard curve (produced from using relevant standards) and the gene abundance of the test samples (Starting Quantity; SQ) were determined. The logarithmic gene abundances (copy number) per DNA extract (Equation 4-2) and per ml of samples (Equation 4-3) were determined as described in section 4.2.10.

Table 5-4 Primer sets used for qPCR analysis of all samples followed by their sequences and appropriate reaction conditions.

Primer	Batch	Sequence	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles[1]
16S (Yu *et al.*, 2005; Huber *et al.*, 2007)	BAC338F	ACTCCTACGGGAGGCAGC	98°C for 3 minutes	98°C for 5 seconds	60°C for 10 seconds		40
	BAC1046R	CGACARCCATGCANCACCT					
Bla-NDM-1 (Naas *et al.*, 2011)	NDM-1-F	ATTAGCCGCTGCATTGAT	95°C for 5 minutes	95°C for 15 seconds	60°C for 1 minute		50
	NDM-1-R	CATGTCGAGATAGGAAGTG					
*IntI*1 (Xu *et al.*, 2007)	IntI 1F	GTTCGGTCAAGGTTCTGG	95°C for 3 minutes	95°C for 10 seconds	63.5°C for 10 seconds	72°C for 30 seconds	30
	IntI 1R	CGTAGAGACGTCGGAATG					

[1]Number of cycles for denaturation, annealing and extension steps

5.2.9 *Preparation of standards for IntI1*

In order to determine the absolute and abundance of *IntI1* genes, standards were prepared by growing a strain of *E. coli* OB3, which contains an 890 base-pair insert of the *IntI1* gene (provided with gratitude from Professor Julian Davies, University of British Columbia). The strain was grown in 50.0 ml of Luria-Bertani (LB) broth (Oxoid Limited, Basingstoke, UK) at 150 rpm at 37°C for 24 hours. To ensure the culture was pure, it was streaked on LB agar plates and incubated at 37°C for 24 hours. Both the LB broth and agar were prepared according to the manufacturer's instructions. All culturing and streaking were done in a class II microbiological safety cabinet. After growth, plasmids were extracted using the Roche High Pure Plasmid Isolation kit (Roche Diagnostics Limited, West Sussex, UK) and a PCR was carried out, as described in section 4.2.7. The reaction conditions consisted of 95°C for 3 minutes, followed by 31 cycles of 95°C for 30 seconds, 60°C for 1 minute and a final 1 minute at 72°C for extension. The products of the PCR reaction were examined by agarose gel electrophoresis, using a 1.50% agarose gel, reference PCR markers and negative and positive controls that were run for 45 minutes at 100v and visualised by UV illumination (Bio-Rad Fluor-S ® MultiImager, Bio-Rad, Hertfordshire, UK) (Section 4.2.8). The PCR product was then cleaned using a QIAquick PCR Purification Kit (Qiagen, Manchester, UK), according to the manufacturer's instructions, and the DNA concentration of each PCR product was quantified using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific, Leicestershire, UK). The copy number per µl of sample was determined using Equation 4-4and the volume of DNA needed to produce a 10^{+9} standard was determined using Equation 3-2.

5.2.10 *Data analysis*

All data was statistically analysed using Minitab (Version 17, Coventry, UK), as described in section 3.2.9. As data was not normal, Mann-Whitney non-parametric test were used, where all tests were conducted by assessing whether sample-pairs were significantly different to each other using 95.0% significance tests. Bi-variate correlation analysis also was performed to assess linear correlations and general trends among measured parameters. The Pearson's coefficient (value of r) signified the strength of the test, where the closer the coefficient to 1, the stronger the correlation, whereas p-values < 0.050 were significantly different.

5.3 Results and discussion

5.3.1 *Preliminary Activated Sludge Respiration Inhibition Test (ASRIT)*

The preliminary ASRIT (Section 5.2.2) study showed that percent inhibition of oxygen concentration increased in flasks amended with 30.0 and 100 mg/l Zn when no meropenem was added. The highest inhibition of oxygen uptake was at 40.0% (EC_{40}; Figure 5-4).
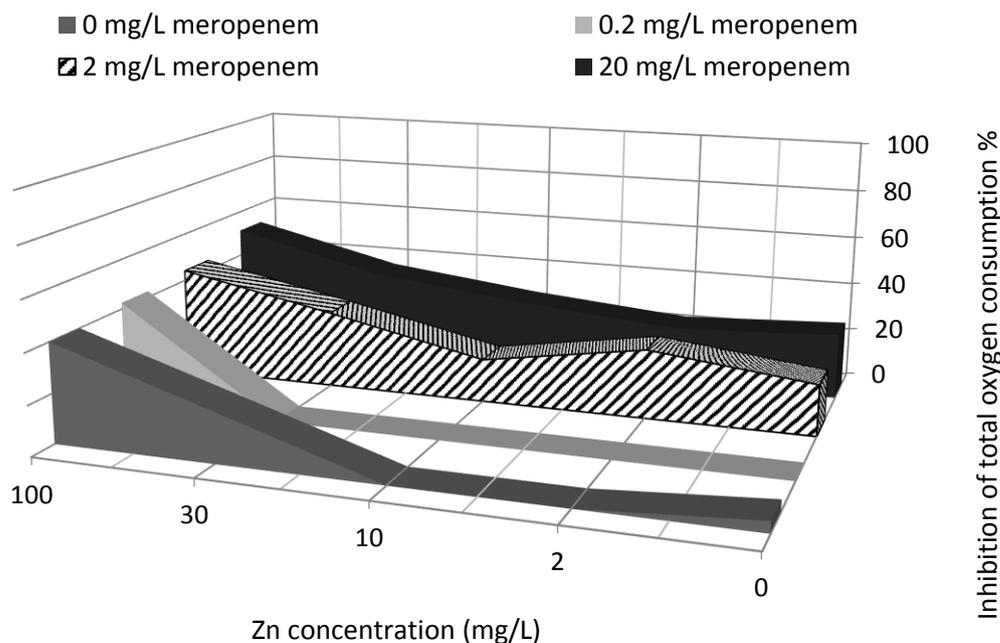
Figure 5-4 The percentage inhibition of total oxygen consumption to concentrations of 0.00, 0.20, 2.00 and 20.0 mg/L of meropenem and 0.00, 2.00, 10.0, 30.0 and 100 mg/L of Zn.

In flasks containing 0.20 mg/L meropenem, inhibition was observed when 100 mg/L Zn was present at 42.5% ($EC_{42.5}$; Figure 5-4). In flasks containing 2.00 mg/L meropenem and 0.00, 2.00, 10.0, 30.0 and 100 mg/L Zn, inhibition was higher than in flasks where lower concentrations or no meropenem was present and the highest inhibition was observed in flasks amended with 100 mg/L Zn at 44.0% (EC_{44}; Figure 5-4). A similar pattern to the latter was observed in flasks containing 20.0 mg/L meropenem and various concentrations of Zn, with the highest inhibition observed in flasks amended with the highest level of Zn at 51.0% (EC_{50}; Figure 5-4). When looking at the averages of flasks amended with 0.00, 2.00, 10.0, 30.0 and 100 mg/L Zn, two sample statistical tests confirmed that inhibition was significantly higher in flasks amended with 100 mg/L Zn compared to those with no Zn amendment, 2.00, 10.0 and 30.0 mg/L Zn (Mann-Whitney; all p-values = 0.030). When considering only the averages of flasks amended with 0.00, 0.20, 2.00 and 20.0 mg/L meropenem, there were trends between flasks amended with 0.20 mg/L meropenem and 2.00 and 20.0 mg/L

meropenem, where the latter two had higher inhibition, compared to flasks amended with 0.20 mg/L meropenem (Mann-Whitney; all p-values = 0.095).

These results suggested that the higher the concentrations of meropenem and Zn in activated sludge, the greater the inhibition of oxygen consumption. From preliminary tests, the suitable concentrations were suggested for meropenem and Zn for the definitive ASRIT test. These were 2.00 and 20.0 mg/L of meropenem and Zn concentrations, ranging from 0.00 to 100 mg-Zn/L. However, a concentration of 20.0 mg/L meropenem is not realistic in a WWTP, therefore 2.00 mg/L meropenem was chosen for subsequent ASRIT testing.

5.3.2 *Definitive Activated Sludge Respiration Inhibition Test (ASRIT)*

The definitive ASRIT (Section 5.2.2) showed that percentage inhibition of total oxygen consumption to Zn and meropenem increased as the level of Zn increased from 2.00 to 100 mg/L (Figure 5-5). The concentrations that inhibited oxygen uptake by 50.0% (EC_{50}) were 60.0 mg/L Zn and 2.00 mg/L meropenem. Addition of 2.00 mg/L meropenem marked the beginning of inhibition at EC_{15}, and that increased to EC_{21} when 2.00 mg/L meropenem and 2.00 mg/L Zn were added in tandem (Figure 5-5).
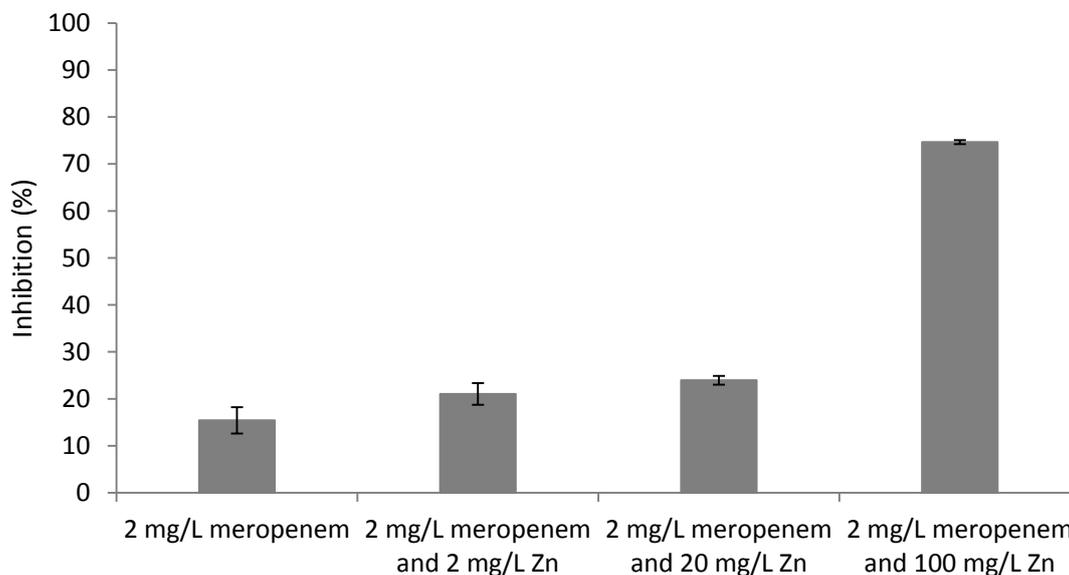
Figure 5-5 The percentage inhibition of total oxygen consumption to: 2.00 mg/L meropenem, 2.00 mg/L meropenem and 2.00 mg/L Zn, 2.00 mg/L meropenem and 20.0 mg/L Zn and, 2.00 mg/L meropenem and 100 mg/L Zn. Values indicated are averages of quintuplicate test vessels and standard error (S.E.) bars are shown. There was no inhibition of total oxygen consumption with the addition of 2.00 mg/L Zn, so this treatment was not added to the bar chart.

Inhibition increased to EC$_{24}$ when 2.00 mg/L meropenem and 20.0 mg/L Zn were added, which was statistically higher than inhibition when only meropenem was added (Mann-Whitney; p-value = 0.031). The highest inhibition of oxygen uptake was at 75.0% (EC$_{75}$) when 2.00 mg/L meropenem and 100 mg/L Zn were added, which was statistically higher than inhibition in flasks amended with only meropenem and, 2.00 mg/l meropenem with 2.00 mg/L and, 20.0 mg/L Zn (Mann-Whitney; all p-values = 0.005; Figure 5-5). It is important to note that the addition of 2.00 mg/L Zn alone had no effect on oxygen uptake, therefore it is apparent that the addition of meropenem to Zn is most influential in reducing oxygen use by microorganisms in the activated sludge (Figure 5-5). As such, Zn/meropenem mixtures were compared with Zn and meropenem to assess the longer term effect of these concentrations on microbial communities in settled sewage and within biofilms in the subsequent rotating tubular reactor experiment.

5.3.3 *DOC removal in the rotating tubular reactors*

DOC levels in each tubular reactor were monitored over the duration of the study. The DOC removal rates before Zn and meropenem additions were commenced were consistently between 70.0 and 80.0%. When meropenem and Zn additions were started, DOC removal rates declined in all reactors, except the control (R1) and the reactor dosed with 2.00 mg/L Zn (R3; Figure 5-6).
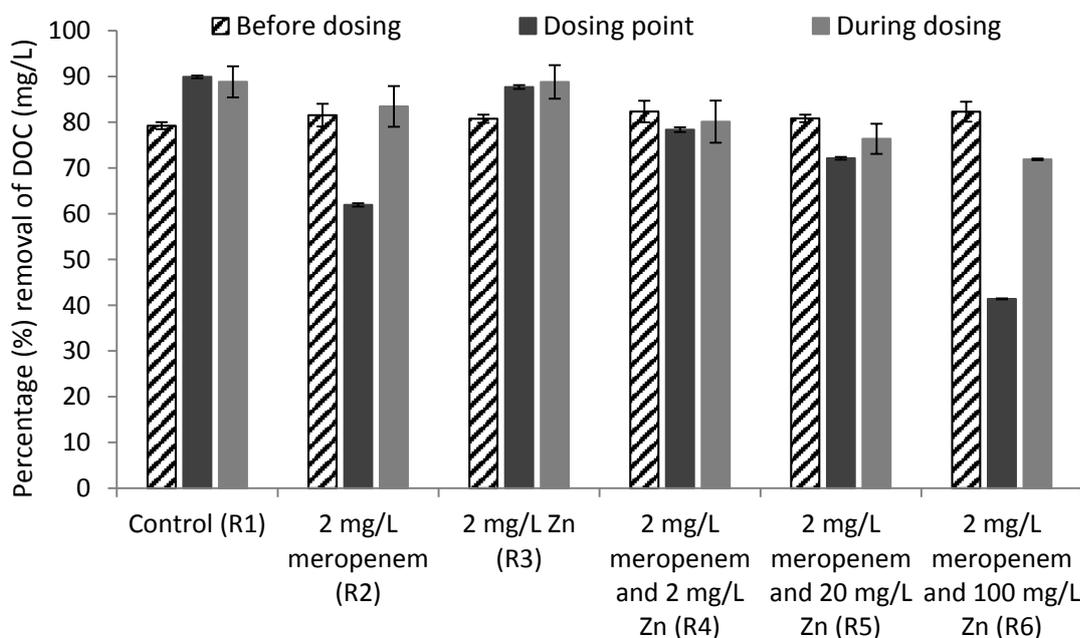
Figure 5-6 Average percentage (%) removal of DOC (mg/L) in the six rotating tubular reactors, before dosing (day 12-22), at the dosing point (day 26) and during dosing (day 33-51). Values indicated are average over days stated above and standard error (S.E.) bars are shown.

However statistically significant changes were only seen in the reactor dosed with 2.00 mg/L meropenem (R2), where removal statistically decreased to 61.9% (S.E. ± 0.37; ANOVA; Tukey's comparison; p-value = 0.025) and in the reactor dosed with 2.00 mg/L meropenem and 100 mg/L Zn (R6), where it significantly decreased to 41.5% (S.E. ± 0.11; ANOVA; Tukey's comparison; p-value = 0.000; Figure 5-6), compared to the removal prior to dosing.

The addition of 2.00 mg/L meropenem alone had more of an effect on organisms than the addition of meropenem plus 2.00 or 20.0 mg/L Zn (ANOVA; Tukey's comparison; R4: p-value = 0.662 and R5: p-value = 0.262 respectively). The dosing of 2.00 mg/L Zn (R3), did not change the DOC removal rates; in the contrary, it increased removal (ANOVA; Tukey's comparison; p-value = 0.189), showing that Zn alone at low levels of 2.00 mg/L did not have a significant or sudden effect on the microbial population, however as indicated above, high concentrations of 100 mg/L Zn and meropenem did have an effect on the DOC (Figure 5-6).

The DOC removal rates recovered throughout the dosing period, as the organisms gained tolerance to the concentrations of meropenem and Zn. However there were only significant differences between the removal measured at the dosing point and during the dosing in R2 and R6 (ANOVA; Tukey's comparison; R2: p-value = 0.017 and R6: p-value = 0.000), where removal rates recovered and increased to 82.0% (S.E. ± 3.31) and 71.9% (S.E. ± 0.29) respectively. Despite recovery, in R6, removal rates were still lower than they were before dosing (ANOVA; Tukey's comparison; p-value = 0.004), showing that high concentrations of 100 mg/L Zn and meropenem still had an effect on the microbial communities (Figure 5-6).

5.3.4 *Observed resistance in the upper and lower biofilms and effluent of each rotating tubular reactor using different selective media*

Culturing of samples from the top end of each reactor (upper biofilm) on agar plates amended with 65.0 mg/L Zn, 2.00 mg/L meropenem, and a combination of both showed that Zn, meropenem and combined resistance all significantly increased in reactors dosed with 2.00 mg/L meropenem plus 20.0 or 100 mg/L Zn (R5 and R6) relative to the un-amended control (R1; Mann-Whitney; both p-values = 0.030; Figure 5-7).
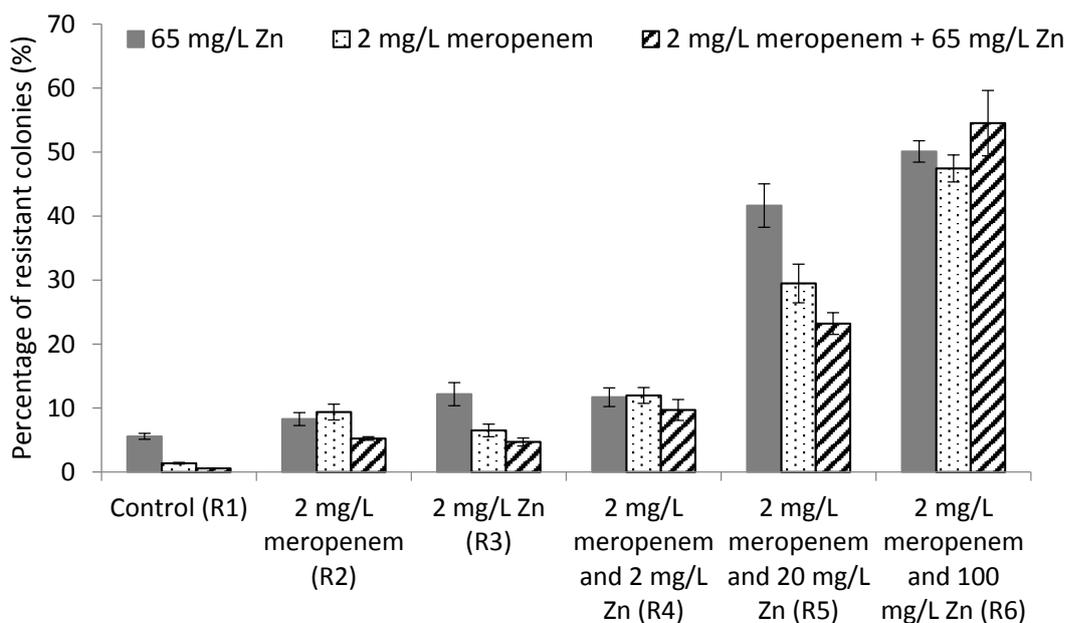
Figure 5-7 Percentage (%) of colonies in the upper biofilm, resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor; Control (R1), 2mg/L meropenem (R2), 2 mg/L Zn (R3), 2 mg/L meropenem and 2 mg/L Zn (R4), 2 mg/L meropenem and 20 mg/L Zn (R5) and 2 mg/L meropenem and 100 mg/L Zn (R6). Values indicated are averages over 4 weeks of sampling (all data shown in Table C-2) and standard error (S.E.) bars are shown.

