The Effect of Heavy Metals on Antibiotic Resistance in the Environment

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract

Antibiotic resistance is a significant clinical problem, with bacterial infections becoming increasingly difficult to treat. Efforts, such as reducing the use of antibiotics, have proved unsuccessful, and we now face the prospect of a future without antibiotics.

The natural environment acts as a reservoir for resistance genes. The selection and maintenance of resistance could counteract clinical efforts to reduce antibiotic resistance. Heavy metals have been linked to antibiotic resistance by genetic mechanisms whereby metals potentially select for and maintain antibiotic resistance, even in the absence of the antibiotic itself. Here, the role of heavy metals in enhancing, or maintaining, antibiotic resistance in the environment is investigated.

Background levels of metals in soil were found to correlate to antibiotic resistance gene abundances, implying the effect heavy metals in the environment have on antibiotic resistance is more intrinsic than anticipated. Using controlled microcosm studies, the influence of pollution levels on antibiotic resistance was further investigated. Copper concentrations influenced tetracycline and β lactamase resistance gene abundances in both soils and simulated wastewater activated-sludge treatment process. Results suggest that copper discharged to wastewater and the environment increase resistance genes.

Additionally surface-water microcosms were used to determine whether copper concentration enhanced retention of antibiotic resistance genes released by wastewater treatment. While no selection effect was observed, further work is still needed.

Effect exhorted by metals on antibiotic resistance is not novel; however, its role in the environment could play a more significant role in the clinical problem than anticipated. Agencies, such as World Health Organisation, call for further investigations to reduce antibiotic resistance in the environment; this thesis highlights how metals, particularly, contributes to the problem.

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Chapter 1: Introduction & Literature Review

1.1 Introduction

The World Health Organisation (WHO) has listed antibiotic resistance in clinically important bacteria as one of the top three threats to human health (1). Despite this, the steps taken to tackle the problem have had limited effect. Bacteria in the wider environment are thought to act as a reservoir for antibiotic resistance genes as resistance genes, like antibiotics, originate in bacteria ubiquitous in the environment such as *Streptomyces* (4). Additionally genes may transfer to other bacteria in the surrounding milieu. In the environment, antibiotic resistance genes are often associated with heavy metal resistance (5-8). As such, the purpose of this thesis is to investigate the role heavy metals play in selecting for antibiotic resistance genes with particular emphasis on wastewater treatment due to its role as an interface between the bacteria in the clinical setting and environment.

1.2 Antibiotic Resistance and its Relationship with Heavy Metals and the Environment

1.2.1 Antibiotics

Antibiotics are compounds used to treat bacterial infections and target bacterial cells in various ways to inhibit growth (bacteriostatic) or lead to cell death (bactericidal) (Table 1-1). Numerous antibiotics originate from bacteria themselves; soil bacteria from the genus *Streptomyces* are responsible for producing a wide range of antibiotics, such as tetracycline (9).

Many of the antibiotics currently in use are semi-synthetic, meaning, they are chemical derivatives of natural antibiotics that have been modified to evade bacterial resistance mechanisms; for example, all β lactams have a conserved β-lactam ring structure with a variable side chain. The β lactam ring is vulnerable to enzymatic degradation by β lactamases; however, modified β lactams such as methicillin are less vulnerable to attack than their predecessor penicillin. Methicillin possesses a bulkier side chain so when it occupies the active site of a β lactamase, hydrolysis is prevented either by displacement of

a water molecule or by blocking access to a serine residue within the active site. As a result, the enzyme is unable to complete degradation of the β lactam ring (10).

Table 1-1 Antibiotic Mechanisms of Action (reproduced from ref (11)).

Antibiotic Target	**Antibiotic Class**
DNA Replication	Quinolones
Transcription	Rifamycins
C1 Metabolism	Pyrimidines
	Sulfonamides
Cell Membrane	Lipopeptides
	Cationic Peptides
Peptidoglycan Synthesis	β lactams
	Glycopeptides
Translation	Aminoglycosides
	Tetracyclines
	Macrolides
	Lincosamides
	Streptogramins
	Oxazolidinones
	Phenicols

Bacteria have now also developed resistance mechanisms to these modified antibiotics, such as methicillin resistant *Staphylococcus aureus*, or MRSA. Consequently, it is therefore necessary to identify novel bacterial drug targets to counteract resistance development; however these new drugs require extensive testing prior to their approval for clinical use. This is a lengthy process; penicillin was first discovered in 1928 (12) but was not introduced for clinical use until 1940 (13). Modern licencing laws are more stringent so the time span from drug discovery, to introduction of the new antibiotic into the clinical setting takes considerably longer - oxazolidinones were discovered in 1978 (12), but it was not until 2000 (13) for a drug from that class to be approved for clinical use. Additionally fewer companies are involved in the discovery of new antibiotics than previously; this is thought to be mainly due to the cost associated with the research and development of new antibiotics not being offset by profits after the drugs are approved (14). As a result, antibiotic resistance is a continuing and significant problem that is compounded by the lack of new drugs coming on to the market.

1.2.2 Antibiotic Resistance

Antibiotic resistance is the possession of mechanisms to evade the action of antibiotics designed to either kill a bacterium or inhibit its growth. The first antibiotic resistance mechanism (penicillinase) was discovered in 1940 (15), the same year penicillin was introduced for clinical use (13). Sir Alexander Fleming, who discovered penicillin, warned during his Nobel Prize lecture in 1945, that "under dosing" patients with penicillin while treating a bacterial infection could easily lead to resistance (16).

The discovery of penicillin revolutionised the method for treating infections and has saved the lives of countless patients since. However, despite Fleming's warning, antibiotic resistance soon made an appearance in clinically relevant strains (15). This problem has escalated and, with the increase multi-drug resistant strains, a post-antibiotic era is now approaching. As such, the problem of antibiotic resistance is now paramount. Linezolid, a drug of last resort for treating multidrug resistant strains such as MSRA, was introduced in 2000, however within a year resistant strains were isolated in intensive care units (14). This was an unexpected outcome as it was entirely synthetic, the first drug of its class and, consequently, no pre-existing resistance genes were anticipated. In the USA, 90 000 infection cases per annum are caused by antibiotic resistant bacterial strains, and as these infections are more difficult to treat, they add 5 billion dollars to US healthcare costs annually (17, 18). Antibiotic resistance could eventually lead to problems in common hospital procedures such as insertion of IVs, catheters and ventilation tubes, as well as making immune suppression and transplants more risky. WHO says we could eventually see that "commonplace procedures once previously taken for granted could be conceivably consigned to medical limbo" as a direct result of antibiotic resistance (19).

While antibiotic resistance genes are extremely diverse, they all confer resistance to antibiotics in one of four ways (see Table 1-2, which also lists biofilm formation as a mechanism of resistance; however, this is not conferred by an antibiotic resistance gene and is discussed further below). Many of the antibiotics in current use originated from bacteria such as *Streptomyces* (4). These same bacteria also harbour resistance genes to protect themselves from the antibiotics they produce (4). It is likely that these resistance genes have disseminated to other bacteria in the wider environment (20, 21), or bacteria that share an ecological niche with these natural antibiotic producers have developed their own resistance mechanisms as a result of antibiotic exposure.

Resistance to quinolones and linezolid (both fully synthetic drugs) is conferred by resistance genes that originated in animals (fish and swine respectively (18, 22)). Whilst it is possible that the use of quinolones in fish farming helped the development of resistance to the drugs clinically (23), it cannot be as easily explained for linezolid. Environmental producers of linezolid have not been found, however resistance to linezolid is provided by the *cfr* gene which confers resistance to several antibiotics with overlapping binding regions on the ribosome (24). It is possible the use of these antibiotics has selected for the *cfr* gene and caused its movement from the environment to the clinical setting, as such this transfer of resistance genes and the factors favouring this process need to be determined.

Antibiotic resistance was thought to arise due to mutation; however, it is now known that random mutations occur less frequently than previously anticipated (19). Mutations alone cannot account for the current levels of resistance, and it is therefore likely that other factors are involved. Nowadays horizontal gene transfer (HGT) is thought to be the main culprit (25, 26), and can occur by one of three mechanisms—transformation, conjugation and transduction. Transformation, involves the "uptake, integration and functional expression of extracellular DNA" (27). Bacteria must be in a competent state to assimilate DNA by transformation. This can be either naturally occurring, or induced by various proteins and environmental conditions. The DNA, taken up by cells, typically has been released by other cells upon lysis; although, some bacteria naturally produce extracellular DNA. Conjugation is the most studied mechanism of HGT (28) and is "mediated by cell to cell junctions and a pore through which DNA can pass" (27). Conjugation frequently involves plasmids as they can be transferred from cell to cell quickly due to their size and stability. Transduction involves the transfer of DNA from cell to cell mediated by a bacteriophage.

HGT, as mentioned, is the main driving force in increasing antibiotic resistance (21, 29). Resistance genes disseminate rapidly through bacterial populations, and HGT can have a significant impact on the genetic evolution of a bacterial community (30, 31).HGT occurs between unrelated bacteria i.e. phylogenetically distant (32), and allows the transfer of resistance genes from antibiotic producers into the wider population, as well as between clinically relevant strains. Thus, it is intrinsic to antibiotic resistance propagation (33). Whole genome sequencing has granted valuable insight into the role that HGT has had in the spread and evolution of antibiotic resistance; genes or genetic segments originating

from plasmids, transposons or phages have been identified within the bacterial chromosome (29). Additionally whole genome sequencing has allowed the identification of several cryptic genes, which are genes contained within the bacterial chromosome not obviously linked to antibiotic resistance and either not expressed or expressed at low levels (30). They form an integral part of the antibiotic resistome (discussed later), which encompasses all antibiotic resistance genes (30).

Antibiotic chemotherapy was originally thought to be essential for the transfer of resistance (R) factors by HGT; this was tested by feeding patients resistant organisms (34). Supplementation with antibiotics prolonged the time R factors were present after cessation of antibiotic administration (35). It was thought that antibiotics somehow increased plasmid transfer, but this was later found to be false (35).

While random mutations may not have a severe impact on antibiotic resistance, it is not to say that mutations do not play a role. Stress-induced mutations have been shown to occur in bacterial genomes when bacterial cells are exposed to stresses such as starvation or UV (36). Double strand breaks in the bacterial chromosomal DNA initiate the SOS response, which utilises a family of DNA enzymes to repair the break; however, this can lead to the introduction of mutations (36). It should be noted that while this is not the only mechanism of introducing mutations; a double strand break is required to activate the SOS response. It has been proven that antibiotics can also cause stress induced mutagenesis via the SOS response – *Escherichia coli* induces an SOS response mutation pathway in response to the DNA gyrase inhibitor ciprofloxacin (36). Further to this, *E. coli* has a resistance gene, *amp*D, which confers resistance to β lactam antibiotics. *amp*D mediated resistance is caused by a mutation, which can also be induced by lactose starvation initiation of the SOS double strand break repair system (37). Resistance to streptomycin can result from the simple mutation of one nucleotide in the gene encoding a ribosomal protein (37) (resistance is conferred by modification of the target site, which is also the case for the *cfr* gene discussed previously). If stressors can lead to the generation of mutations, and a single nucleotide polymorphism (SNP) is sufficient to confer resistance to an antibiotic in some cases, the role of mutations in antibiotic resistance should not be disregarded.

As previously mentioned, many of the antibiotics in current use, as well as their corresponding resistance genes, originated from bacteria such as *Streptomyces* (4). Although the existence of antibiotic resistance has been known since 1940 (15), it far

outdates the discovery of antibiotics themselves. Whilst antibiotics have only been used in clinical situations for about 70 years (38), genes encoding resistance to antibiotics were isolated from organisms found in permafrost sediment over 30,000 years in age (39). However, phylogenetic studies suggest that antibiotics resistance is many millions of years old (23).

As mentioned above the antibiotic resistome encompasses all antibiotic resistance genes i.e. those carried by antibiotic producers, cryptic genes that are not obviously related to antibiotic resistance and precursor genes that have modest antibiotic activity and could therefore potentially evolve and develop significant resistance activity (30). The resistome represents an intrinsic network of resistance genes contained within the natural environment (40) that have the potential to spread and evolve without anthropogenic influence. Additionally, many environmental bacteria are multidrug resistant (40), and therefore, the resistome should be investigated further to determine the role it plays (or could potentially play) in the current resistance issue (41).

1.2.3 Steps to Reduce Antibiotic Resistance

Antibiotics are used to treat infection, both in the clinical and veterinary settings, as well as growth promoters in animal feed. The over-use of antibiotics may be acting as a selection pressure for resistant organisms; for example, a positive correlation was found between the age of a person and the number of resistance genes found in their microflora (35). Steps to reduce antibiotic use, such as banning their use as animal feed additives (AFA) (42) and reducing the levels of antibiotics prescribed, have had limited effect on the abundance of resistance. It had been hoped that reduced drug usage would see a corresponding decrease in antibiotic resistance because the reservoir of resistance genes should disappear (43). However this proved not to be the case. In fact, reduced use resulted in increased resistance. For example, the amount of sulphonamides being prescribed in the UK significantly declined from 320 000 prescriptions per year in 1991 to only 7000 per year in 1999; however, resistance levels to sulphonamides increased from 39.7% of isolates tested in 1991 to 46% in 1999 (44). Analysis of epidemiological and population genetics data, related to cephalosporin use in both Finland and Iceland showed a similar lack of resistance decline despite reduced antibiotic use (45), as have other such studies (46, 47). A one week treatment course of clarithromycin has been shown to generate resistance in the patient's

commensal bacteria that persists for at least 4 years without further exposure to the antibiotic (48). It is, therefore, clear that antibiotic resistance emerges in relation to antibiotic use at a much faster rate than resistance declines following a reduction in (or cessation) of antibiotic use (49).

When bacteria become resistant to an antibiotic via a mutation, it is usually accompanied by some degree of fitness "cost"(50), such as a reduction in growth (51, 52). The fitness cost of resistance and the subsequent removal of these less biologically "fit" resistant strains, may be linked to the delay in resistance decline following reduced antibiotic use. Bacterial populations can generally overcome any resistance associated fitness cost incurred by compensatory mutations that happen to restore some degree of functionality (51, 53). These compensatory mutations occur more commonly than the simple reversion to antibiotic sensitivity (i.e. no longer resistant) (53). This means that following antibiotic exposure, bacteria will facilitate further mutations in hopes to restore partial or full fitness rather than correcting the initial mutation. This is important as it had been believed that healthier susceptible strains would outcompete and eventually remove less fit resistant strains from the population when an antibiotic selection pressure was removed (52). Additionally some compensatory mutations result in increased fitness, so the resistant strains are actually more fit than the normal susceptible population (30).

There are various reasons that might explain why previous measures to combat the antibiotic resistance problem have had limited success. AFA have been banned in the EU since 2006, but other nations such as the United States (54), have yet to follow suit (42). Additionally, based on the most recent figures for antibiotic use in the EU, the amount of antibiotics prescribed is still on the increase (55). There is also a vast difference in antibiotic consumption between countries. Nordic countries tend to use less antibiotics and prescribe more specific narrow range drugs, while southern European countries have substantially higher antibiotic usage and favour more broad spectrum drugs (55). Further, resistance can move from country to country as a result of travel and importation of agricultural goods; the spread of NDM-1 (a relatively new β lactam resistance gene) is an example of this. NDM-1 originated in India, but cases have been reported all over the world, and the frequency of infections caused by NDM-1 is increasing (56).

It is impractical and impossible to try and control resistance spread by limiting such activities, so perhaps no matter what preventative measures are taken, until all countries

take united action against the resistance issue, measures that are currently in place will have limited effect. On a more positive note, antibiotic use has become less seasonal in recent years (55). There have been shifts away from prescribing them for viral infections during the winter months and more towards only using them when appropriate to deal with bacterial infections.

Antibiotic resistance genes are not necessarily the only mechanisms for resistance in their original host. That is, the genes are used for something other than resistance, but when acquired by pathogens with different biochemical conditions, the genes take on a different role (57). Additionally antibiotics may act as inter-cellular signalling molecules (57). In *Pseudomonas aeruginosa*, tobramycin caused an increase in cell motility, while tetracycline induced the expression of a type-three secretion system, which is a mechanism of virulence (58). Other compounds can select for and maintain antibiotic resistance such as quaternary ammonium compounds (QACs) (59) and heavy metals (60). Antibiotic resistance and QAC resistance are carried on class 1 integrons (59) and plasmids together (61). QACs are used in disinfectants and they can be exported via the same efflux pumps as antibiotics (62). The link between antibiotics and heavy metals is discussed in further detail below.

Antibiotics and the issue of antibiotic resistance are clearly complex and involve factors out with the use of antibiotics themselves as therapeutic agents. Antibiotics and their corresponding resistance genes require further understanding, so appropriate actions can be taken to deal with the clinical issue we currently face.

1.2.4 Heavy Metals

Heavy metals are elements with an atomic mass greater than 53 and a density greater than 6g cm^{-3} (63). They have antimicrobial properties and some, such as silver, were used as disinfectants prior to the introduction of antibiotics (64) and are now being used more frequently (65). Some heavy metals, such as copper and zinc, are required in trace amounts by cells as enzymatic co-factors and to carry out biochemical processes; however, at higher levels they are toxic. They can bio-accumulate and cause illness or death to organisms higher in the food chain (66). Levels of heavy metals in the environment have increased due to industrial processes such as metal smelting and fuel combustion (64). Heavy metals are lethal to cells in various ways, mainly through the generation of free radicals, which cause

lipid peroxidation, and may render enzymes non-functional. Silver can displace metals from the centre of enzymes or bind to their active sites with higher affinity than their intended substrate rendering them useless (67).