This suggests that reactors amended with 2.00 mg/L meropenem plus 20.0 or 100 mg/L Zn (R5 and R6) increases all forms of observed resistance. In addition, there also were statistically higher CFUs from these two reactors that were resistant to both Zn and combined meropenem and Zn, compared with R2 where only meropenem was provided (Mann-Whitney; both p-values = 0.030; Figure 5-7). This shows that the presence of elevated Zn in biofilms, makes resident organisms less susceptible to meropenem, especially when Zn was dosed at 20.0 and 100 mg/L, which is consistent with observations in River Tyne sediments (see Chapter 3). Other observations also showed that R6 had higher meropenem and combined resistance than R3, where only Zn was provided (Mann-Whitney; p-value = 0.030; Figure 5-7), indicating meropenem plus high levels of 100 mg/L Zn, increased resistance related to meropenem. The same pattern was seen in R2 (only meropenem), where meropenem and combined resistance were higher than resistance in the control reactor (R1; Mann-Whitney; p-value = 0.030; Figure 5-7). This shows meropenem addition alone (i.e., without Zn) can increase all forms of meropenem resistance, including combined resistance.

Detected resistance levels at the bottom end of the reactor (lower biofilms) had similar, but generally weaker trends than the upper biofilms. For example, higher levels of Zn, meropenem and combined resistance were seen in reactors with 2.00 mg/L meropenem plus 20.0 or 100 mg/L Zn (R5 and R6) compared to the control (R1), although differences were only significant with an 80.0% confidence level (Mann-Whitney; both p-values = 0.194; Figure 5-8).
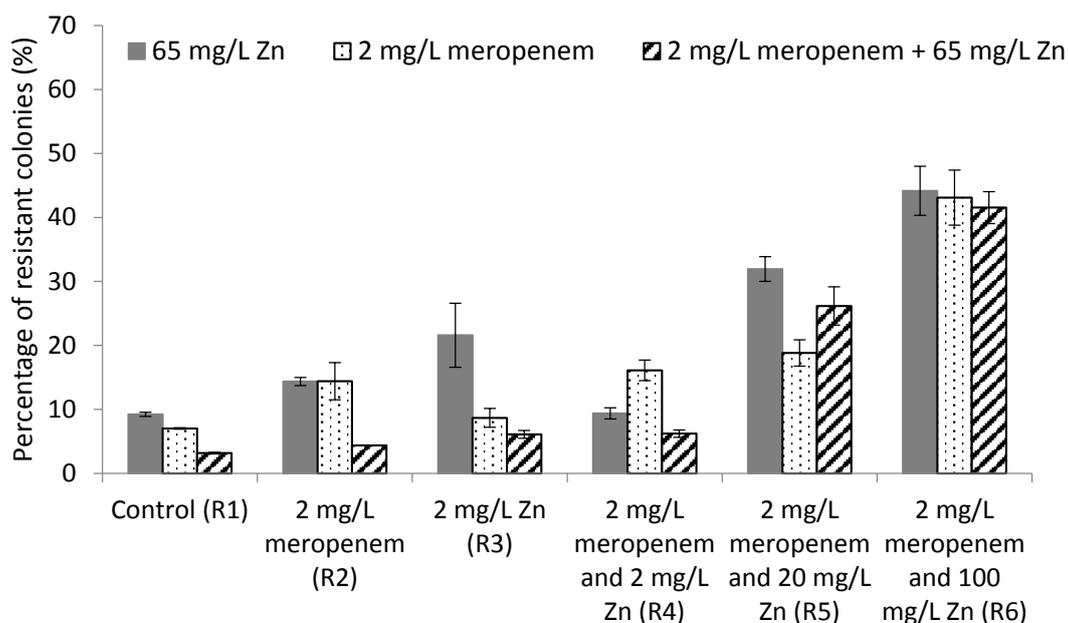
Figure 5-8 Percentage (%) of colonies in the lower biofilm resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor. Values indicated are averages over 4 weeks of sampling (all data shown in Table C-3) and standard error (S.E.) bars are shown.

The analysis of liquid samples taken from the effluent tanks of each reactor provided further evidence that Zn, meropenem and combined resistance were significantly higher in R5 and R6, when compared to the control (R1) and the raw influent (Mann-Whitney; both p-values = 0.030; Figure 5-9). Levels of all forms of resistance were especially high when meropenem and 100 mg/L Zn were provided to the reactors.
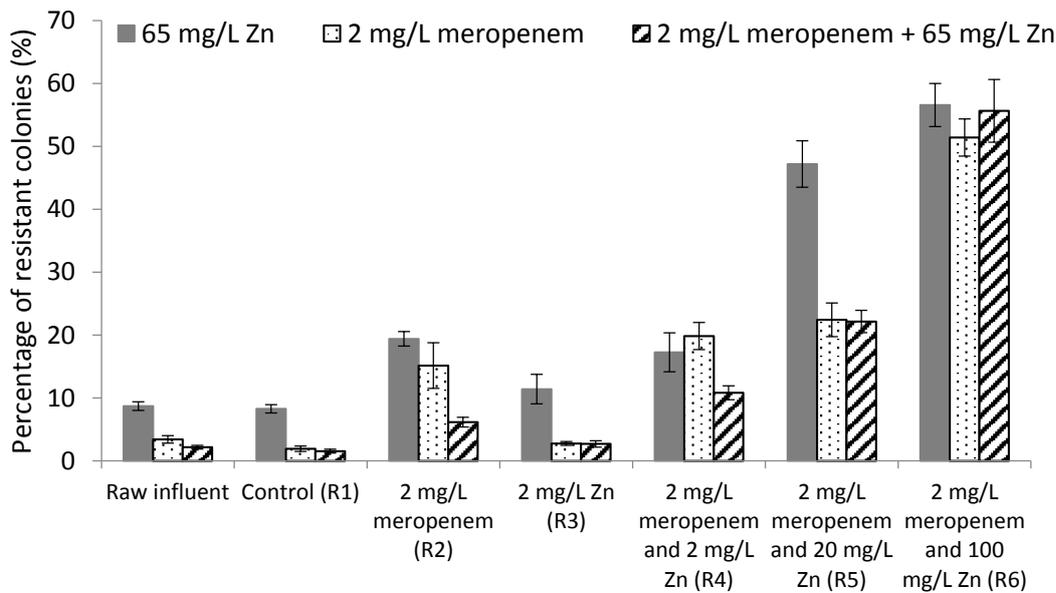
Figure 5-9. Percentage (%) of colonies in the effluent, resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor. Values indicated are averages over 4 weeks of sampling (all data shown in Table C-4) and standard error (S.E.) bars are shown.

In addition, Zn resistance was significantly higher in R5 and R6 compared to Zn alone (R3; Mann-Whitney; both p-values = 0.030) and, meropenem plus 2.00 mg/L Zn (R4; Mann-Whitney; both p-value = 0.030; Figure 5-9). Meropenem and combined resistance also were higher in R4, R5 and R6 relative to R3, which implies meropenem plus Zn increases all forms of detected resistance observed in liquid effluents (Figure 5-9).

5.3.5   *Measured Zn concentrations in biofilms and liquid effluents of each rotating tubular reactor*

The mean concentrations of total Zn in the liquid effluent ranged from below the detection limit of 0.01 mg/L to 2.51 mg/L Zn. The raw influent and R2 (no Zn addition) also were below detection limit relative to Zn (Figure 5-10). Highest mean Zn concentrations in the liquid effluent were detected in the highest Zn dosed reactor (R6; 2.51 mg/L Zn; S.E. ± 0.52), which were significantly higher than levels found in reactors dosed with only 2.00 mg/L Zn (R3; 0.11 mg/L; S.E. ± 0.06; Mann-Whitney; p-value = 0.030; Figure 5-10). The liquid level of total Zn in the reactor dosed with 20.0 mg/L Zn (R5; 0.39 mg/L; S.E. ± 0.05) also was statistically higher than levels observed in R3 (Mann-Whitney; p-value = 0.030).
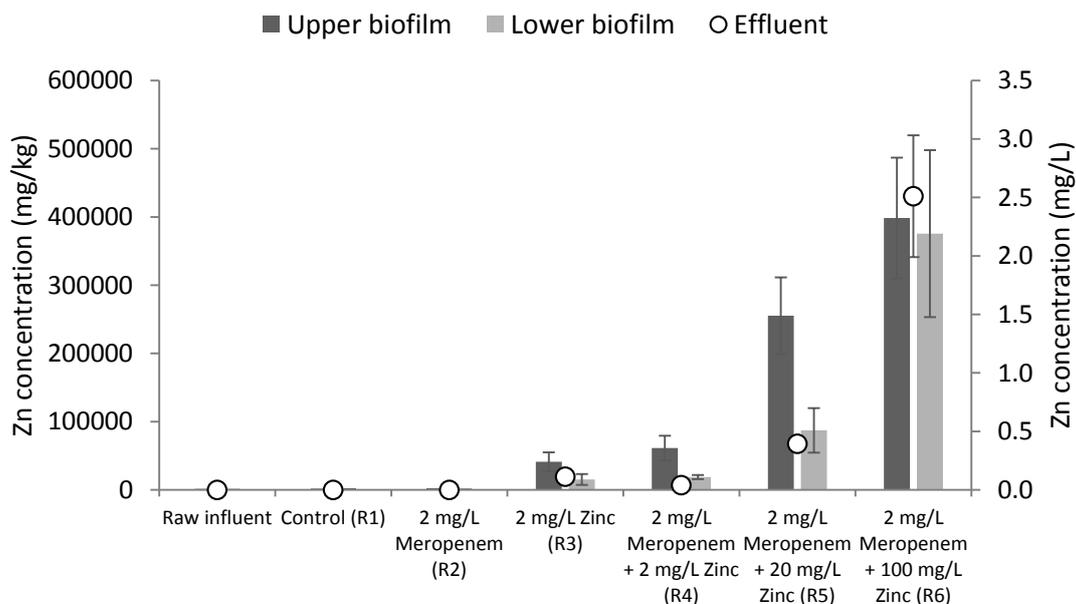
Figure 5-10 The concentrations of Zn in the raw influent and each rotating tubular reactor. The axis on the left represent the upper biofilm and lower biofilm Zn concentrations in mg/kg, while the axis on the right represent the raw influent and liquid effluent Zn concentrations in mg/L. Values indicated are averages (all data shown in Table C-5) over 4 weeks of dosing and standard error (S.E.) bars are shown.

Overall, absolute Zn levels in the liquid effluents were much lower than nominal levels added to the reactors, presumably because of Zn precipitation and accumulation in biofilms within the reactors. Hence, measured Zn levels in the upper and lower biofilms of all reactors were much higher than liquid-phase Zn levels at a statistically significant level (Mann-Whitney; Upper biofilm R1: p-value = 0.030; R2: p-value = 0.029; R3: p-value = 0.030; R4: p-value = 0.029; R5: p-value = 0.030; R6: p-value = 0.030; Lower biofilm- R1: p-value = 0.029; R2: p-value = 0.030; R3: p-value = 0.030; R4: p-value = 0.029; R5: p-value = 0.030; R6: p-value = 0.030; Figure 5-10).

The measured Zn levels in the upper biofilm were significantly higher in R3, R4, R5 and R6 compared to the control (R1) and R2 (Mann-Whitney; all p-values = 0.030). In addition, measured Zn levels in R5 and R6 where both significantly higher than concentrations in R3 (Mann-Whitney; p-value= 0.030) and R4 (Mann-Whitney; p-value= 0.030; Figure 5-10). Further, concentrations of Zn in the upper biofilm of all reactors were generally higher than those in the lower biofilm of all reactors. However, differences were only significant at lower confidence levels and quite variable (Mann-Whitney; R1: p-value = 0.030; R2: p-value = 0.312; R3: p-value = 0.312; R4: p-value = 0.194; R5: p-value = 0.061; R6: p-value = 1.000; Figure 5-10).

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The reasons for these differences are probably due to the Zn being dosed in the top of each reactor, allowing the upper biofilm to retain more Zn and more closely match Zn dose levels. The large standard errors associated with these values are probably due to the fact that Zn accumulated in some areas of the biofilm more than others, which was also reflected by weekly variations in detected Zn levels and differential sloughing of bio-solids out of the reactors. Despite the majority of Zn being retained in the biofilms of the reactors, there still was a strong positive correlation between Zn dose and Zn measured, in the upper and lower biofilms of each rotating tubular reactor (Pearson's correlation coefficient = 0.972; p-value = 0.001).

5.3.6 *Relationships between measured Zn levels and Zn, meropenem and combined resistance in rotating tubular reactors*

Resistance to Zn, meropenem and combined meropenem and Zn was higher in reactors dosed with 2.00 mg/L meropenem plus 20.0 or 100 mg/L Zn (Section 5.3.4). This was observed both in biofilms and in the liquid effluent, and significant correlations were observed between dosed and measured Zn levels (Section 5.3.5). However, these observations are specific to each reactor and it is important determine whether data from all the reactors can show more general trends.

Combining all upper biofilm data (i.e., all reactors), significant positive correlations are apparent between relative Zn, meropenem and combined resistance levels, and biofilm Zn levels (Pearson's correlation; p-value= 0.000; Figure 5-11a-c). Correlations were strongest between the percent abundance of meropenem resistant isolates and total Zn levels (Figure 5-11b). Similar correlations were seen between resistance and measured total Zn levels in the lower biofilms (Figure 5-11d-f), although trends and correlations are weaker, than those observed in the upper biofilms. Furthermore the same pattern between correlations was found, where correlations were strongest between relative percent meropenem resistant isolates and total Zn levels (Figure 5-11e).
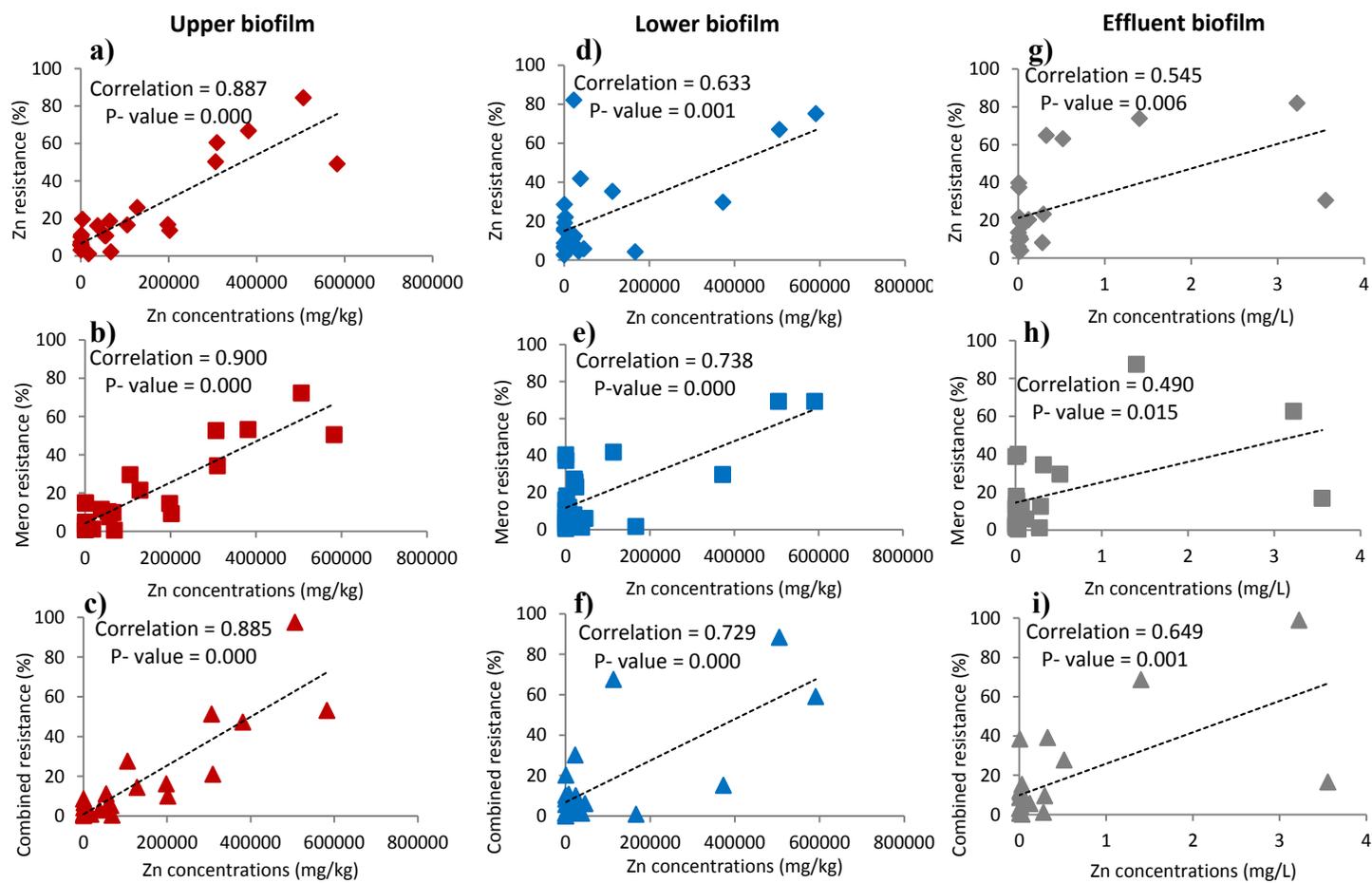
Figure 5-11 Pearson's correlations (r) between total Zn and percent Zn, meropenem (mero) and combined resistance in the upper biofilm (a, b, c), lower biofilm (d, e, f,) and effluent (g, h, i).

When combining data from liquid effluents from all reactors against measured Zn, generally positive trends were also seen (Figure 5-11g-i). However, the shape of data implies relationships may actually be non-linear, although there is not enough data to confirm this observation. These significant positive correlations observed between Zn level and all forms of resistance in the biofilms and the reactor effluents, confirms results seen in the river (Chapter 3) and long-term flasks samples (Chapter 4), and show that the presence of elevated Zn drives increased Zn, meropenem and combined resistance in all settings assessed here. Parallels were especially strong between Zn levels and combined meropenem and Zn resistance in the river sediment studies (Section 3.3.5).