As mentioned above, some heavy metals are toxic; therefore, there are mechanisms in place to control cellular metal concentrations. P-type ATPases are responsible for the efflux of many heavy metals. They are classified as either monovalent or divalent metal transporters (68), but one pump can be capable of transporting more than one metal out of the cell. CopB can excrete both silver and copper from the cytoplasm of a bacterial cell (69). PacS (70) is thought to be a copper resistance mechanism that is active against silver due to the monovalency of both metals. This suggests that efflux proteins are non-specific and can excrete more than one metal. As heavy metals are linked to antibiotic resistance, it is possible these efflux proteins can also confer resistance to antibiotics (this is discussed in further detail below, and specific metal resistance mechanisms are discussed in further detail in Section 1.3).

Table 1-2 Mechanisms of Antibiotic and Heavy Metal Resistance

Antibiotic Resistance Mechanisms	**Heavy Metal Resistance Mechanisms**
Reduction of membrane permeability	Reduced cellular uptake
Drug inactivation	Enzymatically reduced/detoxified
Efflux from the cell	Efflux from the cell
Mutation of cellular target	Metal sequestration
Biofilm formation	Biofilm formation

References used to generate Table 1-2 (67, 71).

1.2.5 The Link Between Antibiotic Resistance and Heavy Metals

Since the 1960s, it has been known that heavy metals and antibiotic resistance were linked. Mercury was used as a component in hospital disinfectants (mercury resistance was considered essential for bacterial survival within the hospital setting (72)), and mercury resistance was commonly found in coagulase-negative bacteria along with resistance to tetracycline, while coagulase-positive bacteria displayed an association of resistance between penicillin and copper (73).

The production of penicillinase (15) was linked to resistance to methicillin and mercury salts. Originally, it was believed that production of penicillinase was needed to acquire resistance to mercury (74), as production of this protein was observed in tandem with various metal resistances and multi-antibiotic resistant (MAR) phenotypes (75). The exposure of hospital *Staphylococcus aureus* to mercuric chloride ($HgCl_2$) induced the production of penicillinase and resistance to bacitracin (76). It was eventually discovered that the genes for both types of resistance were being transferred on a single plasmid (77). This is now referred to as co-resistance, where resistance genes for both the antibiotic and metal are carried on the same molecule (60). Either the metal or antibiotic may be present to select for both resistance traits (78). In 1983, plasmids from the pre-antibiotic era were

profiled and found to contain resistance genes that originated from within the bacterial group they were extracted from; thus, this suggests bacteria were not yet in possession of resistance genes transferred from other genera. Additionally, pre-antibiotic era plasmids had little evidence of mercury resistance (79), which as mentioned previously, is a major feature of clinical strains. This implies that bacteria have acquired both metal and antibiotic resistance genes simultaneously. Other pre-antibiotic era plasmids from around 8000-15000 (60,80) years ago were found to contain metal resistance genes and transposons similar to those found today. However, those isolated in recent years have additional genes for antibiotic resistance that were likely acquired since the antibiotic era began.

Antibiotic resistance and heavy metals are linked by two main mechanisms, cross- or co-resistance. Cross resistance is when the one gene can confer resistance to both an antibiotic and a metal, and it usually involves an efflux pump (81, 82). For example, *tet*(L) can export both tetracycline and cobalt (60) from the cell. Many of the resistance mechanisms to antibiotics and metals are extremely similar (see Table 1-2), so there are potentially other undiscovered cross-resistance mechanisms. β lactam and mercury resistance are often carried together (72, 77), and as previously discussed, this is an example of co resistance. For both mechanisms of resistance, the metal alone is sufficient to select for and maintain the antibiotic resistance phenotype and/or genotype (60).

1.2.6 Role of Environment in Antibiotic Resistance

The role the environment plays in the antibiotic resistance problem was not properly investigated until the 1970s (83). Prior to this, investigations into resistance were conducted almost exclusively in the hospital setting. Now, it is known the environment has a substantial impact on resistance—acting as a reservoir of resistance genes and mediating their dissemination, in addition to being the original source of both antibiotics and resistance genes (28). It has been shown that, when a new antibiotic is released to the market, resistance to the antibiotic is first detected environmentally in enteric bacteria present in both sewage and surface waters; with the resistance gene eventually being detected in clinically relevant strains (85). Whether this is the case for all new antibiotics is unknown. Baquero et al. (57) described four “genetic reactors” involved in antibiotic resistance: 1) human and animal micro flora, 2) hospitals and farms 3) wastewater and biological residues , and 4) soil and surface or ground water environments (57). Human and

animal microflora is discharged into the environment from hospitals or farms, while surface water runoff or wastewater treatment discharge to the environment also. As a result, all genetic reactors are interconnected with the natural environment.

Bacterial DNA has been detected at “biologically significant” levels in aquatic environments in North America (28). While the DNA may be partially degraded, some fragments are often large enough to code for entire functional genes and persist long enough for transformation into another bacterial cell to occur (28). Resistance genes are being released to the environment from anthropogenic sources and then disseminated to the indigenous population, where selections pressures in the environment can then select for and maintain these resistance genes.

The environment also provides a vector by which the human population may become exposed to resistant bacteria. Resistance in the environment is linked to faecal contamination in sea water and fresh water ((85). When enteric organisms enter the environment, they do not “die”, but become dormant and non-culturable (28). They are, therefore, still a potential source of antibiotic resistance genes. If contaminated water is treated and used for bathing or drinking water for animals, enteric bacteria could potentially move into the animal host, introducing novel resistance genes to the microflora of the animal (14, 85). Animal manure is often used to fertilise soil, and thus vegetables become contaminated with resistant bacteria (79). Resistant strains are also found on the surfaces of raw meat products, and if the meat is inadequately cooked, bacteria can persist and transfer resistance genes to human gut microflora (34), which is eventually passed out of the body and enters wastewater treatment plants. Therefore, a cycle of resistance genes from clinical setting to the environment, and *vice versa*, is established.

As mentioned previously, water contaminated with resistance genes can be used as a source for drinking water. Unfortunately, the disinfection process to make water suitable for drinking, such as chlorination, may select for more resistant organisms (86). Healthy adults and children were found to carry antibiotic resistant coliforms in their faeces, despite not having been in hospital or treated with antibiotics recently, implicating another source of resistance genes such as water supply (87). In contrast to this, there is an inverse correlation between antibiotic and chlorine resistance in some hospital strains (88). It has been suggested that the use of UV light could help with the treatment of waste as it damages DNA, including resistance genes (89-91). Wastewater treatment can also select for

antibiotic resistance, releasing a higher proportion of resistant bacteria than what originally entered the plant; however, this is discussed in more detail in Section 1.3.2.

Biofilm formation in the environment can be used as a method of antibiotic resistance (61). Within biofilm, bacteria can exchange genetic information, acquire new resistance genes, and return to a planktonic state to re-colonise a new location bringing newly acquired resistance genes, promoting their spread. It has been shown that biofilms take up antibiotic resistance genes (91) and can help them persist in the environment for longer periods of time (90). While it is possible to reduce antibiotic resistance by changing practices such as the sourcing and treatment of drinking and wastewater, it is not possible to prevent natural movement and retention of antibiotic resistance genes within the environment. However, through reduction in the levels of resistance genes that enter the environment, it may be possible to facilitate a decline in overall resistance abundances.

The antibiotic resistome as discussed above refers to all antibiotic resistance genes contained within the environment (30). Metagenomic analysis has shown that there are significantly higher densities of resistance genes in the environment than previously appreciated (40, 84). Further investigation into the resistome and the role the environment plays in the supply of resistance genes to clinically relevant pathogens is of utmost importance.

1.2.7 Previous Research on Antibiotic Resistance and Heavy Metals

Much research into heavy metals and antibiotic resistance has focused on profiling resistance patterns in both pristine and industrially impacted environments (5, 6, 92-101). Results often contradict each other; for example whether or not the mercury in dental amalgam fillings influences the antibiotic resistance of commensal bacteria (102-104); and while the information obtained is interesting, it does not always contribute to the understanding of the specific mechanisms linking metals and antibiotic resistance. There are several reasons for this; differences in experimental design and the lack of a standard method to determine metal resistance often means studies cannot be directly compared.

A method for determining antibiotic susceptibility was developed in 1966 (105) and is still widely used today. Several governing bodies oversee the methodologies and determine resistance breakpoint values for specific bacteria in terms of antibiotic resistance. However,

in contrast, guidelines for metal resistance are confusing. For example, how much mercury can a dental amalgam contain is not clearly defined (106). In addition, there is not yet a standardised reproducible methodology for assessing susceptibility to metals, as there is no universal definition of resistance or breakpoint metal-resistance concentrations. Methodologies have been adapted for metal resistance (107), and implemented by other studies (88), however generally metal resistance determination remains extremely inconsistent.

Environmental bacteria are difficult to culture, so this is a methodological issue as the antibiotic-resistance standards were generated based (and focused) on clinical isolates. There has been a shift towards the use of molecular approaches to investigate metal resistance that overcome culturability problems (108, 109). However, this is not without issues; for example, PCR primers for metal resistance genes normally only target specific bacterial strains and do not provide much information as to the resistance levels of the population as a whole. Despite this, phenotypic analyses also face the same problems.

While several studies quantify the levels of metal present at the site from which the samples were collected (82, 84), many do not (67, 79, 81). The array of antibiotics and metals that bacterial susceptibility is determined against vary from study to study. More consistent approaches, would perhaps allow associations between specific metals and antibiotics to be identified. These relationships require further investigation to determine the underpinning molecular mechanisms.

1.3 Copper, Ampicillin and Tetracycline Resistance— Background[1]

While a wide range of metal and antibiotic resistance genes have the potential to be linked through the mechanisms already discussed, this thesis focuses on copper and its potential influence on resistance to ampicillin or tetracycline. A brief over view of the resistance mechanisms pertinent to each is detailed below.

1.3.1 Copper

Copper (Cu) is one of the most abundant and widely used metals in the world today. It has been used since prehistoric times (Bronze Age), when it was first combined with metals such as tin to form alloys. These alloys allowed the fabrication of tools and weapons with improved physical properties such as hardness. Its ductile and conductive properties have traditionally provided many uses, such as water pipes and wiring. More recently, it has been used as an antifungal and antimicrobial agent in agriculture (110).

Copper Biochemistry

Copper plays a vital role biologically. It shifts readily between redox states, Cu^{+} to Cu^{2+} and, therefore, acts as an electron donor or acceptor. The redox potential of copper ranges from +200 to +800mV (111), meaning it can be used for the direct oxidation of substrates such as ascorbate or phenolates (112). As such, it plays a vital role both in the electron transport chain and redox active enzymes (2). There are over 30 known copper containing proteins, e.g. cytochrome oxidase, superoxide dismutase and lysyl oxidase (113). While most copper-containing proteins transfer electrons or transport dioxygen (112), copper also contributes to other cellular processes, such as hydrolytic pathways and iron transport (114).

While essential, copper is also toxic even at relatively low concentrations. Copper has a role in cellular Fenton chemistry:

[1] The copper resistance section of this chapter formed the background information of the manuscript "Phylogenetic overview of selected proteins involved in copper resistance" by Alfredo Tello, Seánín McCluskey and Charles W. Knapp. The manuscript is currently in preparation for submission.

$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH\bullet$ (110),

and, as a result, generates hydroxyl radicals that cause oxidative damage to DNA, proteins (denaturation), and lipids (cascading membrane damage) within the cytoplasm (110). While Cu^{2+} is generally regarded less toxic, it can be reduced to Cu^{+} by a superoxide radicals:

$Cu^{2+} + O_2^{-} \rightarrow Cu^{+} + O_2$ (2).

When this process is combined with the above Fenton equation, the Haber-Weiss reaction occurs:

$O_2^{-} + H_2O_2 \rightarrow O_2 + OH^{-} + OH\bullet$ (2).

Again, this results in the generation of toxic hydroxyl radicals.

Copper can, in some cases, displace metal ions such as zinc (2) from proteins or bind to/modify functional groups within proteins, nucleic acids and lipids, which affects their structure and function. In particular, copper covalently modifies the catalytically important residues histidine (110), cysteine and methionine (115). It also reduces cell viability by decreasing the functionality of cellular sulphydryl function groups (114) in sulfur-containing amino acids and coenzymes, due to it preferentially binding with sulphydryl ligands (112).

As previously mentioned, copper is an essential micronutrient with some negative drawbacks; strict regulation systems are required to maintain copper levels in the cell. As such, metal resistance mechanisms and strategies have evolved (71). Bacteria are able to detect copper within the cell and shuttle it using a chaperone to either where it is required, or remove it from the cell. Most proteins requiring copper are found outside the cytoplasm (112) - either the periplasmic space in Gram negative bacteria (e.g., zinc superoxide dismutase in *E. coli*) or in the cytoplasmic membrane (e.g., NADH dehydrogenase-2 in *E. coli* or cytochrome oxidases of Gram positive bacteria (2)). This suggests that copper is either not required in the cytoplasm, or the limited presence of copper-containing enzymes helps limit the risks of copper toxicity. Methanotrophs and cyanobacteria are among the few bacteria to have copper-containing proteins in the cytoplasm, but they are contained within internal membrane structures (116).

How Does it Enter the Cell?

It is not specifically known how copper actually enters cells; however, passive diffusion or "nonspecific metal uptake systems," such as those utilised in the transport of sodium and potassium ions, could play a role (2). In the case of *E. coli*, porins (proteins found in the outer membrane that act like a "pore" controlling the diffusion of specific small molecules into the cell) have been suggested to be the port-of-entry for copper (2).

Certain bacteria, such as *Enterococcus hirae* and *Listeria monocytogenes*, have known specific copper importers such as CopA, a P_{1B} type ATPase, which functions by coupling the transport of the copper ion with the hydrolysis of ATP (2). Under anaerobic conditions, Cu^{2+} is reduced to Cu^{+} in the periplasm, which then diffuses into the cytosol. However, when copper enters the cell it has no beneficial role in the cytosol and must be shuttled elsewhere, such as the periplasm, where it is used in the production of copper containing metalloproteins or enzymes (2).

Regulation of Copper Levels

The cellular copper quota, i.e. how much copper is needed for the cell to function metabolically, is 10^4 atoms (~10µM) for *E. coli* (2). For many bacteria, a two component regulatory system is used were one protein may sense copper within the cell and then signals to a response protein to maintain copper levels. The response protein then induces transcription of other proteins required for either copper import/export or detoxification. These proteins can be energy-dependant ATPases like CopA, which use ATP to power the movement of copper into or out of the cell, or multi copper oxidases such as CueO, which simply convert Cu^{+} to Cu^{2+}- a less harmful form to the cell (2).

In *E. coli*, copper is detected by CueR, a protein from the MerR family of transcriptional activator sensors, which detect a single atom of Cu^{+} in the cytosol (2). This suggests no free copper exists within the cell and is all bound or stored in membranes or the periplasm; once an atom of copper is detected in the cell, systems for its removal are activated. The cue (Cu efflux) system (117) is regulated by CueR, which activates the transcription for proteins CopA and CueO (111) when copper levels become elevated. CopA and CueO then remove Cu^{+} from the cytoplasm and convert it to Cu^{2+} in the periplasm (see Figure 1-1 Ai).

Other bacteria, such as *Pseudomonas putida* and *Salmonella typhimurium*, use CueR-like proteins to detect copper. *S. typhimurium* has two CueR like proteins – SctK and GolS. SctK is 91% similar to *E. coli* CueR, and mediates CuiD a multicopperoxidase (similar to *E. coli*'s CueO), and CopA; GolS regulates another ATPase GolT and a Cop Z-like chaperone in *Bacillus subtilis* (2).

E. coli has an additional system, *Cus* (standing for Cu sensing system (117)). While *Cus* can function in both aerobic and anaerobic conditions, it is activated during aerobic conditions and elevated periplasm-copper levels (i.e. when CueO cannot cope), but it is the predominant system during anaerobic conditions. It involves a two component regulatory system, *CusRS*, which monitors cell envelope stress and induces CusABC, and F proteins to transport copper from the periplasm across the outer membrane of the cell (111) (see Figure 1-1 Aii). *CusCBA* (part of a family of homologous transport complexes involved in export of metal ions, xenobiotics and drugs (118)) form a proton-cation antiporter complex which is thought to span both membranes as well as the periplasm. CusF is a periplasmic protein, which could potentially be functioning as a Cu chaperone, based on the fact it has a unique topology found in copper binding proteins (111)(see Figure 1-1 Aii). CusRS have homologues, PcoR in *E. coli* and CopR in *Pseudomonas syringae*, both of which are 61% similar to CusR. PcoS from *P. syringae* is 38% similar to PcoS, while *E. coli's* CopS is 42% similar (111).

Enterococcus hirae use CopY and CopZ – a copper responsive repressor and antirepressor to regulate its copper homeostasis operon – *copYZAB* (see Figure 1-1 D). CopY is one of the "winged helix" proteins in the helix-turn-helix DNA binding proteins superfamily (111). *CopY* is inactive when there is no copper in the cell and, therefore, binds DNA. When Cu^{+} enters the cell it binds to CopY converting it to a DNA binding repressor, CopY binds the operon promoter region repressing transcription. When Cu levels increase, Cu^{+} binds to CopZ the antirepressor, CopZ-Cu^{+} and CopY-Cu^{+} form a non DNA binding complex, CopY is no longer able to bind DNA therefore allowing transcription of the operon (71). While CopY is not widely distributed, it has been found in other bacteria such as *Streptococcus mutans* (2).

The CsoR family of Cu sensors (originally from *Mycobacterium tuberculosis*) are a large family of Cu^{+} responsive repressors. In *M. tuberculosis,* CsoR forms part of the Cso operon, which also has a P1B type ATPase; it is induced by elevated Cu and to a lesser extent silver

(Ag). CsoR-related sequences are widely distributed. Once copper is detected by the bacterium *B. subtilis*, it mediates expression of CopZ and CopA. The majority of CsoR-related proteins appear to function for Cu sensing, however some more distantly related sequences are potentially used to sense other metals or organic molecules (2).

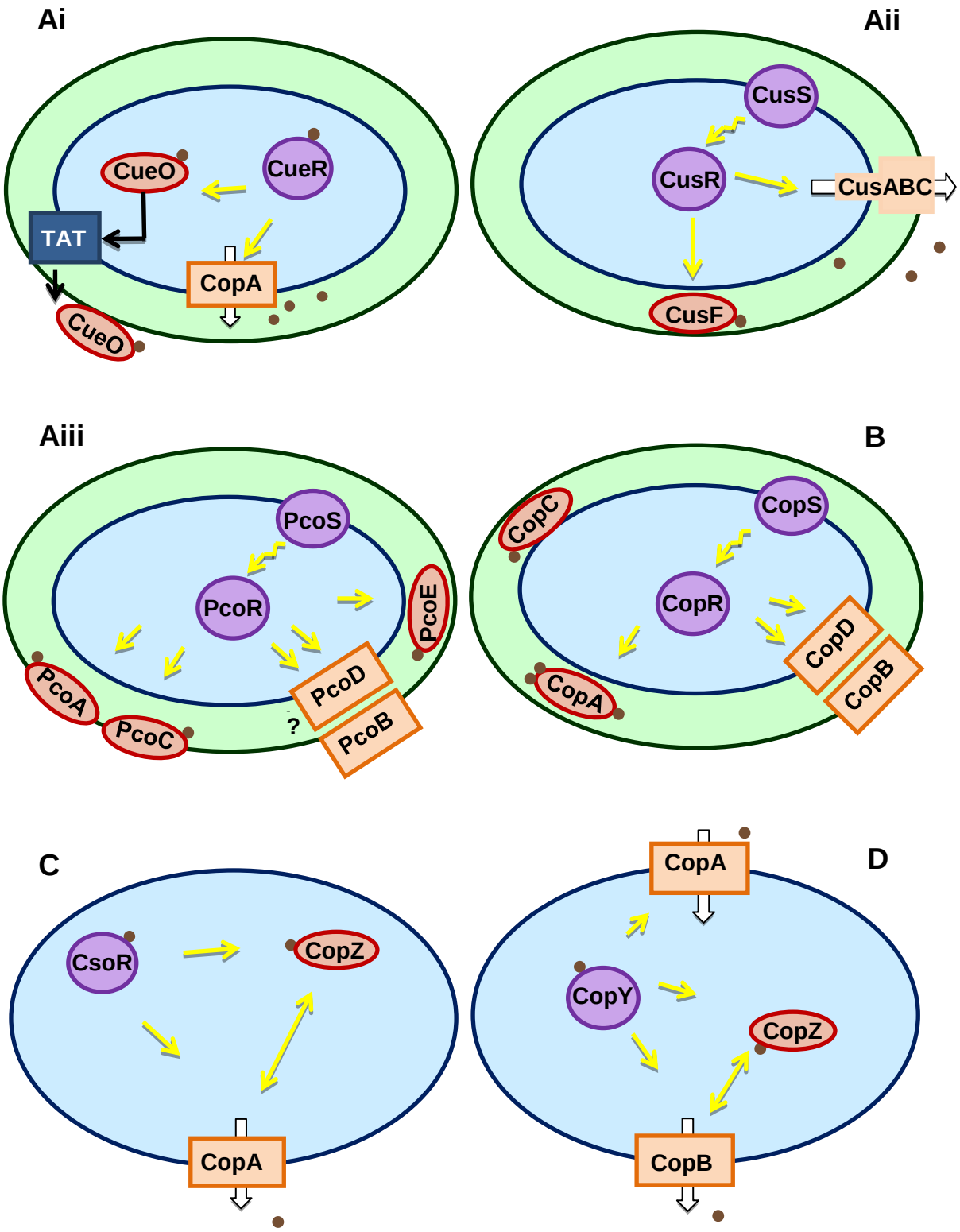

Figure 1-1 Copper homeostasis systems adapted from Osman (2) and Silver & Ji (3). Ai – *E. coli* CueR system Aii – *E. coli* Cus system Aiii – *E. coli* Pco system B – *P. Syringae* system C – *B. Subtilis* system D – *E. hirae* system

Mechanisms for Excess Copper

Bacteria commonly use energy-dependent efflux systems to reduce copper abundance in the cell. These can be either chemiosmotic cation antiporters, such as CusA, which is proposed to form a complex with CusC and CusB (all part of the *E. coli Cus* operon see Figure 1-1 Aii) to transport Cu^{+} from the cytosol across the cell membrane to outside the cell (2), or more commonly ATPase pumps, such as CopA and CopB both found in a wide range of bacteria. Reduction of Cu^{+} to the less toxic Cu^{2+} form, possibly by glutathione (GSH) in the cytoplasm (111), and extracellular sequestration of copper are also used. Regardless of the mechanism, copper itself controls all systems transcriptionally. In general, copper resistance (*cop*) operons are comprised of genes encoding a four polypeptide complex (an inner membrane protein, an outer membrane protein and two periplasmic copper binding proteins) utilising a combination on all the mechanisms mentioned above (71).

ATPases

ATPases, as previously mentioned, couple metal ion transport to the hydrolysis of ATP (2), meaning they use ATP to fuel the transport of the metal into or out of the cell. P_{1B} type ATPases such as CopA are the most common copper trafficking proteins in bacteria and can be involved in either copper export or uptake (mostly export). As a group, these proteins are responsible for the movement of many metal ions (119), and this is likely why many copper homeostasis mechanisms also function for silver (e.g., CopA and CopB). CopA, CopB and CadA (cadmium resistance) are all in the same family of P-type ATPases (120).

Chromosomal *E. hirae* copper resistance is the best understood copper homeostasis system. It has a cop operon comprised of four genes *CopY, CopZ, CopA* and *CopB* (see Figure 1-1 D). *CopA* and *CopB,* for copper uptake and efflux, are regulated in response to cellular copper availability (71).

In *E. coli*, CopA removes Cu^{+} from the cytoplasm by pumping it out of the cell. CopA is an inner membrane protein, functional in both aerobic and anaerobic conditions (119) and is under the control of CueR (see Figure 1-1 Ai).

Multi Copper Oxidases

Multicopper oxidases are part of a family of proteins that catalyse the oxidation of metal ions and other substrates. They transfer liberated electrons to copper clusters (the amount of copper varies from protein to protein; CueO in is a four-copper oxidase, while PcoA is a two-copper oxidase) and are inactive under anaerobic conditions (2).

CueO is regulated by CueR and is found in *E. coli,* where it is proposed to move to the periplasm via the TAT pathway for prefolded proteins. Here it is thought to convert Cu^{+} to the less toxic Cu^{2+} in the periplasm. It is not known whether it binds the copper ions before or after moving from the cytoplasm. CueO in addition to its role in copper homeostasis, protects alkaline phosphatase from Cu induced damage in the periplasm (111).

CueO related proteins are involved in Cu resistance in other bacteria, e.g. copA in *P. syringae* plasmid encoded *cop* operon and CuiD from *S. typhimurium* (2).

Chaperones

Some bacteria have chaperone proteins to help shuttle copper in the cytosol to proteins. For example, *B. Subtilis* and *E. hirae* both have chaperone proteins called CopZ, that are homologues to the eukaryotic chaperone protein AtxI. *B. subtilis* CopZ works with CopA (see Figure 1-1 C), while *E. hirae* CopZ, interacts with its Cu exporting protein CopB (121) (see Figure 1-1 D).

Plasmid Encoded Systems

Most metal resistance systems are found on plasmids. Plasmid systems exist for many metals such as silver (Ag), meta-arsenite (AsO_2^-), arsenate (AsO_4^{2-}), mercury (Hg), Cu and Zn (71) and have been found in most bacteria.

Pseudomonas spp*., Xanthomonas* spp*.* and *E. coli* all possess plasmid-based copper systems that contain similar genes and proteins. *P. syringae* and *E. coli* systems, while homologues of each other, are annotated differently. *P. syringae* have two regulatory genes *copRS* and four structural genes *copABCD* (as originally discovered on plasmid pPT23D (111)) , while *E. coli's* genes are called *pcoRS* and *pcoABCD* (pco which is an abbreviation of Plasmid-borne copper resistance, originally found on plasmid pRJ1004 (110)). While gene clusters similar to *copABCD* have been found in various other bacteria, in many cases only homologues to *copA* and *copB* are found. This may indicate that *copCD* is only required for more extreme copper conditions, as they are not as widely distributed. As mentioned previously, the *Xanthomonas* system is similar to that found in *P. syringae* and *E. coli*; however, its system is controlled by *CopL* instead of *copRS* (111).

While *Pseudomonas, Xanthomonas* and *E. coli* may have similar plasmid-contained systems of copper resistance, they physiologically respond differently to copper. *Pseudomonas* colonies turn blue in media containing high levels of copper (likely due to CopA and CopC

providing resistance to the copper as well as the colour of the colonies), while *Xanthomonas* and *E. coli* colonies turn brown and show no sign of periplasmic copper storage (71). This suggests the systems are less similar than believed.

In *P. syringae*, CopD is an inner membrane protein, CopB an outer membrane protein, and proteins CopA and CopC are both periplasmic "blue" proteins (see Figure 1-1B) - CopA has 2 and CopC 1 Cu^{2+} accounting for the colour. CopA is able to bind 11 Cu^{+} atoms while CopC is only able to bind 1 (122). Storage of copper in the periplasm protects the cell from damage, but exactly how CopB and CopD move Cu across membranes is not understood. CopC may play a role in copper uptake.

For *E. coli*, the Pco operon (see Figure 1-1 Aiii) is controlled by a two component regulatory system, PcoRS, but the mechanism of action for the rest of the operon is largely still unknown. PcoR is a transcriptional activator that binds DNA, while PcoS is a periplasmic histidine kinase that can sense copper. However, in the absence of PcoRS, the system can still be induced by CusRS which also controls the CusFBA copper homeostasis operon (111) (see Figure 1-1 Aii). PcoA is a multicopper oxidase (like the chromosomally encoded CueO), which is thought to be exported to the periplasm along with PcoC (2). When bacteria possess both PcoA and PcoB, they are able to tolerate higher levels of copper, than when they only have PcoA. This suggests that PcoA and PcoB work together, but as the function of PcoB has yet to be determined, we cannot be sure how. PcoC is a periplasmic protein capable of binding both forms of copper, while PcoD is a cytoplasmic membrane protein. While PcoE has its own promoter separate to the rest of the Pco system, it is still needed by the cell for full copper resistance. PcoE binds copper and when it is expressed by itself (i.e. without the rest of the Pco system) copper accumulates in the periplasm, meaning PcoE is likely a periplasmic copper chaperone (111).

Not all *E. coli* possess the *Pco* system (123). A strain, which did not possess a plasmid, was grown in 0.025 and 0.05 µg ml^{-1} of copper and displayed an impaired respiratory chain as a result of the copper exposure. In contrast, another strain which possessed a plasmid, was able to tolerate 20mM of copper with its respiratory chain remaining unaffected. Plasmid-positive strains grew on agar with five times higher concentrations of copper than strains lacking a plasmid (123). This indicates the chromosomal *Cop* (Figure 1-1 Ai) and *Cus* (Figure 1-1 Aii) systems sufficiently provide copper homeostasis at ordinary copper concentration, and the *Pco* (Figure 1-1 Aiii) system is only needed in extreme situations of copper stress.

How Does Copper Resistance Relate to Antibiotic Resistance?

Many metal resistance systems are contained on plasmids, some of which also carry antibiotic resistance genes (120). As a result of this metals may act as a selection pressure for the maintenance of antibiotic resistance through co-resistance mechanisms.

Metal and antibiotic resistance can be linked in other ways. Cross resistance, were one resistance gene conferring resistance to both a metal and an antibiotic (e.g., a non-specific efflux pump), contributes to resistance, but limited evidence and examples exist. *CopY* (the *E. hirae* transcription repressor) shares structural similarities with *blaI* and *mecI*, which are also repressors in the winged helix family of proteins and mediate resistance to β-lactam antibiotics. *CopY, blaI* and *mecI* bind to similar sequences within the promoters of their target genes (2); however, the question remains whether CopY could activate β-lactam resistance operon, or *vice versa*. In addition, it has been suggested CopA (copper efflux ATPase) is under the control of other regulators such as CpxR (2), which responds to cell-envelope stress and can be induced by copper (111). Additionally, CueR (from *E. coli*) has binding sites other than CopA and CueO. In total, there are 129 CueR box-like sites in *E. coli's* genome. While 74 of these sites are associated with genes of known function, the rest of the sites are in unknown genes (111). Copper (as CueR is copper responsive) could potentially activate a variety of genes.

Copper generates free radicals and may stimulate an SOS response, which can lead to DNA mutations and potentially novel resistance traits. While this has been corroborated by *in vitro* studies, an *in vivo* study found copper had no effect on the frequency of mutations in *E. coli* (124). In fact copper may actually protect against both peroxide (H_2O_2) mediated mutations and cell death, as well as against iron mediated-mutations (iron catalyse most DNA damage in *E. coli* (124)). The mechanisms by which this protection occurs, are not clear and may warrant further investigation.

The relationship between metals and antibiotic resistance needs further investigation, in more detail than simply looking at patterns of resistance in polluted environmental sites; however, several obstacles are currently inhibiting the process.

Problems When Investigating Copper Resistance

Genes affected by Menke's or Wilson's disease (human diseases associated with copper homeostasis) encode proteins (ATPases) more similar to bacterial CadA than other eukaryotic ATPases. Therefore, these bacterial copper transport proteins, which are homologues to the human proteins of interest, are often studied by eukaryotic biochemists, not microbiologists. Possibly for this reason, many genes are annotated poorly or incorrectly, making it difficult to compare proteins. For example, CopA is the most common bacterial copper resistance gene however, this refers to many types of proteins. In *Pseudomonas* spp., CopA results in periplasmic sequestration of Cu^{2+} (71). CopA in *E. hirae* imports copper into the cell; however for many bacteria (e.g., *E. coli* and *B. subtilis*), CopA refers to an export ATPase and CopB is used for import of copper. While both of these are chromosomal encoded proteins, for *Pseudomonas*, CopA refers to a plasmid encoded periplasmic protein. Due to inconsistent labelling of genes, bioinformatic analyses, and/or PCR-primer design targeting a wide range of bacteria becomes difficult, as it is difficult to keep track of the various nomenclatures and database accessions.

Besides the issues surrounding the genotypic methods (e.g., confusion over nomenclature), there are problems related phenotypic monitoring. Traditional phenotypic methods can be used, but unfortunately there are no defined standards or metrics for determining susceptibility and resistance to metals (125). This complicates the comparisons of resistance levels between studies and determining resistance levels.

In addition, copper is capable of chelating and binding with many substances. This complicates experiments, since there is uncertainty of its bioavailability, but it also makes comparing copper concentrations almost impossible (111), as copper concentrations can represent anything from the dissolved fraction only, to any copper bound in a matrix.

Overall, while the systems and overall mechanisms of copper homeostasis and resistance for many bacteria are generally known, there is still much to be discovered. Many proteins have only been assigned a predicted function; however, how they interact with each other needs to be better determined. Finally, a better system of gene annotation is needed.

To investigate how copper resistance links to antibiotic resistance, more consistent methodologies are needed. Looking at patterns of resistance in various different settings provides limited information, as it is difficult to compare results between studies. Research

needs to further focus on the specifics of how metal and antibiotics resistance may link to each other, especially if this information is to be used to tackle the problem of antibiotic resistance.

1.3.2 Tetracycline

Background

Tetracyclines, a class of antibiotics, were first discovered in the 1940s and introduced for clinical use in 1949 (13). Their mechanism of action is to inhibit translation (protein synthesis) (11) by preventing the binding of aminoacyl-tRNA to the A-site of the 30S ribosome subunit (126). Tetracyclines are bacteriostatic antibiotics, meaning they inhibit growth of bacteria rather than "killing" them (bacteriocidal) (126); as such, their effect is reversible (9). Tetracycline has been used to treat a range of both Gram-positive and Gram-negative bacterial infections, as well as protozoan parasites (127). As they are inexpensive with limited side effects (126), and they are among the most commonly used antibiotics.

Oxytetracycline has been commonly used as an animal feed additive (AFA), as it helped to promote weight gain in livestock and aquaculture (126). It has been the inappropriate and extensive utilisation of tetracyclines for clinical and veterinary purposes that has likely contributed to the selection and dissemination of tetracycline resistance mechanisms (9).

The first tetracyclines to be discovered in 1948, oxytetracycline and chlortetracycline, are natural products of the *Streptomyces* bacteria *S. rimosus* and *S. aureofaciens* (9). Tetracycline (an antibiotic within the class of tetracyclines) was discovered in 1953 and is produced by *S. rimosus*, *S. aureofaciens* and *S. viridofaciens* (9). While there have been several other natural tetracyclines discovered since then, many within the class are semi-synthetic; meaning natural tetracyclines have been chemically modified to improve their properties—for example, increased solubility to facilitate administration to patients or increase oral adsorption (9).

Tetracycline antibiotics are classified based on the period of discovery: first generation (1948-1963), second generation (1965-1972) and third generation (post 1972) (9). The basic structure for tetracyclines is conserved; all feature a tetracyclic nucleus, with different functional groups attached (126).