5.3.7 *The effect of increased Zn amendments on Zn, meropenem and combined resistance in rotating tubular reactors*

Rotating tubular reactors dosed with 20.0 and 100 mg/L of Zn, as well as 2.00 mg/L meropenem, resulted in higher percent abundances of Zn, meropenem and combined resistant isolates (Section 5.3.4). Further there were also significant relationships between measured Zn levels and all forms of resistance (Section 5.3.6). These results can be compared to a similar study, where wastewater treatment units were set up with different metal and antibiotic inputs (Peltier *et al.*, 2010). Peltier *et al.* (2010) showed that the addition of Zn accompanied with moderate levels of antibiotics caused increased bacterial resistance to ciprofloxacin, oxytetracycline and tylosin. Therefore the pattern observed in the rotating tubular reactors dosed with high concentrations of Zn, provides further evidence that there are strong links between Zn and ambient AR levels.

As described in Chapter 3, possible reasons for this strong relationship include cross resistance and the influence of co-regulation mechanisms with the bacterial community. In the case of the rotating tubular reactors, the development of Zn resistance appears to confer resistance to meropenem due to cross resistance mechanisms, such as the same efflux proteins being used to export or import both meropenem and Zn (Chapman, 2003). This has been reported in many parallel contexts, using *P. aeruginosa*, where resistance to antibiotics have resulted from altered OM permeability as well as multiple RND efflux pumps, which were up regulated due to metal stress (Nikaido, 1994; Saier Jr *et al.*, 1994). Furthermore, a study done by Mata *et al.* (2000) reported that a MDR pump present in *L. monocytogenes* can export both metals and antibiotics from their cells. As data from the definitive ASRIT study shows that bacterial communities in the reactors dosed with 20.0 and 100 mg/L Zn (R5 and R6) were under metal stress, similar mechanisms are possible that would confer resistance to Zn and meropenem.

Another possible mechanism is co-regulation, where resistance is conferred when the exposure of bacteria to one toxic compound, can result in resistance to another compound, due to transcriptionally linked regulatory systems (Baker-Austin et al., 2006) (Section 2.8). An example of this is co-regulatory metal and antibiotic resistance, seen in clinical *P. aeruginosa* isolates (mentioned in section 3.3.7), where selected strains were resistant to both Zn and imipenem (Perron *et al.*, 2004). Therefore, meropenem and combined resistance trends seen in this study could result from similar mechanisms as those identified by Perron *et al.* (2004).

Many reports have demonstrated that biofilms can have effective and additional mechanisms of resistance to both metals and antibiotics, which are presented for cellular protection (Stewart and Costerton, 2001; Teitzel and Parsek, 2003; Harrison *et al.*, 2005). Bacteria within biofilms exhibit different physical and biochemical characteristics than 'free swimming' bacterial cells (i.e., planktonic bacteria), often using variable gene expression to coordinate cell movement and dispersal within these complex multilayered communities (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Furthermore, due to the close proximity of the bacteria in these biofilms, increased HGT could take place (Baker-Austin *et al.*, 2006). Therefore, the presence of biofilms in each rotating tubular reactor, may further contribute to higher levels of Zn, meropenem and combined resistance in the tubular reactors.

Some mechanisms behind this increased resistance to heavy metal and antibiotics include, the ability of the biofilm to accumulate or sequestrate metals or antibiotics, as well as the presence of persister cells (Harrison *et al.*, 2005). These are produced in microbial biofilms at a higher frequency than those in planktonic cells populations and they contribute to the amount of time required for biofilm or planktonic cells to become tolerant to high concentrations of antibiotics (Harrison *et al.*, 2005; Harrison *et al.*, 2007). Furthermore the addition of metal and antibiotics to planktonic cells can also stimulate the production of extracellular polymeric substances (EPS), leading to the eventual formation of a biofilm (Baker-Austin *et al.*, 2006). This was seen in studies where sub-inhibitory concentrations of tetracycline increased the presence of a precursor, needed for biofilm formation (Rachid *et al.*, 2000). The exposure of metals to archaea (Baker-Austin *et al.*, 2006), the cyanobacterium *Phormidium* (Garcia-Meza *et al.*, 2005), and, *Pseudomonas* species (Kidambi *et al.*, 1995) also increased the production of EPS, while EPS has also been observed in biofilms from metal contaminated activated sludge (Wuertz *et al.*, 2001). Therefore cross resistance and co-regulation resistance mechanisms are important in biofilms, where increased levels of metals, can also increase the ability of the cell to adopt AR determinants.

5.3.8 *Abundance of genes in rotating tubular reactors*

To determine whether there was any relationship between resistance levels and selected genes abundances, *Intl1* and *bla*-NDM-1 were quantified using qPCR. Figure 5-12 shows that there was no obvious trend between variation of *Intl1* gene levels and reactor operating conditions, although *Intl1* gene abundances were generally higher in the biofilms, especially the upper biofilm of each reactor, and lowest in the liquid effluent.
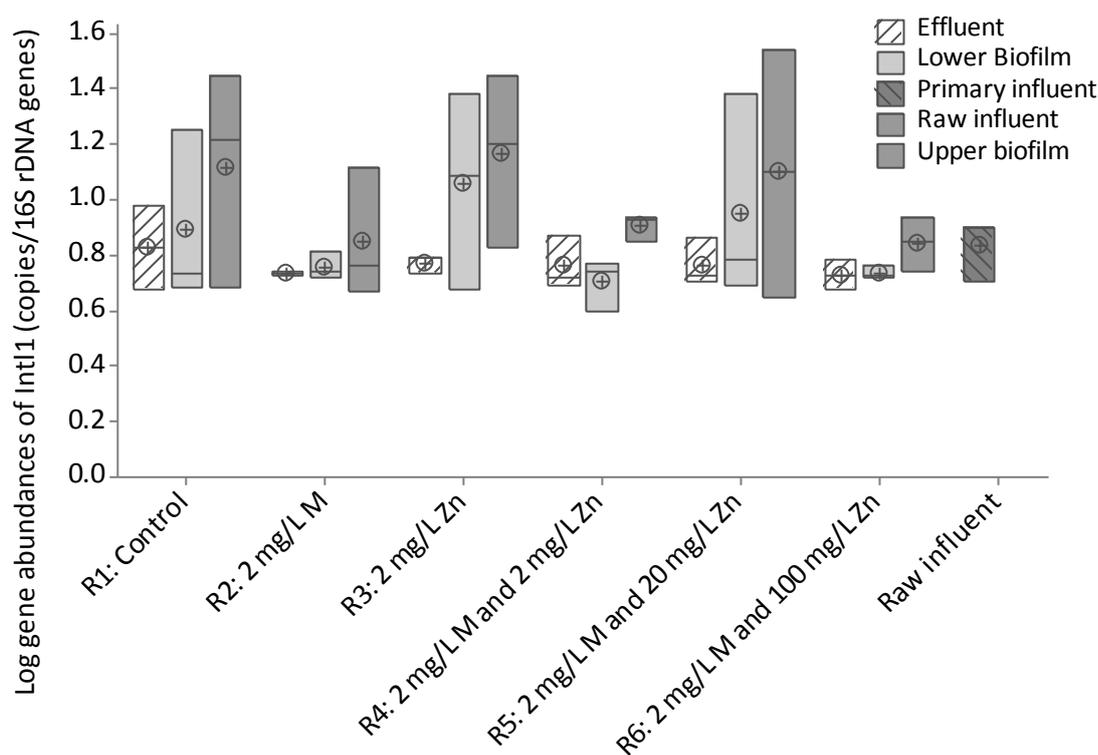
Figure 5-12 Relative abundances of Intl1 genes in the common raw influent, effluent, lower biofilm and upper biofilm of each rotating tubular reactor. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances. Box plots represent median and range values (n= 4, all data shown in Table C-6) and the symbol represents the mean of each sample.

However, relative gene abundances were significantly higher in the upper biofilm of the reactor containing 2.00 mg/L Zn (R3) relative to the 2.00 mg/L meropenem (R2; Mann-Whitney; p-value = 0.030) and the 2.00 mg/L meropenem and 100 mg/L Zn reactor (R6; Mann-Whitney; p-value = 0.030; Figure 5-12). Interestingly, this shows that there were indeed much lower *Intl1* genes in the liquid effluent and, that despite the high addition of 100 mg/L Zn (R6), their abundance was still higher in samples of the top biofilm in R3, containing only 2.00 mg/L Zn. Also, it was expected that the 2.00 mg/L meropenem and 100 mg/L Zn (R6) reactor would have higher genes abundances than other units, but this was not the case, suggesting that the genes responsible for co-selection between Zn and meropenem may not be associated with *Intl1* proteins. However, despite this, *Intl1* genes were present in all reactors,

which implies they are ubiquitous in the domestic wastewater regardless of Zn or meropenem levels.

Intl1 genes may be present in bacterial communities in the natural environment due to (i) there closeness to enriched sources of *Intl1* genes, such as WWTPs, (ii) the dispersal ability of the integron, through mobilization to a plasmid or transposons, (iii) the potential of HGT in the bacterial community and (iv) selective pressure (e.g., presence of heavy metals that can select and maintain genes contained within integrons) (Wright *et al.*, 2008). Therefore, it is possible that co-selection is taking place in natural environments due to co-resistance, where genes with metal and antibiotic resistant determinants are located on the same genetic elements, such as in plasmids, integrons or transposons (Chapman, 2003).

When looking at relative gene abundances of *bla*-NDM-1, an extended spectrum β-lactamase gene, there was no apparent trend between the variation of *bla*-NDM-1 genes and reactor amendments. However, the highest gene levels were generally found in the upper biofilm of each reactor, except for R3, which was dosed with Zn only. The lowest abundances were generally found in the liquid effluent of each reactor (Figure 5-13).
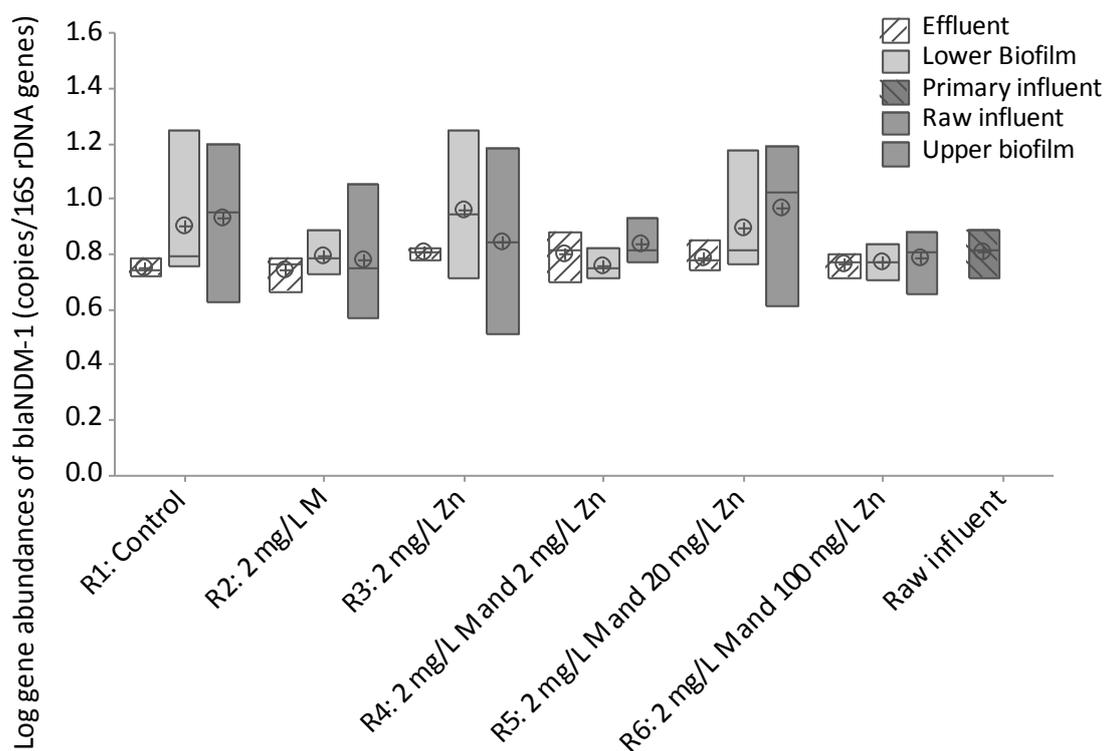
Figure 5-13 Relative abundances of bla-NDM-1, genes in the common raw influent, effluent, lower biofilm and upper biofilm of each rotating tubular reactor. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances. Box plots represent median and range values (n= 4, all data shown in Table C-7) and the symbol represents the mean of each sample.

There was also no general trends between the detection and abundance of *bla*-NDM-1 genes and *Intl1* genes in the reactors. All reactors contained *bla*-NDM-1 genes, however there were no significant differences between reactors suggesting that the different dosages of Zn in the reactors, did not influence the abundance of these genes. In addition, there was a possibility that the abundances shown consisted of similar carbapenem or *bla*-NDM-1 like genes, as the absolute abundance of *bla*-NDM-1 genes was higher than expected. There may be several reasons for this, which are outlined and explained in section 4.3.7. However, the fact that gene abundances were not higher in the reactor dosed with the higher levels of Zn (R5 and R6), one should not dismiss the fact that there may be similar or other genes that are causing the increased meropenem and combined meropenem and Zn resistance in the colonies of these reactors.

5.4   Conclusions

This study assessed culturable Zn, meropenem and combined meropenem and Zn resistance (combined resistance) and *Intl1* and *bla*-NDM-1 gene abundances, in upper and lower biofilms and liquid effluents of rotating tubular reactors provided with different supplemental additions of meropenem and Zn. Sampling over 4 weeks showed significantly higher abundances of bacteria resistant to Zn, meropenem and a combination of both, in the upper biofilm and effluents of rotating tubular reactors dosed with 2.00 mg/L meropenem plus 20.0 or 100 mg/L Zn (R5 and R6), compared to control (R1) reactors. No significant trends were demonstrated between reactor dosages in the lower biofilm; however, trends were similar to those seen in upper biofilms in R5 and R6 and the control reactor. Furthermore, in the upper biofilm, Zn and combined resistance also were higher compared to the reactor with only meropenem addition (R2). This implies that the high dosed concentrations of 20.0 and 100 mg/L Zn, where measured Zn levels were significantly higher, may have had an effect on the level of resistance observed in isolates.

In addition there were greater numbers of culturable meropenem and combined resistant isolates in the upper biofilm of R5 and R6, compared to the reactor dosed with only Zn (R3), suggesting that the presence of 2.00 mg/L meropenem may also have had an effect on resistance observed. This pattern was also seen in the effluent, where meropenem and combined resistance was higher in R4, R5 and R6 compared to R3. Positive correlations were apparent between relative percent Zn, meropenem and combined resistant isolates and measured Zn levels in all parts of the reactor, demonstrating that the dosing of increased Zn, resulted in high measured Zn, which also resulted in higher resistance. Therefore Zn may be

conferring resistance to Zn, meropenem and a combination of both, due to a variety of mechanisms, including cross resistance and co-regulation.

When assessing the abundance of *Intl1* and *bla*-NDM-1 gene levels, there were no apparent trends between the reactor dosages and the abundance of genes, however the highest gene abundances were generally observed in the upper biofilm of each reactor. Despite there not being a relationship between high measured Zn and increased *Intl1* and *bla*-NDM-1 gene abundances, there may be similar or other genes that are facilitating in the co-selection of meropenem and Zn resistance in the colonies of these reactors.

Chapter 6   Conclusions and recommended future work

6.1   Conclusions

This thesis studied relationships between total and soluble Zn levels, and relative levels of meropenem and combined meropenem and Zn resistance (combined resistance) in the environment. Three large studies were performed that used sediments and associated water from local rivers (Chapter 3); experimental bioreactors seeded with sediments and amended with different Zn levels (Chapter 4); and rotating tubular reactors inoculated with wastewater and different levels of Zn and meropenem to assess how Zn and meropenem resistance changed under different exposures (Chapter 5).

The overall aim of this thesis was to assess and determine whether Zn concentrations increased and promoted relative percent meropenem and combined resistance in microbial communities within rivers and wastewaters. The following hypothesis were also made:

a) Ambient Zn conditions influence levels of meropenem and combined resistance in aquatic systems

b) Zn availability influences meropenem and combined resistance

c) The observed resistance relationships translate from reactor to sediment conditions

All three studies met the overall aim and showed that environments with high concentrations of Zn, especially soluble Zn, displayed greater abundances of culturable meropenem and combined meropenem and Zn resistant strains, which is important for understanding how heavy metals, like Zn, influence the extent and characteristics of AR in the environment. It should be noted that many other experiments were performed within this work, but only the most explanatory have been included in the thesis.

Initial work focused on quantitative and phenomenological studies on relationships between *in situ* Zn levels and meropenem resistance along the two arms of the River Tyne (Chapter 3). These studies were performed to determine whether Zn levels and apparent meropenem resistance were related in nature, which would provide the basis for further laboratory investigations. Chapter 3 shows that the relative percent of Zn and combined resistance were significantly higher in sediments and river water from reaches along the river with higher concentrations of Zn (South Tyne), relative to resistance observed in low Zn reaches (North Tyne). Specifically, total, soluble and colloidal Zn levels all were statistically significantly higher at sites within the South Tyne basin (Featherstones; FS, West Allen; WA, and River Nent; RN) relative to sites from the North Tyne basin (Reavehill; RH and Warks Burn; WB), which were associated with significantly higher levels of Zn resistant isolates. Further,

stronger significant correlations were observed between soluble Zn levels and combined resistance, which implies that *in situ* resistance in soluble Zn is more important than total Zn, possibly due to toxicity induced cross resistance in resident strains.

The first hypothesis (a) can therefore be accepted, as ambient Zn conditions influenced levels of meropenem and combined resistance in aquatic systems with high levels of Zn contamination. Further the second hypothesis (b) can also be accepted as it is clear that the availability of soluble Zn influences the level of meropenem and combined resistance seen in river sediments and waters.

Experiments conducted in Chapter 4 also confirm the acceptance of the latter hypothesis (b). Increased relative meropenem resistance was observed in experiments (Chapter 4), where batch reactors were seeded with sediments taken from the North Tyne (WB) and South Tyne basins (WA) and amended with high (100 mg/L) and low levels (2.00 mg/L) of soluble Zn. Interestingly, the original sediment source had no influence on the level of observed meropenem and combined resistance over 309 days. However, meropenem resistance significantly positively correlated with soluble Zn levels in all reactors, confirming that soluble Zn is probably more bioavailable and stress-inducing than total Zn. Therefore, metal form and probably speciation will likely be critical to the observance of *in situ* resistance to meropenem in natural and engineered environments.

Within this context, experiments were performed using continuous-flow rotating tubular reactors treating settled domestic wastewater to assess whether similar observations related to the co-occurrence of Zn and meropenem resistance were apparent in a contrasting environment (Chapter 5). Reactors were acclimated to effectively treat the wastewater and biofilms readily formed on their walls. The reactors were then dosed with 0.00 or 2.00 mg/L meropenem and-or 2.00, 20.0 or 100 mg/L Zn respectively, and compared with a non-amended control reactor.

Similar to the field and batch experimental studies (Chapter 3 and Chapter 4), significantly higher relative abundances of bacteria resistant to Zn, meropenem and a combination of both were observed in the upper biofilm (top end of the reactor) and liquid effluents of the reactors with 2.00 mg/L meropenem added plus 20.0 or 100 mg/L Zn, compared to the control reactor. Positive significant correlations were also apparent between relative percent Zn, meropenem and combined resistant isolates, and measured Zn levels in all parts of the reactor, which confirms dosing with Zn results in higher measured Zn levels and in higher observed resistance. Although it is speculation, Zn appears to select for resistance to Zn, meropenem and a combination of both in bioreactor microbial communities, probably due to a variety of

mechanisms, including cross resistance and co-regulation. Thus this study (Chapter 5) also confirmed the second hypothesis (b), indicating that indeed available soluble Zn plays a major role in meropenem and combined resistance. Further the experiments conducted in Chapter 4 and Chapter 5, where reactor set-ups were used, confirms that the observed resistance relationship can be translated from reactor to sediment conditions. In both these experiments the presence of Zn increased the observed resistance to meropenem and-or combined resistance, confirming the acceptance of the third hypothesis (c).

To investigate quantitative phenotypic observations, molecular biology work using community fingerprinting (DGGE) and qPCR was employed to better understand possible relationships between Zn levels and microbial communities in the seeded batch reactors (Chapter 4) and rotating tubular reactors (Chapter 5). This work revealed that there was a clear shift in observed microbial community diversity over time in the sediment-seeded batch reactors (Chapter 4); i.e., higher levels of observed meropenem resistance were associated with significantly altered microbial communities that were able to withstand and tolerate meropenem exposure in culturable isolates. This increased resistance, could be because of evolutionary change at a genetic level specific to Zn or could simply result from increased community tolerance to stress, which may have occurred due to stringent nutrient conditions in the reactors.

To assess possible direct genetic change versus a community stress response, bla-NDM-1 gene levels were quantified among reactors (a Zn-associated meropenem resistance-related gene) and were found to increase in abundance over time in all reactors (Chapter 4). However this apparent increase could result from factors unrelated to the sediment source or Zn amendments, such as the bla-NDM-1 probe detecting similar genes in the natural samples that do not relate to Zn resistance specifically or generic cell stress in the reactors due to starvation conditions. Delineating mechanisms requires further work. In contrast, there were no apparent trends between the reactor Zn and meropenem dosages and the abundance of bla-NDM-1 genes in the rotating tubular reactors (Chapter 5). However, the highest bla-NDM-1 gene abundances were generally observed in the upper biofilm of each reactor, which is where Zn and nominal meropenem levels are the highest, suggesting there may be some influence of Zn and meropenem on genes where exposure levels are high. It should be noted that in both studies, other possible genes that facilitate co-selection of meropenem and Zn resistance might also be present, which were not tested here. Examples include genes that code for Zn efflux and uptake pumps, which could be induced by Zn, but then confer meropenem resistance due to non-specific restricted efflux or uptake.

In total, the broad conclusion of this work is that the presence of elevated Zn, especially soluble Zn, that is intrinsically more toxic, significantly influences microbial communities in natural environments and experimental bioreactors. This, in turn, results in increased relative meropenem, Zn and combined resistance to locations of high soluble Zn. However, observed meropenem resistance may be Zn-dependent, which implies meropenem resistance will be most apparent when Zn is present and may not translate to other environments when Zn levels are reduced.

6.2   Future work

An array of additional work is possible from this project. Most importantly, further development of culturing and molecular techniques is essential to provide deeper insights on why meropenem resistance is increasing in natural environments and experimental bioreactors, when microbial communities are exposed to high Zn levels. Specifically, when examining relationships between Zn, meropenem and combined resistance in the two river basins with low and high Zn levels (Chapter 3), other heavy metals, including Pb and Cd were also high in the sediments, and further study is required to determine the relative importance of these metals. Areas of improvement to this experiment include:

- Culturing for isolates on Pb and Cd amended agar plates (with and without meropenem) from sediments from each basin to compare with the Zn and meropenem resistance data.
- Testing different concentrations and combinations of Pb, Cd and Zn amendments would also be valuable to assess the impact of metal mixtures on observed meropenem resistance.
- Performing additional cross resistance studies and identification of novel isolates using Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF) and-or isolate sequencing. These isolates could then be assessed on very specific level relative to both gene expression and cross- and multi-resistance traits.

The set-up of batch reactors seeded with sediments from a low and high contaminated Zn area and amended with low and high levels of Zn (Chapter 4), showed that meropenem resistance increased over time (309 days) independent of the original Zn levels in sediments. Areas of further development in context with this experiment include:

- The set-up of similar batch experiments, where batch reactors are amended with a spread of Zn concentrations to determine what level of Zn tends to select for the

highest number of meropenem resistant isolates. An experiment of this type was performed within this project, but operational problems ensued and results were inconclusive. This experiment should be performed again, but additional genetic work should be included, especially qPCR on possible Zn efflux and uptake genes, to contrast their abundance over time and the co-occurrence of combined resistance in isolates from the same systems.

- Distinguish between treatment-specific versus generic starvation effects, with such an experiment to determine whether resistance developed due to Zn specifically, starvation alone, or starvation impacted Zn-specific effects.

Independent of the above specific experiments, quantifying known efflux/uptake pump genes in different Zn-exposed environments are needed to delineate mechanisms of metal-induced resistance, especially over time and across exposures. This work could be coupled with;

- Further analysis of water and sediments samples from the North and South Tyne (Chapter 3), and also from rotating tubular reactors inoculated with wastewater and dosed with varying concentrations of meropenem and Zn (Chapter 5).

- Design new qPCR probes for this work that target co-resistance and efflux/influx genes to determine how Zn levels correlate with these genes and observed meropenem co-resistance.

Chapter 7   References

Abel, P.D. (1996) *Water Pollution Biology*. 2nd edn. London, UK: Taylor and Francis Ltd.

Abenavoli, L. and Almasio, P.L. (2011) 'Chronic hepatitis C infection and insulin resistance: two best friends', *Expert Rev Anti Infect Ther*, 9(8), pp. 555-558.

Abraham, E.P. and Chain, E. (1988) 'An enzyme from bacteria able to destroy penicillin. 1940', *Rev Infect Dis*, 10(4), pp. 677-8.

Ahammad, Z.S., Sreekrishnan, T.R., Hands, C.L., Knapp, C.W. and Graham, D.W. (2014) 'Increased waterborne bla_{NDM-1} resistance gene abundances associated with seasonal human pilgrimages to the upper ganges river', *Environ Sci Technol*, 48(5), pp. 3014-20.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. (2010) 'Call of the wild: antibiotic resistance genes in natural environments', *Nat Rev Microbiol*, 8(4), pp. 251-9.

Alonso, A., Sánchez, P. and Martínez, J.L. (2001) 'Environmental selection of antibiotic resistance genes', *Environ Microbiol*, 3(1), pp. 1-9.

Aminov, R.I. (2011) 'Horizontal Gene Exchange in Environmental Microbiota', *Front Microbiol*, 2, p. 158.

Andersson, D.I. and Hughes, D. (2011) 'Persistence of antibiotic resistance in bacterial populations', *FEMS Microbiol Rev*, 35(5), pp. 901-11.

Anton, A., Große, C., Reißmann, J., Pribyl, T. and Nies, D.H. (1999) 'CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in Ralstonia sp. strain CH34', *J Bacteriol*, 181(22), pp. 6876-6881.

Antunes, P., Machado, J. and Peixe, L. (2006) 'Characterization of antimicrobial resistance and class 1 and 2 integrons in Salmonella enterica isolates from different sources in Portugal', *J Antimicrob Chemother*, 58(2), pp. 297-304.

APHA, A.P.H.A. (2005) *Standard Methods for the Examination of Water & Wastewater*. 21 edn. United States: American Public Health Association.

Archer, D. (2008) 'The influence of river regulation at Kielder Water on the thermal regime of the River North Tyne', Exeter. BHS 10th National Hydrology Symposium.

AstraZenenca (2000) 'AstraZeneca extends manufacturing agreement with Sumitomo Pharmaceuticals to boost 'Merrem/Meronem world-wide supply'. Available at: http://www.astrazeneca.com/Media/Press-releases/Article/20000531--ASTRAZENECA-EXTENDS-MANUFACTURING-AGREEMENT-WITH-SUMI (Accessed: 21/08/2015).

AstraZenenca (2010) *Environmental Risk Assessment Data- Meropenem*. Available at: https://www.astrazeneca.com/content/dam/az/our-company/Sustainability/Meropenem.pdf (Accessed: 08/04/2016).

Auerbach, E.A., Seyfried, E.E. and McMahon, K.D. (2007) 'Tetracycline resistance genes in activated sludge wastewater treatment plants', *Water Res*, 41(5), pp. 1143-51.

Baker-Austin, C., Wright, M.S., Stepanauskas, R. and McArthur, J.V. (2006) 'Co-selection of antibiotic and metal resistance', *Trends Microbiol*, 14(4), pp. 176-82.

Balke, B., Hogardt, M., Schmoldt, S., Hoy, L., Weissbrodt, H. and Haussler, S. (2006) 'Evaluation of the E test for the assessment of synergy of antibiotic combinations against multiresistant Pseudomonas aeruginosa isolates from cystic fibrosis patients', *Eur J Clin Microbiol Infect Dis*, 25(1), pp. 25-30.

Baquero, F., Martinez, J.L. and Canton, R. (2008) 'Antibiotics and antibiotic resistance in water environments', *Curr Opin Biotech*, 19(3), pp. 260-5.

Baranova, N. and Nikaido, H. (2002) 'The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate', *J Bacteriol*, 184(15), pp. 4168-76.

Barkay, T., Miller, S.M. and Summers, A.O. (2003) 'Bacterial mercury resistance from atoms to ecosystems', *FEMS Microbiol Rev*, 27(2-3), pp. 355-84.

Baroud, M., Dandache, I., Araj, G.F., Wakim, R., Kanj, S., Kanafani, Z., Khairallah, M., Sabra, A., Shehab, M., Dbaibo, G. and Matar, G.M. (2013) 'Underlying mechanisms of carbapenem resistance in extended-spectrum beta-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases', *Int J Antimicrob Agents*, 41(1), pp. 75-9.

Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., Thayer, S.G. and Maurer, J.J. (1999) 'Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian Escherichia coli', *Antimicrob Agents Chemother*, 43(12), pp. 2925-9.

Bassetti, M., Nicolini, L., Esposito, S., Righi, E. and Viscoli, C. (2009) 'Current status of newer carbapenems', *Curr Med Chem*, 16(5), pp. 564-75.

Beard, S.J., Hashim, R., Membrillo-Hernandez, J., Hughes, M.N. and Poole, R.K. (1997) 'Zinc(II) tolerance in Escherichia coli K-12: evidence that the zntA gene (o732) encodes a cation transport ATPase', *Mol Microbiol*, 25(5), pp. 883-91.

Bedenic, B., Plecko, V., Sardelic, S., Uzunovic, S. and Godic Torkar, K. (2014) 'Carbapenemases in gram-negative bacteria: laboratory detection and clinical significance', *Biomed Res Int*, 2014, p. 841951.

Benveniste, R. and Davies, J. (1973) 'Aminoglycoside Antibiotic-Inactivating Enzymes in Actinomycetes Similar to Those Present in Clinical Isolates of Antibiotic-Resistant Bacteria', *Proc Natl Acad Sci USA*, 70(8), pp. 2276–2280.

Berg, J., Brandt, K.K., Al-Soud, W.A., Holm, P.E., Hansen, L.H., Sørensen, S.J. and Nybroe, O. (2012) 'Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term cu exposure', *Appl Environ Microbiol*, 78(20), pp. 7438-7446.

Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O. and Brandt, K.K. (2010) 'Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay', *Environ Sci Technol*, 44(22), pp. 8724-8728.

Berg, J., Tom-Petersen, A. and Nybroe, O. (2005) 'Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field', *Lett Appl Microbiol*, 40(2), pp. 146-151.

Bhagat, R. and Srivastava, S. (1993) 'Growth response of Pseudomonas stutzeri RS34 to ethylenediaminetetraacetic acid (EDTA) and its interaction with zinc', *Indian J Exp Biol*, 31(7), pp. 590-4.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A. and Wright, G.D. (2012) 'Antibiotic resistance is prevalent in an isolated cave microbiome', *PLoS One*, 7(4), p. e34953.

Bing (2015) *Totnes, Devon, United Kingdom*. Available at: https://www.bing.com/maps/#Y3A9NTQuOTgwNDk5fi0xLjYxODMwMCZzdmw9NiZzdHk9ciZlbz0wJnE9VG90bmVzJTJDJTIwVW5pdGVkJTIwS2luZ2RvbQ== (Accessed: 22/07/2015).

Birkett, C.I., Ludlam, H.A., Woodford, N., Brown, D.F., Brown, N.M., Roberts, M.T., Milner, N. and Curran, M.D. (2007) 'Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum beta-lactamases', *J Med Microbiol*, 56(Pt 1), pp. 52-5.

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J.V. (2015) 'Molecular mechanisms of antibiotic resistance', *Nat Rev Micro*, 13(1), pp. 42-51.

Blake, K.L. and O'Neill, A.J. (2013) 'Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents', *J Antimicrob Chemother*, 68(1), pp. 12-6.

Boerlin, P. and Reid-Smith, R.J. (2008) 'Antimicrobial resistance: its emergence and transmission', *Anim Health Res Rev*, 9(2), pp. 115-26.

Boucher, Y., Labbate, M., Koenig, J.E. and Stokes, H.W. (2007) 'Integrons: mobilizable platforms that promote genetic diversity in bacteria', *Trends in Microbiology*, 15(7), pp. 301-309.

Bradley, J.S., Garau, J., Lode, H., Rolston, K.V.I., Wilson, S.E. and Quinn, J.P. (1999) 'Carbapenems in clinical practice: a guide to their use in serious infection', *Int J Antimicrob Agents*, 11(2), pp. 93-100.

Brady, F.O. (1982) 'The physiological function of metallothionein', *Trends Biochem Sci*, 7(4), pp. 143-145.

Bush, K., Jacoby, G.A. and Medeiros, A.A. (1995) 'A functional classification scheme for beta-lactamases and its correlation with molecular structure', *Antimicrob Agents Chemother,*, 39(6), pp. 1211-1233.

Cabrero, A., Fernandez, S., Mirada, F. and Garcia, J. (1998) 'Effects of copper and zinc on the activated sludge bacteria growth kinetics', *Water Res*, 32(5), pp. 1355-1362.

Canton, R., Akova, M., Carmeli, Y., Giske, C.G., Glupczynski, Y., Gniadkowski, M., Livermore, D.M., Miriagou, V., Naas, T., Rossolini, G.M., Samuelsen, O., Seifert, H., Woodford, N. and Nordmann, P. (2012) 'Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe', *Clin Microbiol Infect*, 18(5), pp. 413-31.

Caplin, J.L., Hanlon, G.W. and Taylor, H.D. (2008) 'Presence of vancomycin and ampicillin-resistant Enterococcus faecium of epidemic clonal complex-17 in wastewaters from the south coast of England', *Environ Microbiol*, 10(4), pp. 885-92.

Chaiyanan, S., Chaiyanan, S., Huq, A., Maugel, T. and Colwell, R.R. (2001) 'Viability of the nonculturable Vibrio cholerae O1 and O139', *Syst Appl Microbiol*, 24(3), pp. 331-41.

Chapman, J.S. (2003) 'Disinfectant resistance mechanisms, cross-resistance, and co-resistance', *Int Biodeterior Biodegradation*, 51(4), pp. 271-276.

Charif, G., Nitin, R., Amal, A.-S. and Shinoona, A.-A. (2012) 'A Critical Review of Removal of Zinc from Wastewater', *Lecture Notes in Engineering and Computer Science*. Available at: http://www.iaeng.org/publication/WCE2012/WCE2012_pp627-630.pdf.

Charrel, R.N., Pagès, J.M., De Micco, P. and Mallea, M. (1996) 'Prevalence of outer membrane porin alteration in beta-lactam-antibiotic-resistant Enterobacter aerogenes', *Antimicrob Agents Chemother*, 40(12), pp. 2854-2858.

Cheng, J., Hicks, D.B. and Krulwich, T.A. (1996) 'The purified Bacillus subtilis tetracycline efflux protein TetA(L) reconstitutes both tetracycline–cobalt/H(+) and Na(+)(K(+))/H(+) exchange', *Proc Natl Acad Sci USA*, 93(25), pp. 14446-14451.

Chou, A., Archdeacon, J. and Kado, C.I. (1998) 'Agrobacteriumtranscriptional regulator Ros is a prokaryotic zinc finger protein that regulates the plant oncogeneipt (cytokinin)', *Proc Natl Acad Sci USA*, 95, pp. 5293-5298.

Choudhury, R. and Srivastava, S. (2001a) 'Mechanisms of zinc resistance in *Pseudomonas putida* strain S$', *World J Microbiol Biotechnol*, 17(149-153).

Choudhury, R. and Srivastava, S. (2001b) 'Zinc resistance mechanisms in bacteria', *Curr Sci India*, 81(7).

Chung, H.J., Bang, W. and Drake, M.A. (2006) 'Stress Response of Escherichia coli', *Compr Rev Food Sci F*, 5(3), pp. 52-64.

Cielecka-Piontek, J., Zajac, M. and Jelinska, A. (2008) 'A comparison of the stability of ertapenem and meropenem in pharmaceutical preparations in solid state', *J Pharm Biomed Anal*, 46(1), pp. 52-7.

Clarke, K.R. (1993) 'Non-parametric multivariate analyses of changes in community structure', *Aust J Ecol*, 18, pp. 117-143.

Clarke, K.R. and Gorley, R.N. (2006) *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-R.

Cox, G. and Wright, G.D. (2013) 'Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions', *Int J Mech Sci*, 303(6–7), pp. 287-292.

Czekalski, N., Berthold, T., Caucci, S., Egli, A. and Bürgmann, H. (2012) 'Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland', *Front Microbiol*, 3(MAR).

D'Costa, V.M., McGrann, K.M., Hughes, D.W. and Wright, G.D. (2006) 'Sampling the antibiotic resistome', *Science*, 311(5759), pp. 374-7.

Datta, N. and Hughes, V.M. (1983) 'Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics', *Nature*, 306(5943), pp. 616-7.

Davey, P., Brown, E., Fenelon, L., Finch, R., Gould, I., Hartman, G., Holmes, A., Ramsay, C., Taylor, E., Wilcox, M. and Wiffen, P. (2005) 'Interventions to improve antibiotic prescribing practices for hospital inpatients', *Cochrane Database Syst Rev*, (4), p. Cd003543.

Davies, J. and Davies, D. (2010) 'Origins and evolution of antibiotic resistance', *Microbiol Mol Biol Rev*, 74(3), pp. 417-33.