Tetracyclines are also strong chelating compounds (9), meaning they are able to bind metal ions. In fact, their pharmacokinetic and antimicrobial properties are impacted by this ability (9). In order for tetracyclines to exert their bacteriostatic effect on bacteria, they must first enter the cell. Tetracyclines gain entry to Gram-negative bacteria via porin channels OmpF and OmpC as charged cationic complexes, likely formed between tetracylines and magnesium (9). The cation-tetracycline molecules accumulate in the periplasm where they dissociate. The tetracycline then passes into the cytoplasm by diffusion through the inner membrane (9). The uncharged lipophilic form of tetracycline that diffuses from the periplasm in Gram-negative bacteria is believed to be the same form that enters Gram-positive bacteria via the cytoplasmic membrane (9).

When finally in the cytoplasm, tetracylines become re-chelated (again likely by magnesium) as the pH and concentration of divalent metal ions are higher than outside the cell (9). Tetracyclines then bind the ribosome, inhibiting protein synthesis and subsequent bacterial growth. Tetracyclines are able to induce secondary effects as a result of preventing the binding of aminoacyl-tRNA, such as amino acid metabolism or rRNA synthesis (126). Atypical tetracyclines may disrupt the bacterial membrane (128).

Resistance Mechanisms

To evade the action of tetracycline, bacteria have evolved several mechanisms of resistance – tetracycline efflux from the cell, ribosomal protection to prevent tetracycline binding or modification of the tetracycline rendering it ineffective (126).

The natural producers of tetracyclines (i.e., *Streptomyces*) harbour resistance genes to protect themselves from the antibiotics effects (23). Since the introduction of antibiotics, these genes have disseminated throughout the wider bacterial population (9) and are now found in many pathogens. It has been shown that the exposure to sub-therapeutic levels of antibiotics, such as those used in animal husbandry, has increased the transfer and presence of resistance genes (129). Resistance has also evolved as a result of successful mutations (26).

While resistance mechanisms to tetracycline fall into three categories, the resistance genes themselves are significantly more diverse. A website at the University of Washington collates and standardises the nomenclature of resistance genes to tetracycline (*tet*) and

oxytetracycline (*otr*) (127). As of 2012, there were 43 individual resistance genes (130): 27 encoded energy-dependent efflux pumps, 11-12 encoded ribosomal protection proteins, three genes were associated with tetracycline-inactivating enzymes, and one gene had (as of yet) an undetermined mode of action (130).

Gene nomenclature uses the abbreviation *tet (*or *otr*) followed by either a letter or number from the roman alphabet (9). Genes that share ≥79% amino acid sequence homology are considered to be in the same class and are, therefore, designated the same letter/number (9). While there is no differences between tetracycline and oxytetracycline resistance genes, oxytetracycline resistance genes were first identified in oxytetracycline producing bacteria – thus the genes were annotated *otr*. *Tet* genes are found in tetracycline-producing *Streptomyces* spp., while *otr* genes are also found in non-producing *Mycobacterium* spp. (9).

Genes were originally associated with either Gram-negative or Gram-positive bacteria (based on their G+C content), but it is now known that genes can be found on mobile elements such as transposons, and as such, are now found in both Gram-negative and Gram-positive species (9).

Efflux Pumps

Efflux pumps are part of the major facilitator superfamily (MFS) of proteins and have similar structures to efflux pumps which confer resistance to other antibiotics and quaternary ammonium compounds. They are generally membrane-bound and extrude tetracyline from the cell by working against the natural concentration gradient and exchanging the tetracycline for a proton (9). Gene determinants *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(H) all encode for Gram-negative efflux proteins (130) that are normally found on plasmids. These genes are carried in tandem with a gene that encodes a repressor protein. When tetracycline is not present in the cell, the repressor blocks transcription of the efflux protein (9). Approximately 1nM of tetracycline is required to activate transcription of efflux genes. Tetracycline binds to the repressor protein, causing it to move off the DNA allowing transcription of the efflux gene to occur (130).

Currently no known repressors have been found for the Gram-positive efflux genes *tet*(K) or *tet*(L), but it is possible they are regulated by translational attenuation. A putative leader peptide has been found upstream of the plasmid bound *tet*(L) gene. The leader peptide has

two ribosome binding sites (RBS), and when there is no tetracycline present, the ribosome binds the first RBS. However, when tetracycline enters the cell, a stem loop in the peptide uncovers the second RBS allowing translation of the efflux protein (9).

Ribosomal Protection Proteins

Ribosomal protection proteins are found in the cytoplasm and protect the ribosomes from the action of tetracycline. They are able to confer resistance to more tetracylines than efflux pumps, which tend to be more substrate specific (9). The ribosomal protection proteins have homology to elongation factors (128) such as EF-TU (tetracycline inhibits the protein synthesis step catalysed by EF-TU) (131).

In the presence of the *tet*(M) or *tet*(O) protein, tetracycline is removed from the ribosomes (128), but this only occurs when GTP is present (9, 128). It is assumed the other ribosomal protection proteins operate in a similar manner, as they all share amino acid sequence homology (9). The expression of both *tet*(M) and *tet*(O) proteins appears to be regulated by a 400-bp region found upstream of the coding region, however, the exact mechanism of this is yet to be elucidated (9).

Enzymatic Inactivation of Tetracycline

The *tet*(X) gene, which confers resistance through enzymatic modification of the tetracycline, is thought to be of little clinical relevance. Oxygen is essential for the enzyme to function, and the gene has only been identified in anaerobic strains, suggesting that the gene is unlikely functional in its original host (9).

Tetracycline Resistance Associated with Metals and Other Antimicrobials

Tetracycline resistance genes are often associated with mobile genetic elements such as plasmids and transposons, which frequently also carry other antibiotic resistance genes as well as those which confer metal resistance (127). A full list of tetracycline resistance genes and associated gene is available in Roberts, et al. 2012 (130), as well as on the *tet* gene website at University of Washington.

tet(A) has been linked to both the gene bla_{TEM} (which confers β lactam resistance) and the *mer* operon, which confers mercury resistance (130). *tet*(B) is also associated with bla_{TEM}, *tet*(M) and the transposon *Tn*10 (130). *Tet*(M) is associated with *tet*(B) and *erm*(B), an erythromycin resistance determinant quantified in Chapter 3. *Tet*(M) is also associated with

the *mer* operon and uniquely the transposon *Tn*917 and the *Tn*916-*Tn*1545 transposon family (130).

Tetracycline resistance genes are diverse and widely distributed throughout the bacterial population. They are frequently associated with mobile genetic elements such as plasmids and transposons and, as such, are often transferred with other resistance genes. While tetracycline has thus far only been commonly linked with mercury resistance, other associations are possible.

1.3.3 Ampicillin

Background

Ampicillin is one of the penicillins in the β lactam group of antibiotics. Penicillin was discovered in 1929 by Alexander Fleming (132) and introduced for clinical use in 1940 (13). Penicillin and its semisynthetic derivatives were therefore the first β-lactam antibiotics; however, the group now also includes cephalosporins, carbapenems and monobactams (133). β lactams are the most commonly used antibiotics in the world, accounting for 65% of usage (133), and are used to treat Gram-negative and Gram-positive infections.

Penicillins have a conserved structure of 6-aminopenicillanic acid (which contains a β-lactam ring), but each penicillin has a specific acyl side chain (134). Penicillin is produced by the fungi *Penicillium chrysogenum*, and as previously mentioned, many other penicillins are chemical derivatives of penicillin, with replacement side chains to improve their properties, such as an increased spectrum of bacteria or increased resistance to enzymatic attack by bacterial resistance mechanisms (134).

β lactams inhibit enzymes that are essential for cell wall synthesis, which is essential for maintaining cell shape and stability, particularly during osmotic stress (135). The cell wall is composed of peptidoglycan, which is comprised of *N*-acetyl-muramic acid (NAM) and *N*-acetylglucosamine (NAG) subunits that are synthesised inside the cell. They are fused together as alternating units outside the cytoplasmic membrane by transpeptidases (membrane bound enzymes) (136).

Penicillin binding proteins (PBPs) catalyse the joining of two NAM units, which each have a pentapeptide attached (135). The β lactam ring has a similar structure to the D-alanine-D-

alanine found in the NAM pentapeptide, meaning PBPs may try to incorporate a β lactam into the cell wall during synthesis instead of the correct NAM unit (135). The PBP becomes acylated after binding the penicillin and is, therefore, unable to catalyse further reactions. This contributes to the disruption of the cell wall synthesis process (135), meaning the peptidoglycan becomes weakly cross linked (136). This eventually leads to cell lysis by induced osmotic fragility (137), although the exact mechanisms by which penicillin confers its bactericidal effect are still being elucidated (133, 135). β lactams are more effective against Gram-positive bacteria due to their structural differences; however, newer β lactams are also able to target Gram-negative organisms.

Much like the mechanism of action, exactly how β lactams enter the cell is not clearly understood, but for Gram-negative bacteria they are thought to pass through the outer membrane via porins (133, 138, 139).

Resistance Mechanisms

Bacterial resistance to β lactams falls into four main categories: enzymatic degradation of the antibiotic, modification of the drug target site, reduced permeability of the cell and extrusion of the antibiotic from the cell by efflux pumps (135). In general, Gram-negative bacteria produce β lactamases, which inactivate β lactam antibiotics, while Gram positives modify their penicillin binding proteins (PBPs), preventing β lactams from binding to their target (139). Gram negatives carry both PBPs and β lactamases in their periplasm, while Gram positives have PBPs on the outer surface of their cytoplasmic membrane with β lactamases bound here also or excreted extracellularly (139).

β lactamases

The first β-lactamase enzyme was discovered in 1940 (15), before penicillin was introduced for clinical use. β-lactamase enzymes are structurally similar to the PBPs involved in cell wall synthesis and may have evolved from them (133). They confer resistance by hydrolysing the β-lactam ring and disrupting its amide bond (133). Serine β lactamases are categorised by molecular class (133), but two different systems of classification exist: the Ambler system, which uses classes A to D, and the Bush-Jacoby-Medeiros system, which involves groups 1 to 4 (135). The Ambler system will be used within the context of this chapter, as it groups the enzymes based on their protein structure (140).

All serine β lactamases utilize a similar mechanism of action to hydrolyse the β lactam ring. β lactams are electrostatically attracted to β lactamases (139). Once the β lactam ring is non-covalently bound to the serine β lactamase, the β lactam ring is disrupted and becomes covalently acylated to a serine in the active site. Hydrolysis of the acyl enzyme then causes the release of the inactivated β lactam and the enzyme becomes reactivated, ready to accept a new substrate (133). Modified or semi-synthetic β lactams are able to prevent interaction with the β lactamase active site due to the possession of a bulky side chain. This can prevent the β lactam ring from interacting with the serine in the active site, or displace the water molecule preventing hydrolysis (133). Newer β lactams are able to overcome these avoidance techniques and still render the drugs inactive.

Serine β lactamases are found both in the chromosome and on plasmids (139); however, class C lactamases have only been carried on plasmids since the beginning of the antibiotic era, while class A and class D have been plasmid bound for considerably longer (millions of years) (139).

Class A β lactamases are all serine pencillinases, and the various enzymes within this group are able to confer resistance to penicillins-narrow and extended spectrum cephalosporins and carbapenems (135). The class includes the enzymes TEM-1, SHV-1 and CTX-M-15 (140), which are discussed in further detail below. Class B β lactamases are metallo- β lactamases, which are able to confer resistance to many β lactams (including carbapenems) (135) . Class C are cephalosporinases, which confer resistance to cephalosporins (135). Class D β lactamases are all oxacillinases, which are able to confer resistance to penicillins and cloxacillin (135); enzymes in this group include various OXA genes (140), which are also discussed in more detail below.

The structure of class A β lactamases is similar to that of PBPs. There are three groups of class A β lactamases: historical Gram-negative plasmid-associated penicillinases (TEM/SHV), *Pseudomonas aeruginosa* cephalosporins (PER/OXA/TOHO), and CTX-M carbapenemase subclasses (139). Class C β lactamases hydrolyse β lactam ring structures using a similar mechanism to class A β lactamases; however, they differ in the deacylation step, as both classes use opposite faces of the acyl-enzyme when introducing the water molecule for hydrolysis of the β lactam ring (139). Class D β lactamases were originally named oxacillinases due to their ability to hydrolyse the side chain of oxacillin type antibiotics. Class D β lactamases require carbon dioxide to activate hydrolysis. They have horizontally

transferred from *P. aeruginosa* and are increasingly found in other Gram-negative pathogens (139). Class B metallo- β lactamases are metal dependent and normally involve divalent zinc. Discovered in 1967, they are able to hydrolyse a broad spectrum of β lactam antibiotics; however, their mechanism of action differs to that utilised by serine β lactamases as there is no enzyme intermediate involved (139).

The regulation of β lactamase resistance has not yet been fully elucidated, however it is known to involve a repressor - BlaI and a receptor – BlaR, both of which are induced by β lactams (136). The repressor BlaI is immediately upstream of BlaR and BlaP (the β lactamase gene). BlaI blocks expression of all three genes in the *bla* divergon (139). The receptor BlaR is a trans-membrane protein, which is similar to class D β-lactamases (139). BlaR is comprised of a sensor domain, which lies outside the cell, and when it becomes acylated by a β lactam, signals (via its transmembrane domain) to the intracellular metalloprotease domain (136). How the signal is transferred is still unknown (139). The metalloprotease domain cleaves the repressor BlaI which resides in the in the cytosol as a dimer. The BlaI dimer binds an operator, but once BlaI has been cleaved, it prevents the operator from binding to DNA, allowing the *bla* resistance gene of interest to be expressed (136).

Penicillin Binding Proteins

Alteration of the drug target by modification of penicillin binding proteins (PBP), results in resistance to β lactams. This is due to decreased affinity between the β lactam and the PBP, rendering the β lactam less effective (141).

Reduced Permeability and Efflux from the Cell

Outer membrane proteins (OMPS), or “porins” as they are also known, are nonspecific solute channels in the outer membrane that allow movement into and out of the cell. They are essential for serine β lactam entry to the periplasm (133); therefore, reducing their expression or altering them in some way, reduces permeability of the cell to β lactams (133). Resistance to β lactams can be conferred by deletion or reduced expression of OMPs, but this resistance mechanism is usually expressed in conjunction with expression of a β lactamase such as AmpC (a cephalosporinase) (133, 139). Porin deletion has a corresponding fitness cost and is usually accompanied by changes in the outer membrane and peptidoglycan layer (139). Transporters, which are regulated (unlike porins), also allow

movement out of and into the cell. Bacteria, which are multi-drug resistant, often utilise transporters; however, the exact role in resistance remains unclear (139).

Efflux proteins extrude substrates from the cell. While there are five classes of bacterial efflux pumps, the resistance-nodulation-division (RND) class are the most significant in terms of antimicrobial resistance. RND proteins are widely distributed in the chromosome of Gram negative bacteria (133). While both efflux and reduced permeability confer resistance to β lactams, they are not as important or widely studied as β lactamases or PBPs.

β Lactam Resistance Associated with Metals and Other Antimicrobials

Penicillin resistance has been associated with mercury resistance since the 1960s. Mercury was used in many hospital disinfectants, and as a result, exposure to mercury was found to induce penicillinase production and resistance to bacitracin (76), it was eventually determined genes conferring resistance to all were being carried on the same plasmid (77).

Coagulase positive bacteria displayed an association of resistance between penicillin and copper (73). Copper can increase the rate of hydrolysis of benzylpenicillin 100 million fold; however, it binds penicillins less tightly than cephalosporins (142). Therefore while copper may enhance for penicillin resistance, it also aids its enzymatic degradation.

As previously discussed, many β lactam resistance genes are associated with plasmids or other mobile genetic elements such as transposons (143). As such, they are often transferred with other resistance genes. This means other antimicrobials or heavy metals could be selecting for β lactam resistance. As the most important class of antibiotics, it is imperative that specific linkages to other selection pressures are elucidated to develop strategies to increase their longevity.

Both ampicillin and tetracycline resistance genes have been linked to other types of resistance and could potentially be influenced by copper, however this needs to be investigated further using an experimental approach to determine the effect (if any) copper has.

1.4 How Wastewater Treatment May Influence Antibiotic Resistance[2]

Introduction

Wastewater treatment (WWT) acts as the interface between the clinical and environmental settings; and has been implicated in in increasing levels of antibiotic resistance. A literature based investigation into the potential role of WWT in increasing or maintaining antibiotic resistance is presented here, with experimental work presented in a subsequent chapter (Chapter 5).

As previously discussed, antibiotics have been used since the 1940s to treat bacterial infections, both clinically as well as in the veterinary setting, as well as used for growth promotion in agriculture (86, 144). The non-therapeutic use of antibiotics and exposures to sub-inhibitory concentrations create opportunities for pathogenic and commensal bacteria to develop AR, thus decreasing the effectiveness of drug therapy for both clinical and food-supply scenarios. Additionally, evidence has become apparent that antibiotic resistance genes (ARG) increase due to anthropogenic activity in both the clinical setting (e.g., 145) and the environment (e.g., 146). The accumulation of resistance traits creates an enriched gene pool from which other resistant organisms may develop, including multi-resistant pathogens.

In many cases, antibiotic-exposed higher organisms enrich for ARG-containing bacteria, and the resistant bacteria enter the waste stream via faecal and related matter from bacterial hosts. However, waste streams also contain discharged antimicrobial compounds in partially or un-metabolised forms. As such, they often remain biochemically active, albeit at sub-inhibitory concentrations, which can further enhance the presence of resistance traits. Unfortunately current sewage treatment technologies have not been designed to treat ARG and biologically-active pharmaceutical compounds. Therefore, water and sediments downstream of urban activity often have elevated ARG and antibiotic concentrations (78, 147-149).