Davies, J., Spiegelman, G.B. and Yim, G. (2006) 'The world of subinhibitory antibiotic concentrations', *Curr Opin Microbiol*, 9(5), pp. 445-53.

Davies, J.E. (1997) 'Origins, acquisition and dissemination of antibiotic resistance determinants', *Ciba Found Symp*, 207, pp. 15-27; discussion 27-35.

Davis, I.J., Richards, H. and Mullany, P. (2005) 'Isolation of silver- and antibiotic-resistant Enterobacter cloacae from teeth', *Oral Microbiol Immunol*, 20(3), pp. 191-4.

Davison, J. (1999) 'Genetic exchange between bacteria in the environment', *Plasmid*, 42(2), pp. 73-91.

Diels, L., Dong, Q., van der Lelie, D., Baeyens, W. and Mergeay, M. (1995) 'The czc operon of Alcaligenes eutrophus CH34: from resistance mechanism to the removal of heavy metals', *J Ind Microbiol*, 14(2), pp. 142-53.

Dinh, T., Paulsen, I.T. and Saier, M.H. (1994) 'A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria', *J Bacteriol*, 176(13), pp. 3825-3831.

Dintilhac, A., Alloing, G., Granadel, C. and Claverys, J.P. (1997) 'Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases', *Mol Microbiol*, 25(4), pp. 727-39.

Dortet, L., Poirel, L. and Nordmann, P. (2014) 'Worldwide Dissemination of the NDM-Type Carbapenemases in Gram-Negative Bacteria', *Biomed Res Int*, 2014, p. 249856.

Drusano, G.L., Liu, W., Fregeau, C., Kulawy, R. and Louie, A. (2009) 'Differing effects of combination chemotherapy with meropenem and tobramycin on cell kill and suppression of resistance of wild-type Pseudomonas aeruginosa PAO1 and its isogenic MexAB efflux pump-overexpressed mutant', *Antimicrob Agents Chemother*, 53(6), pp. 2266-73.

EA, E.A. (2008) *River Tyne Salmon Action Plan Review* (APEM REF: EA 410230).

EA, E.A.E.W. (2009) *River Basin Management Plan: Northumbria River Basin District.* Bristol, UK: Environment Agency.

EC, E.C. (1976) 'Directive 76/464/EEC- Water pollution by discharges of certain dangerous substances', *OJ*, 19, p. L 129.

Eitinger, T. and Friedrich, B. (1991) 'Cloning, nucleotide sequence, and heterologous expression of a high-affinity nickel transport gene from Alcaligenes eutrophus', *J Biol Chem*, 266(5), pp. 3222-7.

Fagan, M.J. and Saier, M.H., Jr. (1994) 'P-type ATPases of eukaryotes and bacteria: sequence analyses and construction of phylogenetic trees', *J Mol Evol*, 38(1), pp. 57-99.

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J.C., Ghysels, B., Matthijs, S., Cornelis, P., Wiehlmann, L., Tümmler, B., Baquero, F. and Martínez, J.L. (2008) 'The Neglected Intrinsic Resistome of Bacterial Pathogens', *PLoS One*, 3(2), p. e1619.

Fath, M.J. and Kolter, R. (1993) 'ABC transporters: bacterial exporters', *Microbiol Rev*, 57(4), pp. 995-1017.

Fick, J., Soderstrom, H., Lindberg, R.H., Phan, C., Tysklind, M. and Larsson, D.G. (2009) 'Contamination of surface, ground, and drinking water from pharmaceutical production', *Environ Toxicol Chem*, 28(12), pp. 2522-7.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A. and Dantas, G. (2012) 'The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens', *Science*, 337(6098), pp. 1107-1111.

Foster, T.J. (1983) 'Plasmid-Determined Resistance to Antimicrobial Drugs and Toxic Metal Ions in Bacteria', *Microbiol Rev*, 47(3), pp. 361-409.

Garcia-Meza, J.V., Barrangue, C. and Admiraal, W. (2005) 'Biofilm formation by algae as a mechanism for surviving on mine tailings', *Environ Toxicol Chem*, 24(3), pp. 573-81.

Gillings, M.R. (2013) 'Evolutionary consequences of antibiotic use for the resistome, mobilome, and microbial pangenome', *Front Microbiol*, 4(JAN).

Gillings, M.R. (2014) 'Integrons: Past, present, and future', *Microbiol Mol Biol Rev*, 78(2), pp. 257-277.

Gilotra, U. and Srivastava, S. (1997) 'Plasmid-encoded sequestration of copper by Pseudomonas pickettii strain US321', *Curr Microbiol*, 34(6), pp. 378-81.

Girgis, H.S., Hottes, A.K. and Tavazoie, S. (2009) 'Genetic architecture of intrinsic antibiotic susceptibility', *PLoS One*, 4(5), p. e5629.

Goswitz, V.C. and Brooker, R.J. (1995) 'Structural features of the uniporter/symporter/antiporter superfamily', *Protein Sci*, 4(3), pp. 534-7.

Gowri, P.M. and Srivastava, S. (1996) 'Reduced uptake based zinc resistance in Azosprillum brasilense sp7', *Curr Sci India*, 71(2), pp. 139-142.

Gozzard, E., Mayes, W.M., Potter, H.A. and Jarvis, A.P. (2011) 'Seasonal and spatial variation of diffuse (non-point) source zinc pollution in a historically metal mined river catchment, UK', *Environ Pollut*, 159(10), pp. 3113-22.

Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D. and Bowen, E. (2011) 'Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba', *Environ. Sci. Technol.*, 45(2), pp. 418-424.

Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G., Skurray, R.A., Paulsen, I.T., Chater, K.F., Baldwin, S.A. and Henderson, P.J. (1992) 'Membrane transport proteins: implications of sequence comparisons', *Curr Opin Cell Biol*, 4(4), pp. 684-95.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbe, J., Ploy, M.C. and Mazel, D. (2009) 'The SOS response controls integron recombination', *Science*, 324(5930), p. 1034.

Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D. and Andersson, D.I. (2011) 'Selection of resistant bacteria at very low antibiotic concentrations', *PLoS Pathog*, 7(7), p. e1002158.

Harrison, J.J., Ceri, H. and Turner, R.J. (2007) 'Multimetal resistance and tolerance in microbial biofilms', *Nat Rev Micro*, 5(12), pp. 928-938.

Harrison, J.J., Turner, R.J. and Ceri, H. (2005) 'Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa', *Environ Microbiol*, 7(7), pp. 981-94.

Hassan, M.-e.-T., van der Lelie, D., Springael, D., Römling, U., Ahmed, N. and Mergeay, M. (1999) 'Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*', *Elsevier Science*, Gene 238, pp. 417-425.

Hawkey, P.M. and Livermore, D.M. (2012) 'Carbapenem antibiotics for serious infections', *BMJ*, 344, p. e3236.

Henderson, P.J. and Maiden, M.C. (1990) 'Homologous sugar transport proteins in Escherichia coli and their relatives in both prokaryotes and eukaryotes', *Philos Trans R Soc Lond B Biol Sci*, 326(1236), pp. 391-410.

Henriques, I., Moura, A., Alves, A., Saavedra, M.J. and Correia, A. (2006) 'Analysing diversity among beta-lactamase encoding genes in aquatic environments', *FEMS Microbiol Ecol*, 56(3), pp. 418-29.

Hibbing, M.E., Fuqua, C., Parsek, M.R. and Peterson, S.B. (2010) 'Bacterial competition: surviving and thriving in the microbial jungle', *Nat Rev Microbiol*, 8(1), pp. 15-25.

Higgins, C.F., Haag, P.D., Nikaido, K., Ardeshir, F., Garcia, G. and Ames, G.F.-L. (1982) 'Complete nucleotide sequence and identification of membrane components of the histidine transport operon of S. typhimurium', *Nature*, 298(5876), pp. 723-727.

Hirsch, R., Ternes, T., Haberer, K. and Kratz, K.L. (1999) 'Occurrence of antibiotics in the aquatic environment', *Sci Total Environ*, 225(1-2), pp. 109-18.

Holmes, A.J., Gillings, M.R., Nield, B.S., Mabbutt, B.C., Nevalainen, K.M. and Stokes, H.W. (2003) 'The gene cassette metagenome is a basic resource for bacterial genome evolution', *Environ Microbiol*, 5(5), pp. 383-94.

Houndt, T. and Ochman, H. (2000) 'Long-Term Shifts in Patterns of Antibiotic Resistance in Enteric Bacteria', *Appl Environ Microbiol*, 66(12), pp. 5406-5409.

Huber, J.A., Mark Welch, D.B., Morrison, H.G., Huse, S.M., Neal, P.R., Butterfield, D.A. and Sogin, M.L. (2007) 'Microbial Population Structures in the Deep Marine Biosphere', *Science*, 318(5847), pp. 97-100.

Hughes, V.M. and Datta, N. (1983) 'Conjugative plasmids in bacteria of the /ˆpre-antibiotic/' era', *Nature*, 302(5910), pp. 725-726.

Ilves, H., Horak, R. and Kivisaar, M. (2001) 'Involvement of sigma(S) in starvation-induced transposition of Pseudomonas putida transposon Tn4652', *J Bacteriol*, 183(18), pp. 5445-8.

Jain, P.K., Ramachandran, S., Shukla, V., Bhakuni, B. and Sanjay, S.K. (2009) 'Characterization of Metal and Antibiotic Resistance in a Bacterial Population Isolated from a Copper Mining Industry', *Int J Integr Biol*.

Ji, G. and Silver, S. (1995) 'Bacterial resistance mechanisms for heavy metals of environmental concern', *J Ind Microbiol*, 14(2), pp. 61-75.

Jones, A.L., DeShazer, D. and Woods, D.E. (1997) 'Identification and characterization of a two-component regulatory system involved in invasion of eukaryotic cells and heavy-metal resistance in Burkholderia pseudomallei', *Infect Immun*, 65(12), pp. 4972-7.

Jones, J.G., Gardener, S., Simon, B.M. and Pickup, R.W. (1986) 'Antibiotic resistant bacteria in Windermere and two remote upland tarns in the English Lake District', *J Appl Bacteriol*, 60(5), pp. 443-53.

Kahan, J.S., Kahan, F.M., Goegelman, R., Currie, S.A., Jackson, M., Stapley, E.O., Miller, T.W., Miller, A.K., Hendlin, D., Mochales, S., Hernandez, S., Woodruff, H.B. and Birnbaum, J. (1979) 'Thienamycin, a new beta-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties', *J Antibiot (Tokyo)*, 32(1), pp. 1-12.

Kennelly, P.J. and Potts, M. (1996) 'Fancy meeting you here! A fresh look at "prokaryotic" protein phosphorylation', *J Bacteriol*, 178(16), pp. 4759-64.

Kidambi, S.P., Sundin, G.W., Palmer, D.A., Chakrabarty, A.M. and Bender, C.L. (1995) 'Copper as a signal for alginate synthesis in Pseudomonas syringae pv. syringae', *Appl Environ Microbiol*, 61(6), pp. 2172-9.

Kinney, C.A., Furlong, E.T., Zaugg, S.D., Burkhardt, M.R., Werner, S.L., Cahill, J.D. and Jorgensen, G.R. (2006) 'Survey of Organic Wastewater Contaminants in Biosolids Destined for Land Application†', *Environ Sci Technol*, 40(23), pp. 7207-7215.

Klingeman, P.C., Beschta, R.L., Komar, P.D. and Bradley, J.B. (1998) *Gravel-Bed Rivers in the Environment*. USA: Water Resources Publications, LLC.

Knapp, C.W., Engemann, C.A., Hanson, M.L., Keen, P.L., Hall, K.J. and Graham, D.W. (2008) 'Indirect Evidence of Transposon-Mediated Selection of Antibiotic Resistance Genes in Aquatic Systems at Low-Level Oxytetracycline Exposures', *Environ Sci Technol*, 42(14), pp. 5348-5353.

Knapp, C.W., Lima, L., Olivares-Rieumont, S., Bowen, E., Werner, D. and Graham, D.W. (2012) 'Seasonal variations in antibiotic resistance gene transport in the almendares river, havana, cuba', *Front Microbiol*, 3, p. 396.

Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G. and W., G.D. (2011) 'Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils.', *PLoS One*, 6(11).

Knapp, C.W., Zhang, W., Sturm, B.S. and Graham, D.W. (2010) 'Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions', *Environ Pollut*, 158(5), pp. 1506-12.

Koenig, J.E., Sharp, C., Dlutek, M., Curtis, B., Joss, M., Boucher, Y. and Doolittle, W.F. (2009) 'Integron gene cassettes and degradation of compounds associated with industrial waste: the case of the Sydney tar ponds', *PLoS One*, 4(4), p. e5276.

Kollef, M.H. (2008) 'Update on the appropriate use of meropenem for the treatment of serious bacterial infections', *Clin Infect Dis*, 47 Suppl 1, pp. S1-2.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and Buxton, H.T. (2002) 'Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance', *Environ Sci Technol*, 36(6), pp. 1202-11.

Kumarasamy, K.K., Toleman, M.A., Walsh, T.R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C.G., Irfan, S., Krishnan, P., Kumar, A.V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D.L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J.B., Sharma, M., Sheridan, E., Thirunarayan, M.A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D.M. and Woodford, N. (2010) 'Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study', *Lancet Infect Dis*, 10, pp. 597-602.

Kümmerer, K. (2003) 'Significance of antibiotics in the environment', *J Antimicrob Chemother*, 52(1), pp. 5-7.

Kümmerer, K. (2004) 'Resistance in the environment', *J Antimicrob Chemother*, 54(2), pp. 311-20.

Kümmerer, K. (2009) 'Antibiotics in the aquatic environment – A review – Part I', *Chemosphere*, 75(4), pp. 417-434.

Lavigne, J.-P., Sotto, A., Nicolas-Chanoine, M.-H., Bouziges, N., Pagès, J.-M. and Davin-Regli, A. (2013) 'An adaptive response of Enterobacter aerogenes to imipenem: regulation of porin balance in clinical isolates', *Int J Antimicrob Agents*, 41(2), pp. 130-136.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K., Wertheim, H.F., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D. and Cars, O. (2013) 'Antibiotic resistance-the need for global solutions', *Lancet Infect Dis*, 13(12), pp. 1057-98.

Lazo, J.S., Kuo, S.-M., Woo, E.S. and Pitt, B.R. (1998) 'The protein thiol metallothionein as an antioxidant and protectant against antineoplastic drugs', *Chem-Biol Interact*, 111–112(0), pp. 255-262.

Lee, L.J., Barrett, J.A. and Poole, R.K. (2005) 'Genome-Wide Transcriptional Response of Chemostat-Cultured Escherichia coli to Zinc', *J Bacteriol*, 187(3), pp. 1124-1134.

Levy, S.B. (2002) 'Active efflux, a common mechanism for biocide and antibiotic resistance', *Symp Ser Soc Appl Microbiol*, (31), pp. 65s-71s.

Lewis, K. (2013) 'Platforms for antibiotic discovery', *Nat Rev Drug Discov*, 12(5), pp. 371-87.

Li, D., Yang, M., Hu, J., Zhang, J., Liu, R., Gu, X., Zhang, Y. and Wang, Z. (2009) 'Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river', *Environ Microbiol*, 11(6), pp. 1506-17.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M. and Qi, R. (2010) 'Antibiotic Resistance Characteristics of Environmental Bacteria from an Oxytetracycline Production Wastewater Treatment Plant and the Receiving River', *Appl Environ Microbiol* 76(11), pp. 3444-3451.

Liebert, C.A., Hall, R.M. and Summers, A.O. (1999) 'Transposon Tn21, flagship of the floating genome', *Microbiol Mol Biol Rev*, 63(3), pp. 507-22.

Livermore, D.M. (1995) 'beta-Lactamases in laboratory and clinical resistance', *Clin Microbiol Rev*, 8(4), pp. 557-84.

Logan, L.K. (2012) 'Carbapenem-resistant enterobacteriaceae: an emerging problem in children', *Clin Infect Dis*, 55(6), pp. 852-9.

Lu, D., Boyd, B. and Lingwood, C.A. (1997) 'Identification of the key protein for zinc uptake in Hemophilus influenzae', *J Biol Chem*, 272(46), pp. 29033-8.

Lu, S.Y., Zhang, Y.L., Geng, S.N., Li, T.Y., Ye, Z.M., Zhang, D.S., Zou, F. and Zhou, H.W. (2010) 'High diversity of extended-spectrum beta-lactamase-producing bacteria in an urban river sediment habitat', *Appl Environ Microbiol*, 76(17), pp. 5972-6.

MacDonald, R.S. (2000) 'The role of zinc in growth and cell proliferation', *J Nutr*, 130(5S Suppl), pp. 1500s-8s.

Machado, E., Coque, T.M., Canton, R., Sousa, J.C., Silva, D., Ramos, M., Rocha, J., Ferreira, H. and Peixe, L. (2009) 'Leakage into Portuguese aquatic environments of extended-spectrum-beta-lactamase-producing Enterobacteriaceae', *J Antimicrob Chemother*, 63(3), pp. 616-8.

Macklin, M.G., Hudson-Edwards, K.A. and Dawson, E.J. (1997) 'The significance of pollution from historic metal mining in the Pennine orefields on river sediment contaminant fluxes to the North Sea', *Sci Total Environ*, 194–195(0), pp. 391-397.

Macklin, M.G., Ridgway, J., Passmore, D.G. and Rumsby, B.T. (1994) 'The use of overbank sediment for geochemical mapping and contamination assessment: results from selected English and Welsh floodplains', *Appl Geochemi*, 9(6), pp. 689-700.

Manahan, S.E. (2010) *Environmental Chemistry*. 9th edn. USA: CRC press.

Marger, M.D. and Saier, M.H., Jr. (1993) 'A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport', *Trends Biochem Sci*, 18(1), pp. 13-20.

Martinez, J.L. (2009a) 'Environmental pollution by antibiotics and by antibiotic resistance determinants', *Environ Pollut*, 157(11), pp. 2893-902.

Martinez, J.L. (2009b) 'The role of natural environments in the evolution of resistance traits in pathogenic bacteria', *Proc Biol Sci*, 276(1667), pp. 2521-30.

Martínez, J.L. (2008) 'Antibiotics and Antibiotic Resistance Genes in Natural Environments', *Science*, 321(5887), pp. 365-367.

Martinez, J.L. and Baquero, F. (2000) 'Mutation frequencies and antibiotic resistance', *Antimicrob Agents Chemother*, 44(7), pp. 1771-7.

Mata, M.T., Baquero, F. and Perez-Diaz, J.C. (2000) 'A multidrug efflux transporter in Listeria monocytogenes', *FEMS Microbiol Lett*, 187(2), pp. 185-8.

Mazel, D. (2006) 'Integrons: agents of bacterial evolution', *Nat Rev Microbiol*, 4(8), pp. 608-20.

Melechen, N.E. and Go, G. (1980) 'Induction of lambdoid prophages by amino acid deprivation: differential inducibility; role of recA', *Mol Gen Genet*, 180(1), pp. 147-55.

Mendez, A.S.L., Dalomo, J., Steppe, M. and Schapoval, E.E.S. (2006) 'Stability and degradation kinetics of meropenem in powder for injection and reconstituted sample', *J Pharmaceut Biomed*, 41(4), pp. 1363-1366.

Mergeay, M., Houba, C. and Gerits, J. (1978) 'Extrachromosomal inheritance controlling resistance to cadmium, cobalt, copper and zinc ions: evidence from curing in a Pseudomonas [proceedings]', *Arch Int Physiol Biochim*, 86(2), pp. 440-2.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., Van Der Lelie, D. and Wattiez, R. (2003) 'Ralstonia metallidurans, a bacterium specifically adapted to toxic metals: Towards a catalogue of metal-responsive genes', *FEMS Microbiol Rev*, 27(2-3), pp. 385-410.

Mergeay, M., Nies, D., Schlegel, G., Gertis, J., Charles, P. and Gijsegem, V. (1985) 'Alcaligenes eutrophus CH34 Is a Facultative Chemolithotroph with Plasmid-Bound Resistance to Heavy Metals', *J Bacteriol*, 162(1), pp. 328-334.

MHRA, R.M.a.M.D. (2010) *Public Assessment Report: Meropenem 500 mg and 1 g powder for solution for injection or infusion (meropenem trihydrate)* (UK/H/2437/001-2/DC).

MHRA, R.M.a.M.D. (2014) *Public Assessment Report: Meropenem 500 mg and 1 g powder for solution for injection or infusion (meropenem trihydrate)* (UK/K5288/001-02/DC).

Mitchell, R. and Gu, J. (eds.) (2010) *Environmental microbiology*. 2nd edn. New Jersey, USA: Wily- Blackwell.

Morby, A.P., Turner, J.S., Huckle, J.W. and Robinson, N.J. (1993) 'SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene smtA: identification of a Zn inhibited DNA-protein complex', *Nucleic Acids Res*, 21(4), pp. 921-5.

Morita, Y., Kodama, K., Shiota, S., Mine, T., Kataoka, A., Mizushima, T. and Tsuchiya, T. (1998) 'NorM, a putative multidrug efflux protein, of Vibrio parahaemolyticus and its homolog in Escherichia coli', *Antimicrob Agents Chemother*, 42(7), pp. 1778-82.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) 'Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction', *Cold Spring Harb Symp Quant Biol*, 51 Pt 1, pp. 263-73.

Munoz-Aguayo, J., Lang, K.S., LaPara, T.M., Gonzalez, G. and Singer, R.S. (2007) 'Evaluating the effects of chlortetracycline on the proliferation of antibiotic-resistant bacteria in a simulated river water ecosystem', *Appl Environ Microbiol*, 73(17), pp. 5421-5.

Murray, B.E. (1997) 'Antibiotic resistance', *Adv Intern Med*, 42, pp. 339-67.

Muyzer, G. (1999) 'DGGE/TGGE a method for identifying genes from natural ecosystems', *Curr Opin Microbiol*, 2(3), pp. 317-22.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) 'Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA', *Appl Environ Microbiol*, 59(3), pp. 695-700.

Naas, T., Ergani, A., Carrer, A. and Nordmann, P. (2011) 'Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples', *Antimicrob Agents Chemother*, 55(9), pp. 4038-43.

Nakajima, H., Kobayashi, K., Kobayashi, M., Asako, H. and Aono, R. (1995) 'Overexpression of the robA gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in Escherichia coli', *Appl Environ Microbiol*, 61(6), pp. 2302-7.

Nandi, S., Maurer, J.J., Hofacre, C. and Summers, A.O. (2004) 'Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter', *Proc Natl Acad Sci USA*, 101(18), pp. 7118-7122.

Nicolau, D.P. (2008) 'Pharmacokinetic and pharmacodynamic properties of meropenem', *Clin Infect Dis*, 47 Suppl 1, pp. S32-40.

Nies, D., Mergeay, M., Friedrich, B. and Schlegel, H.G. (1987) 'Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in Alcaligenes eutrophus CH34', *J Bacteriol*, 169(10), pp. 4865-4868.

Nies, D.H. (1992a) 'CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (czc system) in Alcaligenes eutrophus', *J Bacteriol*, 174(24), pp. 8102-8110.

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

Nies, D.H. (1995) 'The cobalt, zinc, and cadmium efflux system CzcABC from Alcaligenes eutrophus functions as a cation-proton antiporter in Escherichia coli', *J Bacteriol*, 177(10), pp. 2707-2712.

Nies, D.H. (1999) 'Microbial heavy-meatal resistance', *Appl Microbiol Biotechnol*, 51, pp. 730-750.

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

Nies, D.H. (2003) 'Efflux-mediated heavy metal resistance in prokaryotes', *FEMS Microbiol Rev*, 27(2-3), pp. 313-39.

Nies, D.H., Koch, S., Wachi, S., Peitzsch, N. and Saier, M.H. (1998) 'CHR, a Novel Family of Prokaryotic Proton Motive Force-Driven Transporters Probably Containing Chromate/Sulfate Antiporters', *J Bacteriol*, 180(21), pp. 5799-5802.

Nies, D.H. and Silver, S. (1989) 'Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in Alcaligenes eutrophus', *J Bacteriol*, 171(2), pp. 896-900.

Nies, D.H. and Silver, S. (1995) 'Ion efflux systems involved in bacterial metal resistances', *J Ind Microbiol*, 14(2), pp. 186-99.

Nikaido, H. (1994) 'Prevention of drug access to bacterial targets: Permeability barriers and active efflux', *Science*, 264(5157), pp. 382-388.

Nix, D.E., Majumdar, A.K. and DiNubile, M.J. (2004) 'Pharmacokinetics and pharmacodynamics of ertapenem: an overview for clinicians', *J Antimicrob Chemother*, 53 Suppl 2, pp. ii23-8.

Nogva, H.K., Dromtorp, S.M., Nissen, H. and Rudi, K. (2003) 'Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR', *Biotechniques*, 34(4), pp. 804-8, 810, 812-3.

Nuttall, C.A. and Younger, P.L. (2002) 'Secondary minerals in the abandoned mines of Nenthead, Cumbria as sinks for pollutant metals', *Geol Soc Spec Publ*, 198(1), pp. 241-250.

Ochs, M.M., McCusker, M.P., Bains, M. and Hancock, R.E.W. (1999) 'Negative Regulation of the Pseudomonas aeruginosa Outer Membrane Porin OprD Selective for Imipenem and Basic Amino Acids', *Antimicrob Agents Chemother*, 43(5), pp. 1085-1090.

OECD (2001) 'Test Guideline 303: Simulation Test- Aerobic Sewage Treatment; A: Activated Sludge Units; B: Biofilms', in *Guidlines for the Testing of Chemicals*. Paris: Organisation for Economic Co-operation and Development (OECD).

OECD (2010) 'Test Guideline 209: Activated Sludge, Repiration Inhibition Test (Carbon and Ammonium Oxidation)', in *OECD Guidelines for the Testing of Chemicals, Section 2*. Paris: Organisation for Economic Co-operation and Development (OECD).

OECD (2015a) *About the Organisation for Economic Co-operation and Development (OECD)*. Available at: http://www.oecd.org/about/ (Accessed: 8/07/2015).

OECD (2015b) *OECD Guidelines for the Testing of Chemicals* Available at: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm (Accessed: 8/07/15).

Olesky, M., Zhao, S., Rosenberg, R.L. and Nicholas, R.A. (2006) 'Porin-Mediated Antibiotic Resistance in Neisseria gonorrhoeae: Ion, Solute, and Antibiotic Permeation through PIB Proteins with penB Mutations', *J Bacteriol*, 188(7), pp. 2300-2308.

Oliver, A., Levin, B.R., Juan, C., Baquero, F. and Blazquez, J. (2004) 'Hypermutation and the preexistence of antibiotic-resistant Pseudomonas aeruginosa mutants: implications for susceptibility testing and treatment of chronic infections', *Antimicrob Agents Chemother*, 48(11), pp. 4226-33.

Papp-Wallace, K.M., Endimiani, A., Taracila, M.A. and Bonomo, R.A. (2011) 'Carbapenems: past, present, and future', *Antimicrob Agents Chemother*, 55(11), pp. 4943-60.

Parkhill, J., Dougan, G., James, K.D., Thomson, N.R., Pickard, D., Wain, J., Churcher, C., Mungall, K.L., Bentley, S.D., Holden, M.T., Sebaihia, M., Baker, S., Basham, D., Brooks, K., Chillingworth, T., Connerton, P., Cronin, A., Davis, P., Davies, R.M., Dowd, L., White, N., Farrar, J., Feltwell, T., Hamlin, N., Haque, A., Hien, T.T., Holroyd, S., Jagels, K., Krogh, A., Larsen, T.S., Leather, S., Moule, S., O'Gaora, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. and Barrell, B.G. (2001) 'Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18', *Nature*, 413(6858), pp. 848-52.

Patel, G. and Bonomo, R.A. (2011) 'Status report on carbapenemases: challenges and prospects', *Expert Rev Anti Infect Ther*, 9(5), pp. 555-70.

Pathak, S., Awuh, J.A., Leversen, N.A., Flo, T.H. and Asjø, B. (2012) 'Counting mycobacteria in infected human cells and mouse tissue: a comparison between qPCR and CFU', *PLoS One*, 7.

Patzer, S.I. and Hantke, K. (1998) 'The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli', *Mol Microbiol*, 28(6), pp. 1199-210.

Paulsen, I.T. and Saier, J.M.H. (1997) 'A Novel Family of Ubiquitous Heavy Metal Ion Transport Proteins', *J Membrane Biol*, 156(2), pp. 99-103.

Paulsen, I.T., Skurray, R.A., Tam, R., Saier, M.H., Turner, R.J., Weiner, J.H., Goldberg, E.B. and Grinius, L.L. (1996) 'The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs', *Mol Microbio*, 19(6), pp. 1167-1175.

Paulsen, I.T., Sliwinski, M.K., Nelissen, B., Goffeau, A. and Saier, M.H., Jr. (1998) 'Unified inventory of established and putative transporters encoded within the complete genome of Saccharomyces cerevisiae', *FEBS Lett*, 430(1-2), pp. 116-25.

Pei, R., Kim, S.-C., Carlson, K.H. and Pruden, A. (2006) 'Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG)', *Water Res*, 40(12), pp. 2427-2435.

Peltier, E., Vincent, J., Finn, C. and Graham, D.W. (2010) 'Zinc-induced antibiotic resistance in activated sludge bioreactors', *Water Res*, 44(13), pp. 3829-36.

Pepper, I.L., Gerba, C.P. and Brusseau, M.L. (eds.) (1996) *Pollution Science*. London, UK: Academic Press.

Perron, K., Caille, O., Rossier, C., Van Delden, C., Dumas, J.L. and Kohler, T. (2004) 'CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in Pseudomonas aeruginosa', *J Biol Chem*, 279(10), pp. 8761-8.

Picao, R.C., Poirel, L., Demarta, A., Silva, C.S., Corvaglia, A.R., Petrini, O. and Nordmann, P. (2008) 'Plasmid-mediated quinolone resistance in Aeromonas allosaccharophila recovered from a Swiss lake', *J Antimicrob Chemother*, 62(5), pp. 948-50.

Piddock, L.J. (2006) 'Multidrug-resistance efflux pumps - not just for resistance', *Nat Rev Microbiol*, 4(8), pp. 629-36.

Poirel, L., Hombrouck-Alet, C., Freneaux, C., Bernabeu, S. and Nordmann, P. (2010) 'Global spread of New Delhi metallo-beta-lactamase 1', *Lancet Infect Dis*, 10(12), p. 832.

Poole, K. (2005) 'Efflux-mediated antimicrobial resistance', *J Antimicrob Chemother*, 56(1), pp. 20-51.

Prado, T., Pereira, W.C., Silva, D.M., Seki, L.M., Carvalho, A.P. and Asensi, M.D. (2008) 'Detection of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in effluents and sludge of a hospital sewage treatment plant', *Lett Appl Microbiol*, 46(1), pp. 136-41.

Pruden, A., Arabi, M. and Storteboom, H.N. (2012) 'Correlation between upstream human activities and riverine antibiotic resistance genes', *Environ Sci Technol*, 46(21), pp. 11541-9.

Pruden, A., Pei, R., Storteboom, H. and Carlson, K.H. (2006) 'Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado', *Environ Sci Technol*, 40, pp. 7445-7450.

Psichogiou, M., Tassios, P.T., Avlamis, A., Stefanou, I., Kosmidis, C., Platsouka, E., Paniara, O., Xanthaki, A., Toutouza, M., Daikos, G.I. and Tzouvelekis, L.S. (2008) 'Ongoing epidemic of *bla*VIM-1-positive Klebsiella pneumoniae in Athens, Greece: A prospective survey', *J Antimicrob Chemother*, 61(1), pp. 59-63.

Public Health England (2014) *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR)* (2014362). London, UK: publications, P.

Queenan, A.M. and Bush, K. (2007) 'Carbapenemases: the versatile beta-lactamases', *Clin Microbiol Rev*, 20(3), pp. 440-58, table of contents.

Queenan, A.M., Shang, W., Flamm, R. and Bush, K. (2010) 'Hydrolysis and inhibition profiles of beta-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem', *Antimicrob Agents Chemother*, 54(1), pp. 565-9.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J. and Ziebuhr, W. (2000) 'Effect of Subinhibitory Antibiotic Concentrations on Polysaccharide Intercellular Adhesin Expression in Biofilm-Forming Staphylococcus epidermidis', *Antimicrob Agents Chemother*, 44(12), pp. 3357-3363.

Ramos- Aires, J., Köhler, T., Nikaido, H. and Plésiat, P. (1999) 'Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides', *Antimicrob Agents Chemother*, 43(11), pp. 2624-2628.

Randall, C.P., Mariner, K.R., Chopra, I. and O'Neill, A.J. (2013) 'The target of daptomycin is absent from Escherichia coli and other gram-negative pathogens', *Antimicrob Agents Chemother*, 57(1), pp. 637-9.

Rasmussen, L.D. and Sorensen, S.J. (1998) 'The effect of longterm exposure to mercury on the bacterial community in marine sediment', *Curr Microbiol*, 36(5), pp. 291-7.

Reasoner, D.J. and Geldreich, E.E. (1985) 'A New Medium for the Enumeration and Subculture of Bacteria from Potable Water', *Appl Environ Microbiol*, 49(1), pp. 1-7.

Rensing, C., Mitra, B. and Rosen, B.P. (1997a) 'The zntA gene of Escherichia coli encodes a Zn(II)-translocating P-type ATPase', *Proc Natl Acad Sci USA*, 94(26), pp. 14326-14331.

Rensing, C., Pribyl, T. and Nies, D.H. (1997b) 'New functions for the three subunits of the CzcCBA cation-proton antiporter', *J Bacteriol*, 179(22), pp. 6871-6879.

Roberts, M.C. (2005) 'Update on acquired tetracycline resistance genes', *FEMS Microbiol Lett*, 245(2), pp. 195-203.

Robinson, N.J., Bird, A.J. and Turner, J.S. (1998) *Metal Ions in Gene Regulation*. New York, USA: Springer US.

Rodloff, A.C., Goldstein, E.J. and Torres, A. (2006) 'Two decades of imipenem therapy', *J Antimicrob Chemother*, 58(5), pp. 916-29.

Rosewarne, C.P., Pettigrove, V., Stokes, H.W. and Parsons, Y.M. (2010) 'Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance', *FEMS Microbiol Ecol*, 72(1), pp. 35-46.

Ruiz, N., Montero, T., Hernandez-Borrell, J. and Vinas, M. (2003) 'The role of Serratia marcescens porins in antibiotic resistance', *Microb Drug Resist*, 9(3), pp. 257-64.

Saier Jr, M.H., Tam, R., Reizer, A. and Reizer, J. (1994) 'Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport', *Mol Microbio*, 11(5), pp. 841-847.

Saier, M.H., Jr. (1994) 'Computer-aided analyses of transport protein sequences: gleaning evidence concerning function, structure, biogenesis, and evolution', *Microbiol Rev*, 58(1), pp. 71-93.

Saier, M.H., Jr. (1998) 'Molecular phylogeny as a basis for the classification of transport proteins from bacteria, archaea and eukarya', *Adv Microb Physiol*, 40, pp. 81-136.

Sample, I. (2013) 'Antibiotic-resistant diseases pose 'apocalyptic' threat, top expert says', *The Guardian* edn). [Online] Available at: http://www.theguardian.com/society/2013/jan/23/antibiotic-resistant-diseases-apocalyptic-threat (Accessed: 12/07/2015).

Sarmah, A.K., Meyer, M.T. and Boxall, A.B. (2006) 'A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment', *Chemosphere*, 65(5), pp. 725-59.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W. and Davies, D.G. (2002) 'Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm', *J Bacteriol*, 184(4), pp. 1140-54.

Saxena, D. and Srivastava, S. (1998) 'Short Communication: Carbon source-starvation-induced precipitation of copper by Pseudomonas putida strain S4', *World J Microbiol Biotechnol*, 14(6), pp. 921-923.

Schluter, A., Szczepanowski, R., Puhler, A. and Top, E.M. (2007) 'Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool', *FEMS Microbiol Rev*, 31(4), pp. 449-77.

Schulz-Zunkel, C. and Krueger, F. (2009) 'Trace metal dynamics in floodplain soils of the river Elbe: a review', *J Environ Qual*, 38(4), pp. 1349-62.

Segura, P.A., Francois, M., Gagnon, C. and Sauve, S. (2009) 'Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters', *Environ Health Perspect*, 117(5), pp. 675-84.

Seiler, C. and Berendonk, T.U. (2012) 'Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture', *Front Microbiol*, 3.

Sheikh, W., Pitkin, D.H. and Nadler, H. (1993) 'Antibacterial activity of meropenem and selected comparative agents against anaerobic bacteria at seven North American centers', *Clin Infect Dis*, 16 Suppl 4, pp. S361-6.

Sigma-Aldrich (2013) *Cycloheximide- Product Information Sheet*. Available at: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/c7698pis.pdf.

Silver, L.L. (2011) 'Challenges of antibacterial discovery', *Clin Microbiol Rev*, 24(1), pp. 71-109.

Silver, S. and Phung, L.T. (1996) 'Bacterial heavy metal resistance: new surprises', *Annu Rev Microbiol*, 50, pp. 753-89.

Smith, D.H. (1967) 'R factor infection of Escherichia coli lyophilized in 1946', *J Bacteriol*, 94(6), pp. 2071-2072.

Smith, R.L. and Maguire, M.E. (1995) 'Distribution of the CorA Mg2+ transport system in gram-negative bacteria', *J Bacteriol*, 177(6), pp. 1638-40.

Smith, R.L., Szegedy, M.A., Kucharski, L.M., Walker, C., Wiet, R.M., Redpath, A., Kaczmarek, M.T. and Maguire, M.E. (1998) 'The CorA Mg2+ Transport Protein of Salmonella typhimurium: Mutagenesis of conserved residues in the third membrane domain identifies a Mg2+ pore', *J Biol Chem*, 273(44), pp. 28663-28669.