The link between the roles our individual and societal actions, and the environment, in the propagation and maintenance of AR needs to be better discerned. While research into this

[2] An expanded version of this section has been submitted to Frontiers in Antimicrobials, Resistance and Chemotherapy as “Antimicrobial resistance and the wastewater-treatment process” by Seánín M McCluskey and Charles W Knapp. It is currently in “interactive review”.

topic has recently exploded, how the environment links to the clinical setting and how resistance genes transfer between bacteria remain key questions to address. Wastewater treatment plants (WWTP) act as the interface between the clinical setting, municipal pollution, and the environment. While many researchers have profiled the inflows and outflows, exactly what happens within them, in terms of resistance development and dissemination, remains relatively unclear. WWTP stabilize waste materials and reduce overall bacterial load discharged to receiving waters, but evidence suggests that resistance rates (the ratio of resistant bacteria to total bacteria) may be amplified (26, 87, 147, 150).

Factors Affecting Antimicrobial Resistance

Baquero and Canto (57) refer to wastewater and its biological components as one of four genetic reactors in the development of antibiotic resistance; the other three include human and animal microflora, hospitals and farms, and soil and surface water. WWTP receive waste from humans, hospitals, industries and, in some cases, agriculture. A series of physical, biological and chemical processes treat the wastewater before being discharged into the environment as effluent. Bio-solids (sludge) represent another form of WWTP discharge (57, 151). Without adequate treatment, sewage can dramatically impact surface waters, sediments and soils. As such, WWTP are of key importance in AR and links the outcomes of clinical treatment, consumer behaviour and industrial discharge to the environment.

Many centralised wastewater treatment plants receive wastes from a wide range of sources – hospitals, domestic, storm-water runoff, and industries. As such, the character of the waste differs among municipalities and over time. The site-dependent variations include number of people, industries and hospitals. Seasonal variations occur and reflect pharmaceutical usage (for example, during influenza epidemics). If an industry dominates a service area and pulse-release its waste, the discharge could affect content, even on a daily basis. Constituents of wastewater that contribute to AR include antibiotic residue, metals, bacteria and co-factors that elicit genetic dissemination and exchange. Basically, the AR can be gained by the input of resistant bacteria (resistance gained at the original source), selective growth of resistant bacteria by environmental pressures, or the horizontal genetic transfer of resistance traits. Many factors contribute to the AR problem, and they exist in

the wastewater system; it represents an agglomeration of different bacteria (and their genes) and toxicological stressors.

Antibiotics

Annual consumption of antibiotics has been reported at over 10,000 metric tonnes in the EU, with about 50-50% for human- and veterinary use (152). Similar usage rates have been reported in the USA (e.g., 25,000 tonnes; with 40% to agriculture and 60% for humans) (153). Between 40-90% of antibiotic concentrations pass through the body unaltered (154) and enter the WWTP system. Various antibiotics have been detected in wastewater influent and effluent (Table 1-3). Values vary widely for each plant, due to differences in contributing sources (hospitals or industry) and pharmaceutical use.

Concentrations and distribution of antibiotics depend on consumer behaviour and prescription rates. WWTP influents contain pharmaceutical residues, but it remains difficult to determine if they have originated from hospital or households. Pauwels and Verstraete (152) report that only 26% of antibiotics for human consumption originate in hospitals; while most antibiotics come from out-patient, prescription drug use. Minimal differences have been found in resistant bacteria abundances between hospital effluent and WWTP influent (155, 156). Hospitals, however, contribute for the presence of more multidrug resistant (MDR) strains (156).

Table 1-3 Range of antibiotic concentrations detected in WWTP influent and effluent

Antibiotic	Conc. WWTP Influent ngL^{-1}	Conc. WWTP Effluent ngL^{-1}	References
Cephalexin	670-5600	<2-1800	(157, 158)
Amoxicillin	≤280-6940	<3-50	(157, 159)
Cloxacillin	≤1-320	<1-<9	(157)
Penicillin G	<2-10	<2-300	(157, 159)
Penicillin V	20-13800	≤80-200	(157, 159)
Sulfamethoxazole	0.6-7910	0.31-964	(157, 159, 160)
Trimethroprim	1.1-4300	10-1340	(157, 159, 160)
Doxycycline	<5-2480	<5-915	(157, 159, 160)
Tetracycline	<15-1300	<15-620	(157-160)
Ciprofloxacin	<38-5876	<6-742	(157, 159, 160)
Ofloxacin	22.5-5560	10-991	(157)
Erythromycin	<20-1987	<20-2054	(157)
Clarithromycin	59-1433	12-444	(157)
Clindamycin	20-60	5-70	(159)
Lincomycin	20-500	3-300	(159)

Table 1-3 Concentration ranges are based on those found at various WWTP in different countries. As detection limit varies with method and machine used as well as the fact antibiotics are known to “stick” to organic material making them difficult to quantify, values are not absolute.

There is a difficulty in the chemical extraction and quantification of many antibiotics from the wastewater and sludge matrices. This complicates the ability to accurately determine concentrations and bio-activity. Some antibiotics, such as quinolones, sulphonamides and tetracyclines, tend to adsorb to biosolids (155), thus rendering them more difficult to detect. Additionally, antibiotics can degrade in the treatment stream, further complicating its detection and exposure risk. For example, β lactams are not detected at high levels despite being the most heavily used class of antibiotics (155). The compounds' instability is attributed to the cleavage of the β lactam ring under many environmental conditions.

On the other hand, the partitioning of antibiotics onto solids facilitates the removal of bioactive compounds from the aqueous phase. This dramatically affects effluent quality, but it does potentially concentrate the compounds into the sludge. For example, tetracyclines enter WWTP bound to particles found in sludge, but remain below detection limits in effluent (160). It remains unclear whether the adsorption of antibiotics affects resistance within the WWTP; as once adsorbed onto particles and organic matrices, bioactivity often declines.

Reported concentrations of antibiotics tend to be sub-inhibitory—i.e., will be below typical minimum inhibitory concentrations (MIC) for most bacteria (161). Sub-inhibitory concentrations of antibiotics can still affect AR in bacteria. Some recent evidence suggests that low-level concentrations contribute significantly to the propagation of antibiotic resistance. For example, work by Bernier & Surette (129), showed low-level concentrations of an antibiotic, allow bacteria to become tolerant to that antibiotic and select for resistance genes without actually inhibiting the entire population (129). Kümmerer (155) stated exposure to sub-therapeutic concentrations of antibiotics for long periods of time favours the transfer of resistance genes and increase the speed by which resistance strains become selected. It also increases the emergence and transfer of new combinations of resistance in areas of high bacterial density such as WWTP sludge (155). As antibiotics normally enter WWTP under sub-therapeutic doses (1000 times lower than that needed to inhibit growth of resistant bacteria (162)), they could still be impacting the transfer and maintenance of resistance phenotypes. Sub-inhibitory concentrations of antibiotics act as intracellular signalling molecules (163), promoting gene transfer (164). In addition, several different antibiotics have similar mechanisms. Individual antibiotic abundances may be low,

but overall concentrations of similar antibiotics may be synergistically high, creating additional selective pressure.

Metals

Metals are ubiquitously present in the environment, and at trace concentrations, they provide essential nutrition. At elevated concentrations, they can have toxic effects. Little information exists on the concentration ranges that actually enter the plant, additionally concentrations are likely to be highly variable between plants. However, reported values of metal content in sludge do provide an indication of what has been through the treatment process (165) (Table 1-4).

Metals have an impact on the development of AR, but it remains unclear at which levels. Analytical guidelines are not defined for determining metal susceptibility in bacteria. Probably the biggest difficulty in determining the extent of metals in their role in AR development involves assessing its bioavailability (7). Metal bioavailability depends on factors that dramatically change in wastewater, including concentration and speciation of metals, pH, redox potential, temperature, total organic content (both particulate and dissolved fractions), and velocity and volume of water. The same concentration of metal can have different effects, depending on its availability to the cell. Therefore, one cannot easily infer whether metal concentration levels inhibit bacteria. However, we can hypothesise the various ways that the presence of metals in dense bacterial populations could impact AR.

Metals confer a series of acute and chronic stress responses in bacterial communities along a concentration gradient (166, 167). Assays such as oxygen uptake (168, 169), nitrification inhibition (170) and monitoring of suspended solids have been employed to measure wastewater-related effects. People have studied metal-related effects on bacterial surfaces and the resulting complex formation in the extracellular polymeric substances (170-172). Very few studies have directly examined metal-related effects on wastewater bacteria (7, 64). However, effects have been examined on other systems such as contaminated freshwater systems (5, 95, 173-176), marine and estuary environments (96, 149, 177), contaminated sediment (78), soils (6, 8, 66, 97, 178, 179), and drinking water (180), which provides ample evidence that similar processes could occur in wastewater. It, however, remains unclear whether selective pressures, either via co- or cross-selection (60) (discussed later) or other biological cause an increase of AR.

Table 1-4 Range of metal concentrations detected in sewage sludge

Metal	Conc. Sewage Sludge mg/kg	References
Cadmium (Cd)	2.16-13	(165, 181-183)
Copper (Cu)	131.2-1391.42	(165, 181-183)
Chromium (Cr)	45.8-1120.42	(165, 181-183)
Iron (Fe)	3300-16794	(165, 181, 183)
Manganese (Mn)	77.89-1100	(165, 181, 183)
Nickel (Ni)	23.2-291.53	(165, 181-183)
Zinc (Zn)	515.40-4500	(165, 181-183)
Lead (Pb)	57.5-330	(165, 182, 183)

Table 1-4 Range values are based on those reported from various plants WWTP in various different countries. All are based on results for dewatered sludge.

Certain metals, such as copper and iron, cause the formation of reactive oxygen species, which have been shown to in turn induce the SOS response in bacteria (discussed in detail later). These free radicals are formed during the Fenton reaction (Fe^{2+} oxidised by H_2O_2 to yield $OH^{\bullet}$) or Fenton-like reactions. Hydroxyl radicals (OH•) oxidise proteins, lipids, and DNA (184). While metals have not been shown to induce the SOS response directly, they could possibly increase antibiotic resistance via stress-induced mutations (185).

Metals may also increase the transfer of antibiotic resistance genes in the environment in similar manner that antibiotics, and other stressors, do. Metals could be linked to the dissemination of AR by activating the SOS response and increasing horizontal gene transfer (HGT) of antibiotic resistance genes. For example, integrative conjugative elements (ICEs) related to SXT, an ICE from *Vibrio cholerae* which carries resistance genes for antibiotics

and metals, was transferred to other bacteria at an increased rate as a result of an activated SOS response (164).

Metals provide selective pressures similarly to antibiotics. They select for antibiotic-resistance genes by co-selection or cross-selection processes, or generate resistance via the SOS stress-response processes. One of the challenges with metals is their continuous presence in the environment. Unlike organic antibiotics, metals do not degrade and can maintain selective pressure for longer periods of time.

Bacteria

Many mechanisms exist, by which AR becomes enhanced biologically in a wastewater treatment systems. As mentioned previously, the presence of elevated AR levels could have resulted from A) the influx of pre-selected resistant bacteria; B) physical and chemical conditions within the wastewater treatment selecting populations with resistance traits for survival and subsequent growth; and C) the development of *de novo* resistance traits in the bacterial community.

Bacteria entering the WWTP originate from many different sources. Pathogens from hospitals (152), commensal and enteric bacteria from domestic sewage, and environmental bacteria (186) are present in WWTP influent. Bacteria may have been pre-exposed to antibiotics and other antimicrobial agents, and as a result, they may harbour resistance genes, either innately (e.g. *Streptomyces* having resistance genes to the antibiotics they produce) or via acquired genetic transfer (e.g., plasmids).

Whichever the acquisition method, WWTP creates an environment with dense communities of bacteria. Wastewater systems represent a collection of bacteria from many different sources and environmental conditions, with a wide range of genetic diversity, which are placed in a confined bioreactor/process. Thus the frequency of interaction between bacteria increases, and the processes and the chemical composition of the WWT contents subsequently stress the bacteria, and ultimately trigger additional biological processes as highlighted below.

Horizontal Gene Transfer

One of the biggest challenges in understanding AR is the fact that certain bacteria are capable of exchange genetic material in a process called horizontal gene transfer (HGT) (187). DNA can be transferred between bacterial cells in HGT by three main mechanisms, transformation, transduction and conjugation (28) (discussed above).

Multiple resistance genes can be carried on the same mobile genetic element, which could include genes for multiple antibiotics, metals, and other antimicrobials. Bacteria can share theses multiple resistance genes by horizontal and vertical (to daughter cells) transfer mechanisms, and it requires only a single stressor to be present to selectively maintain the gene cassette in a population. There are essentially two selection mechanisms to maintain these genetic elements via different stressors. Cross-selection mechanisms (60), for example, involves the same gene conferring resistance to multiple compounds (such as an efflux pump, see Table 1-3). The other mechanism is co-selection, which is were different resistance genes are transferred due to their shared presence on a single genetic element (see Table 1-3). Many antibiotic- and metal-resistance genes (for example) are often integrated near each other on a mobile genetic element. In both cases, a metal, for example, can help maintain and confer the resistance for an antibiotic.

Table 1-5 Common bacterial resistance mechanisms against metals and antibiotics

General Resistance Mechanism	Metal	Antibiotic	Linked Resistance	References
Reduction in Permeability	As, Cu, Zn, Mn, Co, Ag	Cip, Tet, Chlor, β lactams	Cu and , β lactams/Tet [1]	(8, 178)
Antibiotic/Metal Alteration	As, Hg	Chlor, β lactams	Hg and β lactams[2]	(8, 60, 119, 120, 178, 188)
Antibiotic/Metal Efflux	Cu, Co, Zn, Cd, Ni, As, Ag	Tet, Chlor, β lactams	Co and Tet[3], Cd/Zn/Co and Imp[4]	(119, 120, 178, 188)
Alteration of Cellular Target	Hg, Zn, Cu	Cip, β lactams, Trim, Rif, Tet	Hg and tet[5]	(120, 188)
Antibiotic/Metal Sequestration	Zn, Cd, Cu	CouA	-	(119, 120, 178, 188)

Table 1-5 (Adapted from Baker- Austin et al. (60)) 1 -Cu concentrations correlated to abundance of genes blaOXA (β lactam resistance), tetM, tetW (both Tet resistance) (8, 178) 2- Hg and β lactam resistance phenotype often observed together (72, 77) 3 - tetL (Tet resistance gene) can export both Tet and Co (60) 4 - Czc operon (export of Cd, Zn and Co) and Mex operon (export of Impinem a β lactam) are transcriptionally linked (60) 5 - Hg and tetM are linked (130). Abbreviations – As, arsenic; Ag, silver; Hg, mercury; Chlor, chloramphenicol; Cip, ciprofloxacin; CouA, coumermycin A; Rif, rifampicin; Tet, tetracycline; Trim, trimethoprim; Imp, Imipenem.

Mosaic Proteins

One result of HGT is the generation of mosaic proteins by combining genes from different bacteria, i.e., the proteins are composed of domains from different original proteins (189). Mosaic tetracycline resistance genes are commonly found in pig and human faecal samples

and have arisen by the recombination of different wild-type genes (190). Additionally, Barile *et al*. (191) describe a novel tetracycline resistance gene *tet*(S/M) found in *Streptococcus bovis* (foodborne bacteria of enteric origin) which, along with *tet*(S/M) genes described by Novais *et al*. (192), seems to have been formed by transposon-mediated recombination. The *tet*(S/M) protein described by Barile et al. is comprised of the N-terminal region from *tet*(S) fused with *tet*(M) coding sequence. The *tet*(S/M) gene confers resistance to tetracycline and is under the control of the *tet*(S) promoter. A region near the *tet*(S/M) shared extensive homology with a multidrug resistance plasmid (191), possibly indicating where the insertion of a genetic element into the genome occurred, generating the mosaic protein. Wastewater treatment plants could allow for greater bacterial and genetic interactions, facilitating generation of mosaic proteins. Ultimately, mosaic proteins could increase antibiotic resistance phenotypes and provide a source of genetic diversity, i.e. novel resistance genes within the population, releasing them to the wider environment upon discharge.

Bacteriophages

Bacteriophages (phages) are extremely common and abundant, and sewage contains a wider range and higher abundance of phages, than found in soil (144). Phages contribute in the evolution of bacterial populations (144) by transferring DNA between bacterial cells (transduction). As previously mentioned transduction is a mechanism of HGT, however unlike transformation and conjugation, transduction does not require cell to cell contact, and protects DNA from degradation, thus it is not dependant on DNA quality (144). A phage infects a bacterial cell, incorporates its DNA into the host chromosome and either becomes dormant, or uses the cell to replicate. Phages can either be broad range or species specific; and can be involved in the transduction of either plasmid or chromosomal genes (28). When the phage is exiting the host genome it may take with it DNA that flanked either end of its insertion site. If this DNA contains resistance genes, these genes are then incorporated into the genome of the next host the phage infects. As the site the phage incorporates is non-specific, it can insert in the middle of a gene, leading to the genetic evolution of resistance genes (see mosaic proteins).

SOS Response

When DNA damage occurs, bacterial cells will induce SOS-response mechanisms to mediate DNA repair (184). The SOS response up-regulates various genes, some of which encode proteins for DNA repair (193) and low-fidelity polymerases, which tend to be error prone with an increased frequency of base mis-pairings. Therefore the SOS response may lead to genomic mutations.

In addition to DNA repair, antibiotic-induced SOS response may increase the transfer of mobilising ICEs. For example, when induced, the response system removes the repressor for SXT-transfer activators, thus enabling SXT transfer and allowing for increased frequency of resistance gene transfer (164).

The SOS response basically increases mutation frequency and genetic change, in an attempt to shift the genetic composition and possibly improve population survivability (194). For example, stress caused by carbon starvation induced mutations that led to a β-lactam resistance phenotype (193); while ciprofloxacin induced a mutation pathway in *E. coli* similar to lactose starvation (36). Alternately, preventing the induction of the SOS response stops *E. coli* from evolving resistance to ciprofloxacin or rifampicin (195).

Mutation

Interactions between the chromosome and certain antibacterial agents, or antibiotic-induced oxidative stress can lead to mutations being generated. While mutations can lead to the development of AR, rates vary with drug concentrations (26). While no work has specifically examined mutation rates within the WWTP system, some principles can be related to previous work with (for example) *Pseudomonas aeruginosa*. Many bacteria within the WWTP (as well as those within sludge treatment process, etc.) form biofilms, and mutation rates in biofilms have been shown to be considerably higher than mutation rates in planktonic cell populations (196-198). Conibear, et al. (197) found a 100-fold increase in mutation frequencies within biofilm populations in comparison to planktonic; additionally Driffield et al. (198) discovered a 105 fold increase in mutation frequency for resistance determinants within biofilm cells after being exposed to rifampicin and ciprofloxacin. It should be noted, however, that some work contradicts this, e.g., García-Castillo (199) found higher mutation frequencies within planktonic populations.

Detergents

AR has been recently linked to a number of biocides, including QAC (quaternary ammonium compounds) (59, 200). Although levels of QAC entering WWTP are normally below its IC_{50} (concentration required to inhibit 50% of bacteria (155)), they can be found at active levels in activated sludge (57, 201). As with antibiotics, sub-inhibitory concentrations can still maintain and select for resistance.

Triclosan, another biocide, is a popular antibacterial compound in the USA, commonly found in soaps and other hygiene products. The biocide has been detected in both WWTP influent and effluent. Triclosan at high concentrations damages microbial cell membranes and disrupts protein and lipid synthesis. At lower concentrations, cross-/co- resistance has been observed between triclosan tolerance and resistance to antibiotics such as tetracycline and β lactams (202).

1.4.1 What Happens During the Wastewater Treatment Process?

The classic, municipal wastewater treatment design is fairly consistent. Preliminary and primary treatments involve the physical and chemical removal of large solids; secondary treatment utilises biological processes to remove remaining organic matter; and finally tertiary treatment further treats the effluent and may include disinfection. However, contemporary treatment strategies are continuously emerging, which may deviate in process design. Additionally, specific treatment strategies may vary according to discharge regulations, plant size, economy, and special design considerations (57, 151), which adds to the number of possible processes. Given the diversity of treatment processes, a wide range of conditions within the wastewater, and the seemingly unpredictable nature of biological systems, it remains difficult to fully understand the processes involved in the development and dissemination of AR.

The main strategy of the wastewater treatment involves the retention of wastes and allows high levels of bacteria to lower BOD (biochemical oxygen demand) levels, which represents the amount of oxygen required to decompose the organic matter. BOD represents the "strength" of the wastewater; as the amount of BOD depends on the concentration and type of organic carbon and reduced nitrogenous compounds present. Other services that WWTP provide include reduction of solids, risk of water-borne diseases, and nutrient levels to prevent eutrophication downstream. Additional treatments may be included as needed,

for example disinfection (151, 203). The wastewater treatment process is tailored to the needs/legal requirements for individual receiving waters.

Preliminary and Primary Treatment

Preliminary treatment involves the physical removal of debris and larger solid items. This is done using a series of bar screens and a period of reduced velocity for the settling of sand, grit and other items that may pose a risk to equipment downstream. Not too many factors in this process influence AR, other than the harvesting of some bacteria, metals and organic compounds that may contribution to sludge quality. Material removed, including debris and bacteria, are either landfilled, incinerated, or disposed as part of sludge processing (discussed later).

This is followed by primary treatment which involves larger, quiescent basins to allow for removal of suspended solids; the process can be enhanced physically or chemically with coagulants (203).The aqueous portion proceeds on to secondary treatment, and solids go to sludge treatment. Similar contributions towards AR are expected as preliminary treatment (mentioned above).

Secondary Treatment

Secondary treatment processes reduce the concentration of any remaining organic matter (as BOD), and it represents the biological aspect of the wastewater treatment process. Secondary treatment must 1) oxidise dissolved and particulate biodegradable constituents; 2) capture and incorporate suspended and non-settleable colloidal solids into a biological floc or biofilm; 3) transform or remove nutrients such as nitrogen and phosphorus; and 4) remove specific trace compounds (e.g., organics or inorganics). Two common approaches include activated sludge (162) or biological filtration. Basically, both processes convert the dissolved or suspended organic matter that has passed through primary treatment into biomass, which is subsequently removed from the system as sludge.

Activated sludge utilises aggregates of bacteria, or flocs, under aerobic conditions to remove the organic matter. In this process, basins detain wastewater long enough for bacteria to oxidise organic matter and ammonia; aeration, via air injection or mechanical mixing, suspends the microorganisms in the water as well as maintaining oxygen levels. The

process maintains a food/microorganism (F/M) ratio to have sufficient levels of active bacteria to rapidly consume large quantities of food (BOD). The wastewater ultimately enters a clarifier where bio-flocs settle into the sludge. A portion of the sludge is returned to the aeration basin to maintain biomass levels. The process ultimately removes about 90-98% of the bacteria.

Attached, or fixed, growth systems utilise a bed of biofilm-covered substrates. Wastewater trickles over the substrate bed and the bacteria breakdown the organic matter (151). Splashing water or airflow through the pore space of the media maintains aerobic conditions. The biofilm accumulate and eventually slough off into the treated effluent and becomes part of the sludge. Like the activated sludge process, this type of secondary treatment also involves a clarifier or sedimentation tank to separate the biofilm aggregates in the effluent.

Secondary treatment relies on high densities of bacteria. The process maintains cells at a balance of starvation and elevated metabolic activity, which create optimal acquisition and assimilation rates. The bacteria compete for nutrients, while at the same time they are exposed to stressors and toxicants, which become conducive for HGT and/or SOS responses. Additionally biofilms and their organic matrix act as a sink for heavy metals and lipophilic organic compounds. As mentioned previously, low concentrations of metals, antibiotics, and certain toxicants are sufficient to stimulate resistance gene expression.

The treatment system creates a natural advantage for certain bacteria having resistance traits and quite possibly selects in favour of resistant phenotypes. Resistance traits in bacteria could improve survivability in the variable environment of the waste stream (e.g., tolerance to heavy metals, antibiotic loads, etc.).

Tertiary Treatment and Disinfection

Tertiary treatment is often employed for additional nutrient removal to prevent eutrophication of receiving waters. This can be in the form of further filter beds, either sand or gravel based, or more natural systems such as wetlands and ponds (151). In general, they are effective means to improve wastewater quality, including further bacterial removal.

Depending on discharge requirements, disinfection of the effluent may also take place after tertiary treatment (151), and a variety of processes such as UV exposure, chlorination/dechlorination or ozone may be used.

Increased relative levels of resistant bacteria have been observed after chlorination. Literature has provided a litany of evidence in drinking water treatment systems, but the effects could translate to sewage systems as well. Chlorine provides oxidative stress to cells ultimately leading to damage to proteins, cell-wall permeability, hydrolysis and mechanical disruption of cells. Chlorination of sewage ultimately has the potential to act as a selection pressure (87, 180) increasing resistance to some drugs, but reducing it for others (26) and may even promote resistance spread (86).

UV radiation photochemically damages the DNA and RNA within the cells, which effectively inactivates the cells by damaging the genetic information necessary for replication and biochemical operation. Exposure to UV radiation has been shown to denature resistance genes (89-91); however, in these systems, there were no selective removal systems – all DNA was affected. Further, Auerbach et al. (204) stated UV neither reduced tetracycline resistance diversity nor abundance.

There is a mixed opinion whether disinfection affects AR. Non-viable bacteria, with their genes intact, still have the potential for genetic exchange in the environment, especially by release and incorporation of extracellular DNA via transformation processes. Several naturally competent bacteria, such as *Bacillus* and *Pseudomonas* spp., could assimilate DNA (187). Treatment strategy should perhaps focus more on gene inactivation rather than just reduction of viable bacteria.

Sludge Disposal and Treatment

The accumulation of cells and other particulates from various stages of the treatment process are combined as sludge for treatment and disposal. Possible sludge treatment processes include anaerobic digestion, aerobic digestion, and/or the sludge is simply dried out to make cakes which are then incinerated (205, 206). If digested, the residual materials (biosolids and/or digestate) are often spread on land or landfilled. Probably the most contentious disposal route, in terms of AR, is land application.

The safety of land application of biosolids (sludge) has long been debated. Possible risks include cross-contamination of food or reduced quality standards; however, there is economic value as a source of nutrients when spread on fields as a fertilizer. The problem arises from the fact that sewage sludge often contains levels of heavy metals and persistent organic pollutants. The application of sludge has resulted in accumulation of metal in soil (167). It has been found that elevated metals resulting from land application can maintain elevated AR potential (178); the other risk factor is the genetic material itself. When sewage is spread on fields, bacteria and their associated genetic information is also applied. Plasmids in soil can survive and potentially be transferred to other bacteria (144, 207, 208). Despite some evidence supporting this theory, the issue has remained rather inconclusive.

While guidelines do exist for the application of sewage sludge (referred to as biosolids after it has been treated), they only consider "pathogens" or specifically *Salmonella*, if the field is to be used for growth of fruit and vegetables. In some cases, a time delay can be observed between application and when the land can be used for agriculture (205). Unfortunately, there is not much information about graze land risks and AR.

1.4.2 Wastewater Treatment Plant Effluent

WWTP reduce bacterial and organic loads, often resulting in between 95% to >99% reduction in comparison to levels found in influent. According to Czekalski et al. (150), a load reduction of 73% according to plate counts, but 42% according to 16S-rRNA qPCR, was observed in the effluent; while Ohlsen et al. (209) found WWTP decreased *E. coli* abundance by 99.5%, while still discharging approximately 100 CFU *E. coli* per ml after treatment. Despite the wide range of values, there are likely to be discrepancies between cell presences, viability and culturability in the methodologies.

In terms of resistant bacteria, Guardabassi et al. (162) found 10-10000 times less resistance in treated sewage than raw influent, while Wiethan et al. (210) found no difference in removal between resistance and non-resistant bacteria. Although there is a reduction of overall bacterial load, the proportion of resistant bacteria to total bacteria tends to be higher in effluent than influent (147). For example, the percentage of resistant coliforms increased from faecal content to urban wastewater to potable waters (80%) (26). Czekalski et al. (150) found increased tetracycline resistance and *sul* (sulphanilamide resistance)

genes (per 16S-rRNA) leaving WWTP than had entered. Other studies have found similar increases in resistance factors (87).

The variety of opinion regarding whether WWTP select for resistance or not, could be attributed to treatment affecting bacteria and their gene content differently. Auerbach et al. (204) detected *tet*(B), *tet*(D) and *tet*(Q) resistance genes in the influent and effluent of WWTP, but not in sludge or biosolids within the plant. They proposed that a dilution effect was the cause, i.e. there are greater abundances of bacteria in sludge, so the resistance genes may have been less noticeable. They also found a greater diversity of tetracycline resistance diversity in WWTP than in surface water.

When the WWTP discharges to receiving waters, diversity and abundances of ARG are found higher in surface waters downstream of treatment plants (147, 148). Heavy-metal resistance has been found to be higher in sediment located near sewage disposal locations (149); for example, ampicillin and mercury resistance was six times higher at sewage disposal sites than control sites in the New York Bight (78) . It remains clear that WWTP reduce the levels of bacteria; however, the relative levels of released resistant bacteria are higher than what would occur naturally at the point of discharge.

The risk of antibiotic resistance spreading has become evident. In a few studies, antibiotic resistant bacteria have also been suggested to move from rivers and end up in food, drinking water and bathing water (85). Resistance genes can transfer from WWTP effluent to intrinsic bacteria in receiving waters, and ultimately into drinking water. *Tet*(W) and *tet*(O) specifically have been detected in drinking water and recycled wastewater (148), but various other AR genes have been found in drinking water and recycled wastewater, as well as surface water and sediments.

While the issue of drinking water may be less of a concern in the developed world, recreation water is still an issue (211-213). Antibiotics that leave WWTP can end up in soil, sediment, or ground water as antibiotics are not fully removed by WWTP (155). WWTP effluent and sewage sludge application to fields acts as a source of antibiotics in surface water (204). They can therefore act as a selection pressure for resistance within receiving waters and further downstream affecting gene transfer (148). As previously discussed, sub-inhibitory concentrations of antibiotics could impact on the transfer and maintenance on AR.

The transfer of resistance genes from WWTP effluent to the environment should be a priority concern. The environment is a reservoir of genes and medium of spread. When enteric bacteria (in effluent) enter the environment, they become dormant and unculturable. However, even if they are being starved they can still express and transfer plasmids. As previously mentioned, WWTP does not treat for resistance genes themselves so any “free” DNA released in the effluent can be taken up by the indigenous bacterial population (28); transduction, transformation and conjugation can all occur in aquatic environments. While plasmids are destroyed in sea water, some remain viable long enough for transformation (28). Pote et al. (214) found plasmid DNA did not completely degrade or have a reduced transformation ability until after ten hours in a saturated soil system. While this may not be the same timeframe in receiving waters, it indicates plasmids can persist long enough for uptake by other bacteria.

We need to know more about the processes that occur within the WWTP. Reducing the total load of bacteria may not be sufficient; we may need to modify the process to specifically target the removal of resistant microorganisms and their genes. Standards relating to effluent release into receiving waters, and sludge application to fields may also need to be reviewed and practices in these areas refined to mitigate potential risks. Sewage treatment is clearly having an impact on the maintenance of AR and may allow increased resistance gene transfer and spread. Further work is needed to determine specifically what and how this may be occurring.

1.5 Conclusion

Antibiotic resistance is an issue of clinical concern. While the overuse or abuse of antibiotics has exacerbated the problem, and likely sped up the dissemination of resistance genes, antibiotic resistance is intrinsic to bacteria and other selection pressures such as heavy metals may have contributed to the maintenance of the environmental resistance gene reservoir. Steps to limit the resistance problem by reducing the use of antibiotics have had limited effect. It is, therefore, necessary to examine other contributing factors such as heavy metals, in order to identify new targets and develop more effective strategies to deal with this problem.

Antibiotic resistant bacteria are found in drinking water (180) and wastewater treatment plants (42, 67), which receives domestic and hospital waste, as well as agricultural runoff. This may select for antibiotic resistance and enrich for antibiotic resistance bacteria in the environment. Rethinking current practices in treatment of water, wastewater or both; may prove effective in reducing the resistance gene load in the environment and subsequently impact the clinical resistance problem.

Heavy metals may also contribute significantly to the maintenance of antibiotic resistance genes. Investigations into the link between metals and antibiotic resistance need to become more focused and attempt to identify associations between specific metals and antibiotics. The cycling of resistance genes from the clinical setting to the environment, and *vice versa*, needs to be fully understood if we are to break the chain and reduce the impact the environment has on the resistance problem.

1.6 Research Aims and Objectives

As discussed, heavy metals could potentially be selecting for, or maintaining antibiotic resistance in the environment, therefore the aim of this thesis is to quantitatively investigate the relationship between metals and antibiotic resistance. To do so, a combination of literature and laboratory based investigations, which are outlined below, have been utilised.

- Determine whether background metal concentrations in soil affect antibiotic resistance levels in the environment – Chapter 3.
- Based on specific correlations between metals and antibiotic resistance genes found in natural conditions, (Chapter 3), identify potential mechanisms that could link the resistance types based on literature evidence – Chapter 1, Section 1.3.
- Carry out a literature based review on the potential of wastewater treatment in selecting/maintaining antibiotic resistance, as wastewater treatment acts as the interface between the clinical setting and the environment – Chapter 1, Section 1.4.
- Examine the effect increased metal levels, e.g. industrial pollution scenario, have on antibiotic resistance levels. Experimentally determine whether causation can be

attributed to metal exposure, rather than being a statistical relationship. This is done both in soil (Chapter 4) and aquatic systems (Chapter 5).

- Determine whether metals maintain antibiotic resistance levels in environmental systems, and provide pressure to possibly promote antibiotic-resistance gene retention – Chapter 6.

The research question this thesis aims to address is whether heavy metals in the environment can select for or maintain antibiotic resistance. Steps taken thus far to stem the resistance problem by limiting the use of antibiotics have had minimal success. As such, the environment could potentially be acting as a resistance gene reservoir, negating the impact such practices are able to exhort. Overall a better understanding of the interactions between heavy metals and antibiotic resistance in the environment, will allow for better predictions of epidemiological risk and development of strategies for mitigation and prevention of antibiotic resistance in the environment.

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Chapter 2: Materials and Methods

2.1 Introduction

This chapter contains the general materials and methods utilised for experimental chapters within this thesis. Specific methodologies and experimental design are described in detail within the relevant chapters. Experimental outlines and concepts are described below so that subsequent sections of this chapter are placed in context.

Chapter 3: preliminary experiment which correlated background/basal heavy metal levels in soil collected from various locations in Scotland, to abundances of antibiotic resistance genes.

Chapter 4: soil microcosm experiment which compared antibiotic resistance gene levels between soil microcosms amended with various concentrations of copper.

Chapter 5: wastewater treatment activated sludge bioreactor experiment which investigated differences in antibiotic resistance gene retention between bioreactors amended with copper and/or antibiotics.

Chapter 6: antibiotic resistance gene fate experiment which investigated whether the conditions of surface water receiving sewage discharge; in terms of copper and antibiotic content; influence antibiotic resistance gene retention.