Smith, R.L., Thompson, L.J. and Maguire, M.E. (1995) 'Cloning and characterization of MgtE, a putative new class of Mg2+ transporter from Bacillus firmus OF4', *J Bacteriol*, 177(5), pp. 1233-8.

Srivastava, S., Choudhury, R. and Joshi, N. (eds.) (1999) *Active efflux — a mechanism for metal ion resistance in bacteria.* New Delhi: APH Publishing Corporation.

Srivastava, S., Mago, R. and Bhagat, R. (eds.) (1998) *Genetics of heavy metal resistance in soil bacteria.* New Delhi: MPH.

Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J. and McArthur, J.V. (2006) 'Coselection for microbial resistance to metals and antibiotics in freshwater microcosms', *Environ Microbiol*, 8(9), pp. 1510-4.

Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H. and McArthur, J.V. (2005) 'Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments', *Environ Sci Technol*, 39(10), pp. 3671-8.

Stewart, P.S. and Costerton, J.W. (2001) 'Antibiotic resistance of bacteria in biofilms', *Lancet*, 358(9276), pp. 135-8.

Stokes, H.W., Holmes, A.J., Nield, B.S., Holley, M.P., Nevalainen, K.M., Mabbutt, B.C. and Gillings, M.R. (2001) 'Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA', *Appl Environ Microbiol*, 67(11), pp. 5240-6.

Stokes, H.W., Nesbø, C.L., Holley, M., Bahl, M.I., Gillings, M.R. and Boucher, Y. (2006) 'Class 1 Integrons Potentially Predating the Association with Tn402-Like Transposition Genes Are Present in a Sediment Microbial Community', *J Bacteriol*, 188(16), pp. 5722-5730.

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002) 'Biofilms as complex differentiated communities', *Annu Rev Microbiol*, 56, pp. 187-209.

Storteboom, H., Arabi, M., Davis, J.G., Crimi, B. and Pruden, A. (2010) 'Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources', *Environ Sci Technol*, 44(6), pp. 1947-53.

Summers, A.O. (2002) 'Generally Overlooked Fundamentals of Bacterial Genetics and Ecology', *Clin Infect Dis*, 34(Supplement 3), pp. S85-S92.

Summers, A.O., Wireman, J., Vimy, M.J., Lorscheider, F.L., Marshall, B., Levy, S.B., Bennett, S. and Billard, L. (1993) 'Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates', *Antimicrob Agents Chemother*, 37(4), pp. 825-834.

Tamber, S. and Hancock, R.E. (2003) 'On the mechanism of solute uptake in Pseudomonas', *Front Biosci*, 8, pp. s472-83.

Tangden, T., Adler, M., Cars, O., Sandegren, L. and Lowdin, E. (2013) 'Frequent emergence of porin-deficient subpopulations with reduced carbapenem susceptibility in ESBL-producing Escherichia coli during exposure to ertapenem in an in vitro pharmacokinetic model', *J Antimicrob Chemother*, 68(6), pp. 1319-26.

Teitzel, G.M. and Parsek, M.R. (2003) 'Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa', *Appl Environ Microbiol*, 69(4), pp. 2313-20.

Tello, A., Austin, B. and Telfer, T.C. (2012) 'Selective pressure of antibiotic pollution on bacteria of importance to public health', *Environ Health Perspect*, 120(8), pp. 1100-6.

Tennstedt, T., Szczepanowski, R., Krahn, I., Puhler, A. and Schluter, A. (2005) 'Sequence of the 68,869 bp IncP-1alpha plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn402-like integron and other transposable elements', *Plasmid*, 53(3), pp. 218-38.

Thelwell, C., Robinson, N.J. and Turner-Cavet, J.S. (1998) 'An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter', *Proc Natl Acad Sci*, 95(18), pp. 10728-10733.

Thiele-Bruhn, S. (2003) 'Pharmaceutical antibiotic compounds in soils – a review', *J Plant Nutr Soil Sc*, 166(2), pp. 145-167.

Torsvik, V.L. (1980) 'Isolation of bacterial DNA from soil', *Soil Biol. Biochem*, 12, pp. 15-21.

Trust, T.R. (2012) *A natural history of the Tyne*. Available at: http://tyneriverstrust.org/about-the-river/history/.

Turner, P.J., Greenhalgh, J.M., Edwards, J.R., McKellar, J. and Abbasi, S.A. (1999) 'The MYSTIC (meropenem yearly susceptibility test information

collection) programme', *Int J Antimicrob Agents*, 13, pp. 117 - 125.

Vaara, M. (1992) 'Agents that increase the permeability of the outer membrane', *Microbiol Rev*, 56(3), pp. 395-411.

Van Bambeke, F., Balzi, E. and Tulkens, P.M. (2000) 'Antibiotic efflux pumps', *Biochem Pharmacol*, 60(4), pp. 457-470.

van der Lelie, D., Schwuchow, T., Schwidetzky, U., Wuertz, S., Baeyens, W., Mergeay, M. and Nies, D.H. (1997) 'Two-component regulatory system involved in transcriptional control of heavy-metal homoeostasis in Alcaligenes eutrophus', *Mol Microbiol*, 23(3), pp. 493-503.

van Elsas, J.D. and Bailey, M.J. (2002) 'The ecology of transfer of mobile genetic elements', *FEMS Microbiol Ecol* 42(2), pp. 187-197.

Waksman, S.A. and Woodruff, H.B. (1940) 'The Soil as a Source of Microorganisms Antagonistic to Disease-Producing Bacteria', *J Bacteriol*, 40(4), pp. 581-600.

Walsh, F. (2013) 'Investigating antibiotic resistance in non-clinical environments', *Front Microbiol*, 4, p. 19.

Walsh, T.R. (2010) 'Emerging carbapenemases: a global perspective', *Int J Antimicrob Agents*, 36, Supplement 3(0), pp. S8-S14.

Walsh, T.R., Weeks, J., Livermore, D.M. and Toleman, M.A. (2011) 'Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study', *Lancet Infect Dis*, 11, pp. 355-62.

Wang, C.L., Michels, P.C., Dawson, S.C., Kitisakkul, S., Baross, J.A., Keasling, J.D. and Clark, D.S. (1997) 'Cadmium removal by a new strain of Pseudomonas aeruginosa in aerobic culture', *Appl Environ Microbiol*, 63 (10), pp. 4075–4078.

Water, N. (2010) *Catchment Management: River Tyne*. Available at: http://www.nwl.co.uk/your-home/environment/river-tyne.aspx.

Weast, R.C. (ed.) (1984) *CRC handbook of chemistry and physics*. 69 edn. Boca Raton, Florida: CRC Press.

Webb, V. and Davies, J. (1993) 'Antibiotic preparations contain DNA: A source of drug resistance genes?', *Antimicrob Agents Chemother*, 37(11), pp. 2379-2384.

Webber, M.A. and Piddock, L.J. (2003) 'The importance of efflux pumps in bacterial antibiotic resistance', *J Antimicrob Chemother*, 51(1), pp. 9-11.

Wellington, E.M.H., Boxall, A.B.A., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M. and Williams, A.P. (2013) 'The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria', *Lancet Infect Dis*, 13(2), pp. 155-165.

WHO (2000) *World Health Organization Report in Infectious Diseases*. Geneva: World Health Organization (WHO).

WHO (2015) *Antimicrobial resistance: Fact sheet N°194*. Available at: http://www.who.int/mediacentre/factsheets/fs194/en/ (Accessed: 12/07/2015).

Wireman, J., Liebert, C.A., Smith, T. and Summers, A.O. (1997) 'Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates', *Appl Environ Microbiol*, 63(11), pp. 4494-503.

Wright, M.S., Baker-Austin, C., Lindell, A.H., Stepanauskas, R., Stokes, H.W. and McArthur, J.V. (2008) 'Influence of industrial contamination on mobile genetic elements: Class 1 integron abundance and gene cassette structure in aquatic bacterial communities', *ISME J*, 2(4), pp. 417-428.

Wright, M.S., Loeffler Peltier, G., Stepanauskas, R. and McArthur, J.V. (2006) 'Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams', *FEMS Microbiol Ecol*, 58(2), pp. 293-302.

Wuertz, S., Spaeth, R., Hinderberger, A., Griebe, T., Flemming, H.C. and Wilderer, P.A. (2001) 'A new method for extraction of extracellular polymeric substances from biofilms and

activated sludge suitable for direct quantification of sorbed metals', *Water Sci Technol*, 43(6), pp. 25-31.

Xu, H., Davies, J. and Miao, V. (2007) 'Molecular characterization of class 3 integrons from Delftia spp', *J Bacteriol*, 189(17), pp. 6276-83.

Yang, Y., Zhang, T., Zhang, X.X., Liang, D.W., Zhang, M., Gao, D.W., Zhu, H.G., Huang, Q.G. and Fang, H.H. (2012) 'Quantification and characterization of beta-lactam resistance genes in 15 sewage treatment plants from East Asia and North America', *Appl Microbiol Biotechnol*, 95(5), pp. 1351-8.

Yigit, H., Queenan, A.M., Anderson, G.J., Domenech-Sanchez, A., Biddle, J.W., Steward, C.D., Alberti, S., Bush, K. and Tenover, F.C. (2001) 'Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae', *Antimicrob Agents Chemother*, 45(4), pp. 1151-61.

Yong, D., Toleman, M.A., Giske, C.G., Cho, H.S., Sundman, K., Lee, K. and Walsh, T.R. (2009) 'Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India', *Antimicrob Agents Chemother*, 53(12), pp. 5046-54.

Yu, Y., Lee, C., Kim, J. and Hwang, S. (2005) 'Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction', *Biotechnol Bioeng*, 89(6), pp. 670-679.

Zhanel, G.G., Simor, A.E., Vercaigne, L., Mandell, L. and the Canadian Carbapenem Discussion, G. (1998) 'Imipenem and meropenem: Comparison of in vitro activity, pharmacokinetics, clinical trials and adverse effects', *Can J Infect Dis Med Microbiol*, 9(4), pp. 215-228.

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J.D. and Song, H. (2013) 'Higher Isolation of NDM-1 Producing Acinetobacter baumannii from the Sewage of the Hospitals in Beijing', *PLoS One*, 8(6), p. e64857.

Zhang, T., Zhang, M., Zhang, X. and Fang, H.H. (2009a) 'Tetracycline resistance genes and tetracycline resistant lactose-fermenting Enterobacteriaceae in activated sludge of sewage treatment plants', *Environ Sci Technol*, 43(10), pp. 3455-60.

Zhang, T., Zhang, X.X. and Ye, L. (2011) 'Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge', *PLoS One*, 6(10), p. e26041.

Zhang, W., Sturm, B.S., Knapp, C.W. and Graham, D.W. (2009b) 'Accumulation of tetracycline resistance genes in aquatic biofilms due to periodic waste loadings from swine lagoons', *Environ Sci Technol*, 43(20), pp. 7643-50.

Ziha-Zarifi, I., Llanes, C., Köhler, T., Pechere, J.C. and Plesiat, P. (1999) 'In vivo emergence of multidrug-resistant mutants of Pseudomonas aeruginosa overexpressing the active efflux system mexA-mexB-OprM', *Antimicrob Agents Chemother*, 43(2), pp. 287-291.

Appendix A

Table A-1 Measured total Zn (mg/kg dry sediment) levels over six months for each sampling site; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN).

Sampling sites	Total Zn (mg/kg dry sediment)					
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Reaverhill (RH)	34.3	88.2	47.9	42.7	51.0	57.5
Warks Burn (WB)	26.0	17.6	11.8	14.2	20.8	19.7
Featherstone (FS)	781.1	2880	1100	1290	1700	15600
River West Allen (WA)	2710	5570	2990	1590	5370	3030
River Nent (RN)	2830	7110	4400	3000	666	3380

Table A-2 Measured soluble Zn (mg/L) levels over six months for each sampling site; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN).

Sampling sites	Soluble Zn (mg/L)					
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Reaverhill (RH)	0.01	0.01	0.01	0.01	0.01	0.01
Warks Burn (WB)	0.01	0.01	0.01	0.01	0.01	0.01
Featherstone (FS)	0.06	0.10	0.23	0.12	0.15	0.11
River West Allen (WA)	0.66	0.60	1.19	1.26	1.19	1.73
River Nent (RN)	4.66	4.90	2.72	4.32	3.47	5.59

Table A-3 Measured colloidal Zn (mg/L) levels over six months for each sampling site; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN).

Sampling sites	Colloidal Zn (mg/L)					
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Reaverhill (RH)	0.01	0.01	0.01	0.01	0.01	0.02
Warks Burn (WB)	0.01	0.01	0.01	0.00	0.01	0.02
Featherstone (FS)	0.01	0.08	0.20	0.10	0.13	0.08
River West Allen (WA)	0.35	0.48	0.90	0.66	0.86	1.56
River Nent (RN)	0.39	1.46	1.86	3.73	2.22	3.11

Table A-4 Correlations (first value) and p-values (second value) between all total (mg/kg dry sediment cadmium (Cd), cobalt (Co), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) data.

Total metals (mg/kg dry sediment)	Cadmium (Cd)[*]	Cobalt (Co)[*]	Copper (Cu)[*]	Nickel (Ni)[*]	Lead (Pb)[*]
Cobalt (Co)	0.451				
	0.191				
Copper (Cu)	0.747	0.539			
	0.013	0.108			
Nickel (Ni)	0.137	0.863	0.515		
	0.707	0.001	0.128		
Lead (Pb)	0.864	0.663	0.716	0.305	
	0.001	0.037	0.020	0.392	
Zinc (Zn)	0.973	0.553	0.754	0.207	0.951
	0.000	0.097	0.012	0.567	0.000

[*] First value: Pearson's correlation; Second value: p-value.

Table A-5 Percentage (%) of colonies in sediment resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn (combination of both) from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN).

Month	Agar amendments	Percentage (%) of colonies showing resistance				
		Reaverhill (RH)	Warks Burn (WB)	Featherstone (FS)	River West Allen (WA)	River Nent (RN)
1	65 mg/L Zn	6.23	7.54	22.07	43.34	47.95
	2 mg/L meropenem	5.19	6.09	5.95	4.34	3.64
	Combination of both	2.57	3.76	3.85	3.44	3.22
2	65 mg/L Zn	7.61	3.12	16.04	21.96	21.62
	2 mg/L meropenem	3.62	3.84	3.27	5.44	4.88
	Combination of both	2.60	1.98	1.54	4.12	5.83
3	65 mg/L Zn	14.22	3.02	8.57	36.05	25.35
	2 mg/L meropenem	11.83	5.00	4.45	5.25	10.95
	Combination of both	4.41	0.70	1.05	4.78	12.35

Table continued on next page.

Month	Agar amendments	Reaverhill (RH)	Warks Burn (WB)	Featherstone (FS)	River West Allen (WA)	River Nent (RN)
3	65 mg/L Zn	3.82	5.19	17.39	11.63	39.49
	2 mg/L meropenem	13.15	3.05	9.72	6.20	5.85
	Combination of both	0.97	0.71	1.14	4.61	5.97
4	65 mg/L Zn	1.08	5.97	27.04	61.67	44.86
	2 mg/L meropenem	3.67	9.20	10.55	10.69	24.45
	Combination of both	0.55	7.93	5.09	4.83	16.98
5	65 mg/L Zn	8.77	15.37	21.62	16.19	27.63
	2 mg/L meropenem	8.54	12.11	5.54	15.61	9.43
	Combination of both	2.71	3.10	4.12	11.70	11.79

Table A-6 Percentage (%) of colonies in river water resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn (combination of both) from each of the sites; Reaverhill (RH), Warks Burn (WB), Featherstone (FS), River West Allen (WA) and River Nent (RN).

Month	Agar amendments	Percentage (%) of colonies showing resistance				
		Reaverhill (RH)	Warks Burn (WB)	Featherstone (FS)	River West Allen (WA)	River Nent (RN)
1	65 mg/L Zn	8.37	13.65	12.96	26.19	31.72
	2 mg/L meropenem	5.65	12.88	2.96	16.19	3.45
	Combination of both	1.41	5.77	2.22	13.33	6.90
2	65 mg/L Zn	1.97	2.28	4.77	7.72	7.20
	2 mg/L meropenem	1.10	1.02	0.89	1.28	0.91
	Combination of both	0.32	0.05	0.66	2.72	0.83
3	65 mg/L Zn	3.29	3.92	2.92	8.67	5.58
	2 mg/L meropenem	3.34	3.00	2.29	0.50	3.18
	Combination of both	1.02	0.71	1.85	3.08	1.36

Table continued on next page.

Month	Agar amendments	Reaverhill (RH)	Warks Burn (WB)	Featherstone (FS)	River West Allen (WA)	River Nent (RN)
4	65 mg/L Zn	0.56	1.33	4.81	8.12	10.30
	2 mg/L meropenem	0.37	0.56	0.39	0.71	2.69
	Combination of both	0.05	0.20	0.74	0.49	1.19
5	65 mg/L Zn	1.36	1.21	6.67	8.17	10.96
	2 mg/L meropenem	0.73	0.38	2.63	1.48	0.77
	Combination of both	0.10	0.03	1.87	0.87	1.92
6	65 mg/L Zn	3.30	2.13	8.26	13.51	9.11
	2 mg/L meropenem	3.06	3.59	3.31	4.68	5.31
	Combination of both	0.38	1.13	2.11	4.94	4.33

Table A-7 Correlations (first value) and p-values (second value) between all total (mg/kg dry sediment), soluble (mg/L) and colloidal (mg/L) cadmium (Cd), cobalt (Co), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) data and all Zn, meropenem and combined resistance (%) data.

Metal	Analysis	Relative Zn resistance (%)[*]	Relative meropenem resistance (%)[*]	Relative combined resistance (%)[*]
	Total Cd (mg/kg dry sediment)	0.926; 0.000	-2.04; 0.571	0.761; 0.011
Cd	Soluble Cd (mg/L)	0.880; 0.001	-0.049; 0.893	0.971; 0.000
	Colloidal Cd (mg/L)	0.865; 0.001	-0.005; 0.989	0.957; 0.000
	Total Co (mg/kg dry sediment)	0.555; 0.096	-0.182; 0.614	0.540; 0.107
Co	Soluble Co (mg/L)	0.253; 0.481	0.179; 0.620	-0.001; 0.999
	Colloidal Co (mg/L)	0.565; 0.089	0.326; 0.358	-0.355; 0.314
	Total (mg/kg dry sediment)	0.676; 0.032	-0.386; 0.271	0.322; 0.364
Cu	Soluble (mg/L)	-0.252; 0.483	-0.407; 0.243	-0.207; 0.566
	Colloidal (mg/L)	0.249; 0.487	-0.441, 0.202	0.282; 0.429
	Total (mg/kg dry sediment)	0.189; 0.602	-0.306; 0.390	0.063; 0.863
Ni	Soluble (mg/L)	0.516; 0.126	-0.054; 0.882	0.682; 0.030
	Colloidal (mg/L)	0.865; 0.001	--0.229; 0.524	0.852; 0.002

[*]First value: Pearson's correlation; Second value: p-value.

Table continued on next page.