2.2 Soil Classification

In order to determine soil type and composition, soil was classified using particle size distribution (dry sieve analysis) according to established methods (1).

Soil was air dried for several days, weighed, and then passed through a series of sieves that contained mesh with decreasing pore diameter. The soil retained on each sieve was weighed and the fraction of the total clay, silt or sand, was determined using the particle size ranges listed in Table 2-1 (2). These values, along with a soil texture triangle (Figure 2-1 (2)), were used to determine soil type according to the established standard method (3).

Table 2-1 Particle size classification table (2).

Particle Class	Particle Size (mm)
Clay	<0.002
Silt	0.002-0.06
Sand	0.06-2

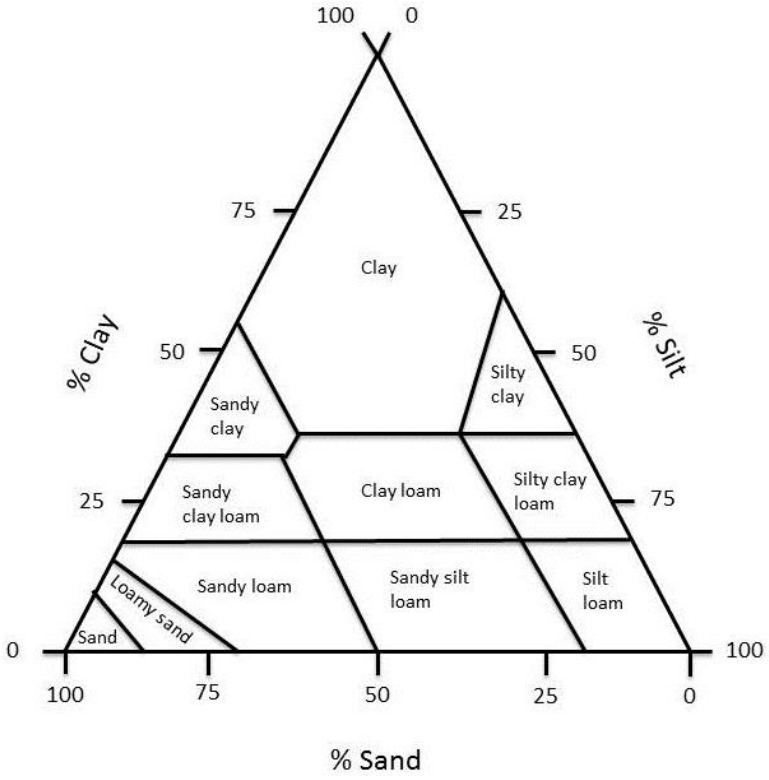

Figure 2-1 Soil texture triangle , adapted from (2).

2.3 Synthetic Wastewater

Synthetic wastewater media was used to feed the wastewater system. This was chosen for chemical consistency, as real wastewater treatment plant influent is extremely variable. Additionally, it reduced the health and safety risk associated with pathogens in untreated waste water.

2.3.1 Synthetic Wastewater Media

The 100x Synthetic Wastewater Media Stock Solution was based on the methods of Knapp and Graham (4). The stock solution was made by adding the components listed in Table 2-2 to 1L of distilled water. The stock solution was autoclaved and then stored at 4°C.

1000X trace element solution was used based on the methods of Knapp & Graham (4). The trace element solution was made by adding the components listed in Table 2-2 to 1L of distilled water. The solution was then sterile filtered and stored in the dark at room temperature.

The final 1x Synthetic wastewater media was produced by adding 10ml of 100x synthetic wastewater media stock solution to 989ml of distilled water. The solution was autoclaved, and following this, 1ml of 1000X trace element solution was added aseptically along with $0.5gL^{-1}$ sodium bicarbonate.

Table 2-2 Synthetic wastewater preparation

Reagent	**Component**	**gL^{-1}**
Synthetic Wastewater Media 100X Stock Solution	Peptone	32
	Lab Lemco Powder (Oxoid)	19
	Yeast Extract	3.0
	Urea	3.0
	$(NH_4)_2SO_4$	6.7
	K_2HPO_4	1.4
	KH_2PO_4	1.1
	$CaCl_2.6H_2O$	0.4
	$MgSO_4.7H_2O$	0.2
1000X Trace Element Solution	$FeCl_3.6H_20$	0.75
	H_3BO_3	0.075
	$CuSO_4.5H_20$	0.015
	KI	0.09
	$MnCl_2.4H_20$	0.06
	$NaMoO_4.6H_20$	0.03
	$ZnSO_4.7H_20$	0.06
	$CoCl_2.6H_20$	0.075
	EDTA	0.5
	Conc. HCl	1ml

2.3.2 Antibiotics and Copper Stock Solutions for Synthetic Wastewater Media Amendment

Activated sludge bioreactors were fed with synthetic wastewater. Individual reactors had amendments – ampicillin, tetracycline, or copper - added to supplement their feed and challenge bacterial populations.

Amendments were made as 1000x stock solutions by adding the appropriate mass (listed in Table 2-3) per 1ml of sterile water (for ampicillin and copper) or 70% ethanol (for tetracycline), then sterile filtering. Stock solutions were stored at -20°C in 1.5ml aliquots. Aliquots were added to 1.5L reactors to give working concentrations listed in Table 2-3.

Table 2-3 Synthetic Wastewater Media Amendment Stock Solutions

Amendment	Stock Concentration	Working concentration
Ampicillin[a]	200mg/ml	0.2mg/L
Tetracycline[b]	200mg/ml	0.2mg/L
Copper[c]	3000mg/ml	3mg/L

[a]Ampicillin 96.0-100.5% (Sigma Aldrich), [b]tetracycline >98.0% (Sigma Aldrich), [c]copper (II) sulphate >99.90% (Sigma Aldrich)

2.4 Sample Collection for Experimental Set Up and DNA Extraction

2.4.1 Inocula for Microcosm Experiments

The inoculum for the soil microcosm experiment in Chapter 4 was a low-clay content soil provided by Raeburn Drilling. The soil sample was collected as a cross sectional core from an uncontaminated site in Montrose, Scotland. Due to confidentiality contracts with Raeburn Drilling, exact information regarding the sample site is not available. The sample was coned and quartered to give a composite sample, then placed in a double layer plastic bag that was then sealed to maintain the moisture content. The soil sample was stored at 4° in the dark prior to addition to the soil microcosms.

Scottish Water kindly provided the activated sludge sample that was added to the bioreactors in Chapter 5. The bioreactors, in turn, provided the inoculum for the gene fate experiment in Chapter 6. The sludge was collected from Shieldhall Wastewater Treatment Plant, which receives domestic waste from much of Glasgow. The sample was collected

using a fill and withdraw method, with a bucket on a rope. The sludge was transferred to 1L plastic bottles and stored at -20°C short term and -80°C long term.

The water for the gene fate experiment in Chapter 6 was collected from Loch Ard, a fresh water lake in the Loch Lomond and Trossachs National Park, Scotland. The sample was collected (as above) and transferred to a 5L plastic drum for transport and temporary storage prior to addition to the microcosms.

2.4.2 Sampling for DNA Extraction

The soil samples for preliminary experiment (Chapter 3), which investigated the correlation between background geochemical conditions and antibiotic resistance gene abundances, were provided by the James Hutton Institute. Samples were collected as part of a previous study, dried, and placed in long-term storage. Soil was transferred to pre-weighed DNA extraction vials using a sterile spatula and the tubes reweighed. Between 0.2 and 0.3g of soil was used for DNA extraction.

The soil microcosm experiment (Chapter 4), which compared antibiotic resistance gene abundances between soil microcosms amended with various concentrations of copper, involved soil samples being collected periodically over a 64 week period. Soil samples were removed from soil columns at specific time points by moving along the column at 10cm intervals (as discussed in Chapter 4) using a sterile spatula and transferred to sterile 1.5ml centrifuge tubes. Samples were stored at -80°C prior to DNA extraction to preserve DNA.

Bioreactor liquor was withdrawn; from the bioreactors utilised in Chapter 5; biweekly as part of routine feeding. 1ml activated sludge samples were aseptically transferred from the settled bioreactor liquor (chapter 5) to sterile 1.5ml centrifuge tubes using a pipette. The 1ml aliquot was centrifuged and 500µl of the supernatant removed, with the remaining 500µl being retained for DNA extraction and stored at -80°C.

Water samples (100ml) were collected from each microcosm for the gene fate experiment (Chapter 6) at specific time points in two 50ml sterile sample tubes. This was then vacuum filtered using sterile Büchner funnels and conical flasks. Bacterial cells were retained on cellulose nitrate filters - 0.2µm pore size and diameter 47mm (Whatman, Buckinghamshire, UK). Filters were placed directly into the Binding Matrix E tubes used for DNA extraction

and processed for DNA extraction. 50ml of the flow through was then passed through a DNA spin column (NBS Biologicals, England) filter using a vacuum manifold to collect any free DNA that was present, the DNA was then eluted from the filter using 200µl of nuclease free water. To harvest RNA at selected time points, 100ml of water was vacuum filtered as described above to harvest bacterial cells. The filters were placed in Binding Matrix E tubes (MP Biomedicals UK, England). 1ml of Qiazol (Biorad) was added and an MP Bio Fast 24 machine used to disrupt the filter. The manufacturers' instructions for using Qiazol to preserve RNA were used from this point forward.

2.5 Molecular Analysis

2.5.1 DNA Extraction

FastDNA Spin Kits for Soil (MP Biomedicals, Cambridge, UK) were used to extract DNA from all samples. Samples were aseptically added to the Binding Matrix E tubes supplied with the kit. For activated sludge samples, 500µl of concentrated sample was added, while for gene-fate experiments, filters were added to the Binding Matrix E tubes. For soil samples, the tube was weighed prior to soil addition. Soil was added to the line on the tube and tapped against the lab bench to settle the sample and remove any air pockets. Tubes were then reweighed to verify there was between 200 and 300mg of soil in each tube.

The variability in sample size was taken into account during analysis by normalising gene abundances to 16S rRNA gene values (a measure for total bacteria). A FastPrep 24 machine (MP Biomedicals, Cambridge, UK) was used to homogenise the samples with buffers in the Binding Matrix E tubes. All steps were carried out following the manufacturers' instructions. DNA was eluted in 100µl of elution buffer and stored at -20°C short term and -80°C long term.

2.5.2 Primers

All primers were ordered from Sigma Aldrich (Dorset, UK) based on the sequences provided in the references listed in Table 2-4. Primers were hydrated using nuclease free water to the volume indicated on each tube to generate a concentration of 100µM. Primers were then stored at -80°C. A working concentration of 10µM for each set of primers (this concentration was used so that 1µl of primer stock could be added per PCR/qPCR reactions)

was generated by adding 30µl each of the forward and reverse primer stocks to a centrifuge tube with 240µl nuclease free water. Primer working stocks were stored at -20°C.

Table 2-4 Primers for PCR and qPCR

Gene	Primer sequence 5'-3'		Amplicon size (bp)	Reference
16S	BAC338F	ACTCC TACGG GAGGC AG	468	(5)
	BAC805R	GACTA CCAGG GTATC TAATC C		
***tet*(M)†**	Forward	GGTTTCTCTTGGATACTTAAATCAATCR	88	(6)
	Reverse	CCAACCATAYAATCCTTGTTCRC		
***tet*(Q)†**	Forward	AGGTGCTGAACCTTGTTTGATTC	158	(7)
	Reverse	GGCCGGACGGAGGATTT		
***tet*(W)**	Forward	GCAGAGCGTGGTTCAGTCT	65	(7)
	Reverse	GACACCGTCTGCTTGATGATAAT		
***tet*(B)†**	Forward	ACACTCAGTATTCCAAGCCTTTG	205	(6)
	Reverse	GATAGACATCACTCCCTGTAATGC		
***erm*B**	Forward	AAAACTTACCCGCCATACCA	694	(8)
	Reverse	TTTGGCGTGTTTCATTGCTT		
***erm*C**	Forward	GAAATCGGCTCAGGAAAAGG	644	(8)
	Reverse	TAGCAAACCCGTATTCCACG		
***erm*E**	Forward	TGTTCGAGTGGGAGTTCGT	662	(8)
	Reverse	GGTACTTGCGCAGAAGCGA		
***erm*F**	Forward	TCGTTTTACGGGTCAGCACTT	751	(8)
	Reverse	CAACCAAAGCTGTGTCGTTT		
***tet*(A)**	Forward	GCTACATCCTGCTTGCCTTC	210	(9)
	Reverse	CATAGATCGCCGTGAAGAGG		
***tet*(B)‡**	Forward	TTGGTTAGGGGCAAGTTTTG	659	(9)
	Reverse	GTAATGGGCCAATAACACCG		
***tet*(C)**	Forward	CTTGAGAGCCTTCAACCCAG	418	(9)
	Reverse	ATGGTCGTCATCTACCTGCC		
***tet*(D)**	Forward	AAACCATTACGGCATTCTGC	787	(9)
	Reverse	GACCGGATACACCATCCATC		
***tet*(E)**	Forward	AAACCACATCCTCCATACGC	278	(9)
	Reverse	AAATAGGCCACAACCGTCAG		
***tet*(G)**	Forward	GCTCGGTGGTATCTCTGCTC	468	(9)
	Reverse	AGCAACAGAATCGGGAACAC		
***tet*(K)**	Forward	TCGATAGGAACAGCAGTA	169	(9)
	Reverse	CAGCAGATCCTACTCCTT		
***tet*(L)**	Forward	TCGTTAGCGTGCTGTCATTC	267	(9)
	Reverse	GTATCCCACCAATGTAGCCG		
***tet*(M) ‡**	Forward	GTGGACAAAGGTACAACGAG	406	(9)
	Reverse	CGGTAAAGTTCGTCACACAC		
***tet*(O)**	Forward	AACTTAGGCATTCTGGCTCAC	515	(9)
	Reverse	TCCCACTGTTCCATATCGTCA		
***tet*(S)**	Forward	CATAGACAAGCCGTTGACC	667	(9)
	Reverse	ATGTTTTTGGAACGCCAGAG		
***tet*(A)(P)**	Forward	CTTGGATTGCGGAAGAAGAG	676	(9)
	Reverse	ATATGCCCATTTAACCACGC		
***tet*(Q) ‡**	Forward	TTATACTTCCTCCGGCATCG	904	(9)
	Reverse	ATCGGTTCGAGAATGTCCAC		

***tet*(X)**	Forward	CAATAATTGGTGGTGGACCC	468	(9)
	Reverse	TTCTTACCTTGGACATCCCG		
***bla*$_{TEM-1}$**	Forward	TCGGGGAAATGTGCG	153	(8)
	Reverse	GGAATAAGGGCGACA		
***bla*$_{SHV-1}$**	Forward	TGATTTATCTGCGGGATACG	94	(8)
	Reverse	TTAGCGTTGCCAGTGCTCG		
***bla*$_{CTX-M}$**	Forward	ATGTGCAGYACCAGTAARGTKATGGC	300	(8)
	Reverse	ATCACKCGGRTCGCCNGGRAT		
***bla*$_{OXA-1}$**	OXA1B14	CACTTACAGGAAACTTGGGGTCG	79	(8)
	BlaOXA1-R	AGTGTGTTTAGAATGGTGATC		
***Cop*A**	CopAUF	GGTGCTGATCATCGCCTG	750	(10)
	CopAUR	GGGCGTCGTTGATACCGT		
DGGE 16S	primer 1	CCTACGGGAGGCAGCAG	193	(11)
	primer 2	ATTACCGCGGCTGCTGG		

†individual gene assay; ‡ multiplex gene assay

2.5.3 PCR

All PCR reactions had a total volume of 20µl and contained 3 µl of template DNA, 1 µl of working stock primers, 10 µl of Mega Mix Blue (Microzone, CamBio, Cambridge, UK), and 6 µl of nuclease free water (Qiagen, Manchester, UK). Assays were run using 96 well plates covered with film in an iCycler (BioRad, Hertfordshire, UK) according to manufacturers' instructions. Details of primer annealing temperatures are listed in Table 2-4.

Table 2-5 PCR conditions for generating qPCR Standards

No. cycles	Process	Temperature (°C)	Time (minutes: Seconds)
1	Denaturing	94	5:00
35	Denaturing	94	1:00
	Annealing	55/56[a]	1:00
	Extension	72	1:30

[a]55°C annealing temperature used for all tetracycline genes, 56°C used for *copA*. This gene also required a final extension step at 72°C for 7mins.

2.5.4 Gel Electrophoresis

1% (w/v) agarose gels were used for verification of PCR products. Molecular grade agarose was dissolved in 1x TAE buffer (from a 10x stock supplied already prepared – Sigma Aldrich,

Dorset, UK), ethidium bromide was added (final concentration of 100 μg ml^{-1}) to allow DNA visualisation later, and the gel was left to solidify. The gel was then placed in a BioRad (Hertfordshire, UK) Electrophoresis tank (used according to the manufacturer's instructions); PCR products were mixed with loading buffer (Table 2-6) and loaded onto the gel. Gels were electrophoresed at 70V for 60 – 90 minutes in 1x TAE buffer. Gels were visualised by UV transillumination using a 2UV transilluminator (UVP, Cambridge, UK) at a wavelength of 365nm for agarose gels or 302nm for polyacrylamide gels. Molecular size markers were included on each gel to verify DNA fragment size. The ladders used were λ DNA/*Hin*dIII, λ DNA/*Eco*RI +*Hin*dIII or a 100bp ladder (see **Error! Reference source not found.**); they were selected based on their suitability for the anticipated band size of the PCR product being resolved.