Metal	Analysis	Relative Zn resistance (%)[*]	Relative meropenem resistance (%)[*]	Relative combined resistance (%)[*]
	Total (mg/kg dry sediment)	0.926; 0.000	-0.211; 0.559	0.845; 0.002
Pb	Soluble (mg/L)	0.859; 0.001	-0.272; 0.448	0.814; 0.004
	Colloidal (mg/L)	0.695; 0.026	0.038; 0.918	0.662; 0.037
	Total (mg/kg dry sediment)	0.972; 0.000	-0.220; 0.542	0.838; 0.002
Zn	Soluble (mg/L)	0.777; 0.008	0.014; 0.969	0.936; 0.000
	Colloidal (mg/L)	0.765; 0.010	0.021; 0.955	0.914; 0.000

[*]First value: Pearson's correlation; Second value: p-value

Appendix B

Table B-1 Measured total Zn (mg/kg dry sediment) levels over 309 days, in all batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and amended with low (2.00 mg/L) and high (100 mg/L) Zn.

Batch reactor	Total Zn (mg/kg dry sediment)								
	14	28	84	112	169	204	253	295	309
	38.3	23.9	134	849	652	456	464	458	298
Low Zn Warks Burn	38.3	158	309	331	234	210	206	179	211
	38.3	186	422	522	529	502	3866	397	384
	636	1810	171	1790	1610	2800	1520	1870	1560
High Zn Warks Burn	636	307	1070	3350	2020	1220	2170	2330	1740
	636	123	1670	3380	1410	1740	1650	2000	1390
	11600	11000	17400	12100	11800	12300	12500	14500	14800
Low Zn West Allen	11600	11100	11000	12400	1170	12600	15800	13600	13100
	11600	11200	8260	14900	13500	12300	15300	16300	10900
	7780	12300	12700	19400	16900	13200	5460	19100	17500
High Zn West Allen	7780	10200	10100	11200	10600	10800	11600	12900	22600
	7780	7030	14700	16500	16600	11300	17300	18100	10600

Table B-2 Measured soluble (mg/L) levels of zinc (Zn) over 309 days, in all batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn.

Batch reactor	Soluble Zn (mg/L)								
	14	28	84	112	169	204	253	295	309
	0.15	0.16	0.12	0.16	0.10	0.01	0.01	0.01	0.01
Low Zn Warks Burn	0.15	0.03	0.03	0.59	0.08	0.01	0.01	0.01	0.01
	0.15	0.05	0.10	0.40	0.61	0.01	0.01	0.01	0.01
	0.09	0.47	1.14	1.17	1.62	0.94	0.92	0.80	0.81
High Zn Warks Burn	0.09	0.62	2.09	3.01	3.06	2.32	2.34	2.05	2.06
	0.09	0.52	1.50	1.84	1.76	0.83	1.06	0.74	0.81
	0.15	0.25	0.07	0.77	0.57	0.07	0.11	6.72	0.14
Low Zn West Allen	0.15	0.27	0.06	0.64	0.23	0.07	0.09	0.09	0.12
	0.15	0.21	0.15	0.81	0.96	0.63	1.93	2.42	3.03
	0.60	0.41	0.10	0.44	0.76	0.03	0.18	0.09	0.16
High Zn West Allen	0.60	0.32	0.04	0.67	0.67	0.06	0.08	0.13	0.24
	0.60	0.42	0.17	0.77	0.60	0.06	2.17	3.20	4.42

Table B-3 Percentage (%) of colonies resistant to 2.00 mg/L meropenem over a time period of 309 days, in reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and respectively amended with low (2.00 mg/L) and high (100 mg/L) Zn.

Batch reactor	Day								
	14	28	84	112	169	204	253	295	309
	0.25	0.05	6.68	14.25	15.61	4.89	8.20	6.53	8.89
Low Zn Warks Burn	0.25	0.24	1.12	1.16	2.53	4.51	7.57	13.33	14.83
	0.25	0.08	2.47	5.03	9.30	19.14	14.06	13.93	17.41
	2.77	7.09	6.58	8.19	11.68	12.28	19.47	27.42	30.42
High Zn Warks Burn	2.77	5.40	7.15	14.70	27.36	28.41	33.55	34.30	35.53
	2.77	2.29	1.46	1.63	9.83	10.29	16.98	29.88	25.65
	0.38	0.49	6.50	8.50	11.36	18.28	21.91	22.40	20.05
Low Zn West Allen	0.38	0.41	2.40	4.51	5.00	5.21	6.98	7.72	14.50
	0.38	0.29	0.72	0.81	3.71	3.67	4.14	6.54	15.55
	3.59	3.08	6.59	6.52	6.42	7.80	11.20	15.10	24.81
High Zn West Allen	3.59	3.95	4.73	7.92	7.70	8.10	11.11	16.40	19.91
	3.59	4.29	5.21	2.28	4.45	7.30	11.63	12.01	19.20

Table B-4 P-values, showing significant differences at a 95.0% level, for comparisons between meropenem resistance at each time point in batch reactors seeded with Warks Burn (WB) sediment and amended with high (100 mg/L) Zn. P-values were determined from using ANOVA statistical tests and Tukey simultaneous tests for differences of means.

Factors (days)	253	295	309
14	0.002	0.000	0.000
28	0.010	0.000	0.000
84	0.011	0.000	0.000
112	-	0.000	0.000

*Table B-5 Relative abundances of bla-*CTX-M *genes in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and dosed with high (100 mg/L) and low (2.00 mg/L) Zn on days 0, 112, 204 and 309. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances.*

| Batch reactor | Logarithmic gene abundances of *bla*-CTX-M (copies/16S rDNA gene level) | | | |
	0	112	204	309
	1.03	0.85	1.01	0.86
Low Zn Warks Burn	0.82	0.88	0.79	0.86
	0.81	0.86	0.89	0.85
	0.82	0.84	0.84	0.79
High Zn Warks Burn	0.73	0.79	0.84	0.77
	0.76	0.77	0.95	0.83
	0.76	0.89	0.77	0.71
Low Zn West Allen	0.81	0.82	0.94	0.92
	1.07	0.87	0.75	0.76
	0.74	0.84	0.91	0.81
High Zn West Allen	0.83	0.81	0.73	0.80
	0.89	0.74	0.91	0.88

*Table B-6 Relative abundances of bla-*NDM-1 *genes in batch reactors seeded with Warks Burn (WB) and West Allen (WA) sediment and dosed with high (100 mg/L) and low (2.00 mg/L) Zn on days 0, 112, 204 and 309. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances.*

Batch reactor	Logarithmic gene abundances of *bla*-NDM-1 (copies/16S rDNA gene level)			
	0	112	204	309
	0.70	0.82	1.00	1.00
Low Zn Warks Burn	0.69	0.84	0.80	0.92
	0.61	0.84	0.93	0.98
	0.69	0.77	0.83	0.89
High Zn Warks Burn	0.61	0.72	0.92	0.91
	0.68	0.70	1.03	0.93
	0.63	0.79	0.66	0.82
Low Zn West Allen	0.68	0.75	0.95	0.95
	0.84	0.78	0.93	0.75
	0.66	0.70	1.10	0.93
High Zn West Allen	0.67	0.70	0.84	0.82
	0.79	0.68	0.99	0.88

Appendix C

Table C-1 Concentrations of meropenem and Zn used in the preliminary ASRIT study.

Test vessel number	Test substance	Test vessel	Test substance concentration (mg/L)	Solids concentration (g/L)
1		Blank	0.00	1.50
2		1.00	1.00	1.50
3	3,5-dichlorophenol	3.2	3.20	1.50
4		10.0	10.0	1.50
5		32.0	32.0	1.50
6		Control	32.0	0.00
7		Control	0.00	0.00
8		Blank	0.00	1.50
9	Zn only	0.00	2.00	1.50
10		0.00	10.0	1.50
11		0.00	30.0	1.50
12		0.00	100	1.50

Table continued on next page.

Test vessel number	Test substance	Test vessel	Test substance concentration (mg/L)		Solids concentration (mg/L)
13		Control	0.00		0.00
14		Blank	0.00		1.50
15	Meropenem only	0.20	0.20		1.50
16		2.00	2.00		1.50
17		2.00	2.00		1.50
18		2.00	2.00		1.50
			Meropenem	Zn	
19		Blank	0.00	0.00	0.00
20		0.20	0.20	0.00	1.50
21	Meropenem & Zn	0.20	0.20	2.00	1.50
22		0.20	0.20	10.0	1.50
23		0.20	0.20	30.0	1.50
24		0.20	0.20	100	1.50

Table continued on next page.

Test vessel number	Test substance	Test vessel	Test substance concentration (mg/L)		Solids concentration (mg/L)
			Meropenem	Zn	
25		Blank	0.00	0.00	1.50
26		2.00	2.00	0.00	1.50
27	Meropenem & Zn	2.00	2.00	2.00	1.50
28		2.00	2.00	10.0	1.50
29		2.00	2.00	30.0	1.50
30		2.00	2.00	100	1.50
			Meropenem	Zn	
31		Blank	0	0.00	1.50
32		20.0	20.0	0.00	1.50
33	Meropenem & Zn	20.0	20.0	2.00	1.50
34		20.0	20.0	10.0	1.50
35		20.0	20.0	30.0	1.50
36		20.0	20.0	100	1.50

Table C-2 Percentage (%) of colonies in the upper biofilm resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor on days 6, 12, 19 and 27.

Day	Agar amendments	Percentage (%) of colonies showing resistance					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
6	65 mg/L Zn	7.31	6.16	10.8	1.09	13.5	16.7
	2 mg/L meropenem	0.74	14.9	10.2	1.07	9.10	14.5
	Combination of both	0.39	8.56	11.2	0.93	10.1	16.2
12	65 mg/L Zn	5.67	11.2	19.5	18.6	66.8	50.2
	2 mg/L meropenem	0.83	14.6	3.86	9.80	53.2	52.6
	Combination of both	0.39	6.69	4.07	5.29	47.3	51.3

Day	Agar amendments	Percentage (%) of colonies showing resistance					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
19	65 mg/L Zn	3.10	5.64	16.1	16.5	60.5	49.1
	2 mg/L meropenem	3.22	4.78	11.5	29.5	34.20	50.5
	Combination of both	0.94	4.10	3.08	27.6	21.0	53.2
27	65 mg/L Zn	6.24	10.1	2.19	10.7	25.8	84.3
	2 mg/L meropenem	0.73	3.32	0.49	7.53	21.4	72.2
	Combination of both	0.61	1.63	0.45	4.91	14.4	97.5

Table C-3 Percentage (%) of colonies in the lower biofilm resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor on days 6, 12, 19 and 27.

Day	Agar amendments	Percentage (%) of colonies showing resistance					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
6	65 mg/L Zn	16.2	15.3	10.5	5.91	5.87	4.58
	2 mg/L meropenem	16.0	10.2	12.1	6.00	6.04	4.14
	Combination of both	8.33	10.2	10.7	6.30	6.13	3.31
12	65 mg/L Zn	19.2	7.12	11.9	10.8	35.4	67.1
	2 mg/L meropenem	37.3	6.48	3.20	7.97	41.9	69.3
	Combination of both	20.2	1.62	3.33	6.86	67.6	88.5

Table continued on next page.

Day	Agar amendments	Percentage (%) of colonies showing resistance					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
19	65 mg/L Zn	2.76	28.6	22.1	12.4	82.3	75.2
	2 mg/L meropenem	3.82	40.4	18.2	23.1	25.7	69.3
	Combination of both	1.15	5.71	8.90	10.1	30.2	59.1
27	65 mg/L Zn	8.75	6.44	41.8	8.41	4.30	29.7
	2 mg/L meropenem	1.26	0.55	1.27	27.3	1.66	29.7
	Combination of both	0.04	0.01	1.43	1.65	0.83	15.3

Table C-4 Percentage (%) of colonies in the liquid effluents resistant to 65.0 mg/L Zn, 2.00 mg/L meropenem and 2.00 mg/L meropenem and 65.0 mg/L Zn for each rotating tubular reactor on days 6, 12, 19 and 27.

Day	Agar amendments	Percentage (%) of colonies showing resistance					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
6	65 mg/L Zn	4.81	6.01	13.6	21.5	37.5	39.8
	2 mg/L meropenem	1.42	11.5	3.10	17.8	13.3	38.7
	Combination of both	0.79	8.65	2.99	11.3	12.2	38.4
12	65 mg/L Zn	14.8	19.1	3.55	10.1	63.1	82.0
	2 mg/L meropenem	3.92	13.1	1.10	9.60	29.5	62.8
	Combination of both	4.62	4.94	1.10	10.8	27.8	99.0

Table continued on next page.

Day	Agar amendments	Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
		Percentage (%) of colonies showing resistance					
19	65 mg/L Zn	9.53	41.2	20.3	19.0	65.0	73.9
	2 mg/L meropenem	1.93	20.6	5.77	40.0	34.5	87.5
	Combination of both	0.60	7.05	5.63	15.3	39.2	68.6
27	65 mg/L Zn	4.00	11.3	8.21	18.5	23.3	30.6
	2 mg/L meropenem	0.49	15.5	1.12	12.0	12.5	16.7
	Combination of both	0.20	4.02	1.11	5.99	9.6	16.6

Table C-5 The concentrations of Zn in the raw influent[2], upper and lower biofilms (mg/kg dry sludge) and the liquid effluents (mg/L) of each rotating tubular reactor on days 6, 12, 19 and 27.

Day	Sample	Concentration of Zn (mg/kg for biofilms and mg/L for liquid effluents)					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper biofilm	409	265	54100	17600	202000	198000
6	Lower biofilm	623	634	7770	9880	46100	33800
	Liquid effluent	<0.01[1]	<0.01[1]	0.01[1]	0.03	0.52	3.22
	Upper biofilm	390	1410	3870	65700	381000	307000
12	Lower biofilm	713	569	12000	19800	113000	505000
	Liquid effluent	<0.01	<0.01	0.12	0.03	0.33	1.40

[1]Below detection limit of 0.01 mg/ L Zn; [2]Raw influent below detection limit of 0.01 mg/L Zn.

Table continued on next page.

Day	Sample	Concentration of Zn (mg/kg for biofilms and mg/L for liquid effluents)					
		Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper Biofilm	393	446	38200	105000	310000	583000
19	Lower biofilm	279	563	2810	24200	22700	591000
	Liquid effluent	0.03	0.02	0.28	0.07	0.29	3.56
	Upper Biofilm	565	421	68500	57000	128000	506000
27	Lower biofilm	643	543	38200	21000	167000	373000
	Liquid effluent	<0.01	<0.01	0.04	0.04	0.44	1.86

[1]Below detection limit of 0.01 mg/ L Zn; [2]Raw influent below detection limit of 0.01 mg/L Zn.

Table C-6 Relative abundances of Intl1 genes in the raw influent, upper and lower biofilms and liquid effluent of each rotating tubular reactor on days 6, 12, 19 and 27. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances.

Day	Sample	Logarithmic gene abundances of *Intl1* (copies/16S rDNA genes)						
		Raw influent	Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper Biofilm	0.00E+00	1.45E+00	7.60E-01	1.45E+00	8.48E-01	1.54E+00	9.31E-01
6	Lower biofilm		1.25E+00	7.39E-01	1.38E+00	7.72E-01	1.38E+00	7.15E-01
	Liquid effluent		0.00E+00	0.00E+00	7.34E-01	0.00E+00	7.22E-01	7.82E-01
	Upper Biofilm	7.02E-01	0.00E+00	0.00E+00	8.28E-01	0.00E+00	6.46E-01	0.00E+00
12	Lower biofilm		0.00E+00	8.09E-01	7.97E-01	5.94E-01	7.80E-01	7.60E-01
	Liquid effluent		6.77E-01	7.28E-01	0.00E+00	7.17E-01	0.00E+00	0.00E+00

Table continued on next page.

Day	Sample	Logarithmic gene abundances of *Intl1* (copies/16S rDNA genes)						
		Raw influent	Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper Biofilm	8.90E-01	6.83E-01	6.70E-01	1.26E+00	9.27E-01	1.26E+00	7.37E-01
19	Lower biofilm		7.32E-01	0.00E+00	6.75E-01	0.00E+00	6.93E-01	7.18E-01
	Liquid effluent		9.78E-01	7.39E-01	7.88E-01	8.71E-01	8.64E-01	0.00E+00
	Upper Biofilm	8.97E-01	1.21E+00	1.12E+00	1.13E+00	9.31E-01	9.43E-01	8.47E-01
27	Lower biofilm		6.83E-01	7.18E-01	1.38E+00	7.39E-01	0.00E+00	7.40E-01
	Biofilm Lower 4		0.00E+00	0.00E+00	7.92E-01	6.88E-01	7.01E-01	6.73E-01

*Table C-7 Relative abundances of bla-*NDM-1 *genes in the raw influent, upper and lower biofilms and liquid effluent of each rotating tubular reactor on days 6, 12, 19 and 27. All values were normalised to 16S rDNA gene abundances to account for differences in background local gene abundances.*

Day	Sample	Logarithmic gene abundances of *bla*-NDM-1 (copies/16S rDNA genes)						
		Raw influent	Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper Biofilm	8.54E-01	1.19E+00	8.14E-01	1.18E+00	8.25E-01	1.19E+00	8.50E-01
6	Lower biofilm		1.24E+00	8.36E-01	1.25E+00	8.16E-01	1.17E+00	7.74E-01
	Liquid effluent		7.82E-01	7.84E-01	8.05E-01	8.45E-01	7.92E-01	7.95E-01
	Upper Biofilm	7.73E-01	7.31E-01	6.81E-01	6.66E-01	7.68E-01	6.11E-01	6.55E-01
12	Lower biofilm		7.81E-01	7.24E-01	7.08E-01	7.14E-01	8.41E-01	8.35E-01
	Liquid effluent		7.44E-01	7.67E-01	8.17E-01	7.78E-01	7.56E-01	7.75E-01

Table continued on next page.

Day	Sample	Logarithmic gene abundances of *bla*-NDM-1 (copies/16S rDNA genes)						
		Raw influent	Control (R1)	2 mg/L meropenem (R2)	2 mg/L Zn (R3)	2 mg/L meropenem and 2 mg/L Zn (R4)	2 mg/L meropenem and 20 mg/L Zn (R5)	2 mg/L meropenem and 100 mg/L Zn (R6)
	Upper Biofilm	7.06E-01	7.06E-01	5.62E-01	5.04E-01	7.99E-01	1.12E+00	7.50E-01
19	Lower biofilm		7.95E-01	8.83E-01	7.33E-01	7.12E-01	7.57E-01	7.65E-01
	Liquid effluent		7.31E-01	6.56E-01	8.07E-01	8.75E-01	8.48E-01	7.67E-01
	Upper Biofilm	8.82E-01	1.17E+00	1.05E+00	1.01E+00	9.26E-01	9.22E-01	8.73E-01
27	Lower biofilm		7.56E-01	7.21E-01	1.14E+00	7.75E-01	7.83E-01	7.06E-01
	Biofilm Lower 4		7.13E-01	7.48E-01	7.75E-01	6.95E-01	7.41E-01	7.08E-01