Table 2-6 6X Loading Buffer Composition

Component	Quantity
0.05%(w/v) Bromophenol Blue	0.05g
40% (w/v) sucrose	40g
0.1M EDTA, pH 8	20ml 0.5M EDTA
0.5% (w/v) SDS	0.5g
dH_2O	To final vol. of 100ml

2.5.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a more sensitive method of DNA separation than normal gel electrophoresis, as it can resolve base-pair differences in composition as opposed to size of fragment. An acrylamide gel (25% v/v 40% BIs/acrylamide) is cast so that it contains a chemical gradient of 30% to 60% denaturant, an electric current forces the DNA to move down the gel, and eventually DNA becomes denatured and separated into two partial strands. DGGE can detect single-base differences between two copies of the same gene, meaning it can detect mutations or single nucleotide polymorphisms (SNPs).

All equipment used for the DGGE gel preparation and running, were supplied as part of the DCode System (BioRad, Hertfordshire, UK) and used according to manufacturer's instructions.

PCR Preparation of DGGE Samples

Samples analysed by DGGE were amplified using the PCR method previously described (specific cycle conditions Table 2-7) with the DGGE 16S primers found in Table 2-4. Gel electrophoresis was used to verify the reactions.

Table 2-7 DGGE PCR Conditions

No. cycles	Process	Temperature (°C)	Time (minutes: Seconds)
1	Denaturing	95	1:00
24	Denaturing	95	0:30
	Annealing	65[a]	1:00
	Extension	72	1:00
15	Denaturing	95	0:30
	Annealing	53	1:00
	Extension	72	1:00
1	Final Extension	72	10:00

Conditions as those described by Muyzer et al. (11). [a]Temperature reduced by 1°C every 2nd cycle.

Gel Preparation

The gradient gel "sandwich" was assembled by layering petroleum jelly (Vaseline, US) and foam spacers between 2 pieces of glass (which had been cleaned and polished using ethanol) along the shorter vertical edges. After ensuring the edges were aligned, clamps were fixed to the horizontal edges and the sandwich placed in the gel casting unit. The gradient gel itself was prepared by adding 15ml of the high concentration solution (Table 2-8) and 15ml of the low concentration solution (Table 2-8) to two separate beakers. 200µl of ammonium persulfate (APS) and 20µl of tetramethylethylenediamine (TEMED) (both Table 2-8) were added to both the high and low solution beakers. The high solution mix was added to a syringe and placed in the "HIGH" side of the gel casting pump, this was repeated for the low solution mix. Tubing was connected to both syringes and a needle fixed to the other end of the tubing. The needle was placed at the top of the gel sandwich between the two panes of glass and the handle of the gel casting pump slowly turned until both syringes were empty. A comb was inserted and the gel was left to polymerise for 2 hours. In the

meantime, 7 litres of 1X TAE buffer was added to the electrophoresis tank and heated to 65°C.

When the gel had set, it was removed from the casting apparatus and the comb removed from the top of the gel. The wells were cleaned out with distilled water and the gel then fitted into the core unit. The core was then placed in the electrophoresis tank.

Table 2-8 Solutions used for DGGE gel preparation

Solution	Component	Quantity
Low Concentration Solution 30%	40% Bis/Acrylamide	50ml
	50xTAE buffer	4ml
	Formamide (deionised)	32ml
	Urea (molecular grade)	42g
	dH_2O	To final vol. of 200ml
High Concentration Solution 60%	40% Bis/Acrylamide	50ml
	50xTAE buffer	4ml
	Formamide (deionised)	56ml
	Urea (molecular grade)	67.2g
	dH_2O	To final vol. of 200ml
10% APS solution[a]	APS	1g
	Nuclease free water	10ml
50X TAE Buffer	Tris Base	242g
	Glacial Acetic Acid	57.1ml
	0.5M EDTA	100ml
	dH_2O	To final vol. of 1L

[a]0.5ml aliquots were then stored at -20°C.

DGGE Electrophoresis

The PCR samples, verified using gel electrophoresis, were mixed with 6X loading buffer (Table 2-6) and loaded into the wells of the gel using gel loading filter tips (VWR, Leicestershire, UK). Markers (DNA from a single bacterial colony) were added to the wells at the centre and ends of the gel as this would give one single band at intervals of the gel so it could be verified the gradient had been cast consistently and all samples had been subject to identical denaturing conditions and could be directly compared. The temperature control module was added to the top of the tank and a power pack connected. The gel was run at 200V, 60°C, for 4.5 hours.

Gel Visualisation

When electrophoresis was complete, the gel was removed from the tank and core. The glass plates were carefully separated and the gel removed from the glass, then placed in a tank for staining with 20µl ethidium bromide and 200ml of 1X TAE. The tank was covered in tinfoil to prevent ethidium bromide degradation and gently agitated on a shaker for 30 minutes. After this, UV transillumination was used to visualise the gel.

2.5.6 qPCR Standards Generation

qPCR involves the use of a fluorescent chelator, SYBR-Green I, to detect PCR products; the machine detects the fluorescence and counts the number of cycles it takes for fluorescence to reach a threshold level. As PCR is an exponential reaction process, the starting quantity (i.e., number of resistance genes in the sample to start with) can be determined by comparing threshold values to a set of standards (known concentrations of target gene).

The standards for each gene assay were prepared from a master stock at a concentration of 10^9 copies of the gene per µl, which was serially diluted using nuclease free water. The 10^9 master stocks were either prepared used a plasmid which carried the resistance gene, or a PCR product which had been verified using gel electrophoresis and UV transillumination (see Section 2.5.4), then purified using a QIAquick PCR purification kit (Qiagen, Manchester, UK). In the case of plasmid generated standards, the bacteria containing the plasmids were grown (according to Section 2.5.7) and the plasmids then extracted using a QIAprep Spin Miniprep Kit (Qiagen, Manchester, UK). The plasmids were then quantified using a Nanodrop2000c (Thermo Scientific, Leicester, UK) according to the manufacturer's instructions and prepared to a 10^9 concentration. All the standards for assays relating to the preliminary experiment (Chapter 3) were prepared this way (PCR product), with the exception of bla_{OXA}. All 10^9 stocks were already in the lab and did not need prepared, with the exception of bla_{OXA}, which was obtained on a plasmid (pCH26) kindly donated by Dr. M R Rodicio, University of Oviedo, Spain. The plasmid was quantified using a Nanodrop and based on its size in base pairs (6942bp) and the concentration obtained, the copy number per µl was calculated and then diluted to give a 10^9 master stock.

Additional standards required for experiments in Chapters 4, 5 and 6 were prepared using purified PCR products. PCR screening of environmental samples was carried out for each gene (using primers listed in Table 2-4). Agarose gel electrophoresis was used to verify the

PCR (product sizes for corresponding genes are listed in Table 2-4). The band was visualised using a Benchtop 2UV Transilluminator (UVP, Cambridge, UK) and excised using a scalpel, then cleaned up with a QIAquick Gel Extraction Kit (Qiagen, Manchester, UK) according to manufacturers' instructions. The excised PCR product amplified by another round of PCR, and then cleaned up using a QIAquick PCR Purification kit (Qiagen, Manchester, UK) according to manufacturers' instructions. This was quantified using a nanodrop and diluted to a 10^9 master stock concentration. For multiplex qPCR assays, only one gene per group was used as a standard.

2.5.7 Bacterial Growth in Liquid Media and Preparation for Plasmid Extraction

2.5ml of L Broth (Table 2-9) was added to a bijou bottle which was then inoculated with a colony of *E. coli* containing the relevant plasmid. Bottles were placed on a shaker (37°C, 220rpm) and left for 16 hours. The *E. coli* culture was then processed for plasmid extraction.

Table 2-9 L Broth preparation (12)

Component	**g/L^{-1}**
Difco Bacto Tryptone	10
Difco Bacto yeast extract	5
NaCl	5
Glucose	1

Components added to 1L of dH_20, then autoclaved.

2.5.8 qPCR Sample and Standards Preparation

DNA samples can contain contamination not removed by the extraction process, such as humic acids from soils. These contaminants may inhibit the qPCR process and affect the results obtained. Diluting the sample reduces the contaminant and therefore minimises the inhibition effect, but it is important the DNA is not diluted too much as this will affect

detection levels. For each experiment, several samples were assayed at neat, 10^{-1}, 10^{-2} and 10^{-3} concentrations. The optimum dilution factor was then selected (based on the dilution factor that gave the highest copy number for each gene when assayed), and all samples were diluted accordingly. This was repeated for each individual gene to be assayed.

For each run, four "no template controls" (NTC) were assayed to ensure that background signal or contaminant levels were low. If any of the NTC indicated fluorescence had been detected, the run was rejected and the assay repeated. Thus ensuring false positive results were kept to a minimum. Additionally, reaction efficiencies were verified using positive controls. Samples were spiked with known amounts of DNA template (same DNA used to generate standards); and the results were compared with efficiencies of "neat" standards. Correlation coefficients (r^2) for all standard curves were >0.99; and log gene-abundance values (except those below detection limits) were within the linear range of the calibration curves.

The standard curve for each assay needed to cover the range of gene copy numbers present in each set of samples. A selection of samples was assayed with a standard curve of concentrations ranging from 10^1 to 10^9. The standards were selected based on which concentration range the samples fell into, normally 10^3 to 10^9 for 16S, 10^1 to 10^6 for resistance genes.

2.5.9 CopA Primer Design

In order to quantify copper resistance by genetic methods (phenotypic analysis only gives an indication of the resistance levels in the culturable fraction of a sample), generic PCR primers needed to be designed. While there were already primers targeting copper resistance genes published in literature, they were usually specific to a single bacteria and would offer little more information that the phenotypic methods. BLAST searches and alignments for the copper resistance genes *Cop*A, *Cop*B, *Cop*C, *Cop*D and *Cue*O (therefore targeting a wide variety of resistance mechanisms and proteins) were carried out to try to find areas of homology suitable for targeting with primers. Sequence data from a selection of both Gram negative and Gram positive bacteria across several genera were used but a long enough consensus sequence could not be found for any of the genes. Using amino acid sequences the design of degenerate primers was attempted, but this also proved

unsuccessful. It was decided to use *copA* primers (10), which targeted a protein binding motif conserved across several bacterial groups, however the PCR product obtained yielded fragments of multiple sizes, none of which corresponded with the size given in the reference article. As the primers from de la Iglesia, et al. (10) did not work, *cop*A quantification analysis was not carried out, but should be revisited in future work, perhaps as a multiplex qPCR assay with primers targeting individual bacteria combined into one assay.

2.5.10 qPCR Reaction Mix and Cycles

qPCR reactions had a total volume of 20µl containing 3 µl template DNA at optimum dilution factor, 1 µl working stock primers, 10 µl of iQ Supermix (Biorad) and 6 µl of nuclease free water. 0.2 µl of 100x SYBR Green, was also added to each reaction. 96-well plates covered with film were used (both BioRad, Hertfordshire, UK) with an iCycler and iQ fluorescence detector and software (all BioRad, Hertfordshire, UK) according to the manufacturer's instructions. qPCR conditions, annealing temperatures are in Table 2-10. Each sample was assayed in triplicate for each gene unless stated otherwise, with standards in duplicate to generate a standard curve. Four wells of each plate contained reaction mix, but 3µl of nuclease free water instead of DNA to act as a negative control. Each gene was assayed in isolation, with the exception of the tetracycline genes quantified in Chapters 4, 5 and 6 which were screened in groups as a multiplex qPCR, to allow quantification of a maximum number of tetracycline genes, while keeping costs to a minimum. Any significant positive results were re-assayed as individual genes. ART barrier pipette tips (VWR, Leicestershire, UK) were used for all processes related to qPCR as they came sealed and certified RNAse-free. Additionally, they prevented any contamination of the sample reactions reducing the risk of fluorescence being generated by anything other than the sample DNA, resulting in more accurate results.

Table 2-10 qPCR conditions - all assays

No. cycles	Process	Temperature (°C)	Time (minutes: Seconds)
1	Denaturing	95	10:00
40	Denaturing	94	0.20
	Annealing & Extension [a]	50/55/60	1:00
1	Further Extension[b]	72	0:30/1:30[c]
1	Post analytical temperature melt curve	50-95	ΔT=0.1°C/sec

[a] 50°C - *bla*$_{TEM}$, 55°C – all other *bla* genes, all multiplex tetracycline genes, 60°C – all individual tetracycline genes, all *erm* genes and 16S. [b] further extension step required for *bla* genes and multiplex tetracycline genes only. [C] 0:30 further extension for *bla* genes, 1:30 further extension for multiplex tetracycline genes.

2.5.11 qPCR Data Analysis

A post analytical melt curve was carried out for every assay so the reaction quality could be verified. Reactions that were of poor quality, or displayed high levels of background fluorescence, were repeated. Triplicate values for each sample were adjusted to reflect the dilution factor of the DNA sample used, these values were then averaged. Any samples that had large standard error values (i.e., >10%) were repeated. The resistance gene values were standardised to 16S-rRNA gene abundances to account for any differences in sample size.

2.5.12 Sequencing

A portion of the 16S rRNA gene was amplified using the method previously described (primers Table 2-4). PCR products were purified using the QIAquick PCR Purification kit and quantified using a Nanodrop to ensure the concentration was appropriate for analysis. Samples and primers were then sent to GATC (GATC, Germany) for sequencing by the Sanger method.

2.6 Plate Count Analysis of WWTP Bioreactors

2.6.1 Spread Plating

Plate Count Agar (Oxoid, Basingstoke, UK) was prepared according to manufacturer's instructions. For plates amended with antibiotics, they were added after autoclaving when

the agar had cooled to around 50°C (stock concentrations Table 2-11). For copper, a 10x copper sulphate solution was sterile filtered and added to the bioreactor samples to provide a 1X (1 mM) copper sulphate exposure. This was then spread onto agar plates. It was found, in preliminary examinations, that higher than expected numbers of Cu-resistant bacteria formed when adding copper directly to media (presumably due to phosphate concentrations). . 100µl of sample (diluted if necessary) was pipetted onto the plate surface and spread using a sterile L shaped spreader. Plates were inverted and incubated at 30°C for 7 days.

Table 2-11 Plate count Agar Amendment Stock Concentrations

Amendment	Stock Concentration
Ampicillin	16mg/ml
Tetracycline[a]	16mg/ml
Copper	1M

[a]Tetracycline dissolved using 70% ethanol.

2.6.2 Colony Counting

Plates with 30-300 colonies were counted using an electronic colony counter pen with permanent marker (Fisher Scientific, Leicester, UK), to improve reliability of counting.

2.6.3 Cross Resistance Plating

A sterile square of velvet was fixed to a replica plating block (Bel-Art Products, VWR, Leicestershire, UK) and a previously incubated plate was gently placed on top of the velvet and then removed. A sterile plate was then placed on top of the velvet to gain the colony imprint left by the previous plate (all under aseptic conditions). The velvet was then removed and soaked in Virkon overnight, rinsed thoroughly with distilled water, wrapped in tinfoil, autoclaved and then left to dry before reuse.

Unfortunately, the colonies on replica plates became smeared and indistinguishable, making accurate counting extremely difficult; therefore, a “patching method” was adopted. A 50 square grid was drawn on the base of a sterile plate, and sterile matchsticks used to “patch” or transfer a resistant colony picked from one plate; e.g. agar amended with

tetracycline; onto each square on the new agar plate (the new plate being amended with something other than tetracycline). Colonies that grew after being patched onto the new plate were therefore considered cross resistant, having been able to grow i.e. show resistance, to both compounds having grown on plates supplemented with them.

The time delay between switching from the velvet replica plating method to the patching method, meant samples that had been stored in a freezer had to be used for plating instead of fresh samples. Due to the length of time samples had been in the freezer, cell viabilities were reduced and low numbers of colonies were being cultivated. Samples were placed in quarter-strength Ringer's solution (Table 2-12) and left for an hour to try to revive the bacteria. This was then plated on normal Plate Count Agar, but had no effect.

Table 2-12 Quarter-strength Ringers Solution Composition

Component	**gL^{-1}**
NaCl	2.250
KCl	0.105g
$CaCl_2$	0.120g
Chapter 2: $NaHCO_3$	0.050

2.7 Copper Analysis

Samples were acid digested using *aqua regia* with a methodology modified from the standard method (13).

20ml of sample was added to a 100 ml flat bottom flask (Schott, Stafford, UK) using a pipette. Flasks were placed in a fume cupboard and 30 ml of hydrochloric acid dispensed into each. 10 ml of nitric acid was then added to each drop by drop. The flask was swirled gently to mix and a watch glass then placed on the top to act as a lid. Samples were left to stand for 16 hours to allow oxidation of organic matter. When oxidation was complete, the flasks were heated on a hot plate (60°C) until no further reaction was observed. The temperature of the hot plate was then increased to 100°C and samples left to heat for 2 hours (they were monitored periodically to ensure reaction was not too vigorous resulting in displacement of the watch glass). Flasks were then left for several hours to cool

completely. Ashless, quantitative filter paper (Whatman, Buckinghamshire, UK) was folded and placed inside a filter funnel, which was inserted into the top of a labelled 100ml volumetric flask. The reaction mix was then passed through the filter paper. The flat bottom flasks were rinsed three times using de-ionised water and the washings added to the filter paper. The volume was made up to 100ml using de-ionised water, the flask then capped and inverted 5 times. 15ml of digest was then transferred using a pipette to a labelled 15ml centrifuge tubes. Samples were then analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), by Dr. Tanya Peshkur at the Scottish Environmental Technology Network (SETN), at the University of Strathclyde.

2.8 Statistical Analysis

Genes quantified by qPCR were assayed in triplicate. Values were adjusted to account for the dilution factor (DNA was diluted to reduce inhibition effect, as discussed above) and then log transformed. Mean and standard deviations were calculated; analysis was repeated if relative error (standard deviation per mean) was greater than 10%.

Often, resistance gene abundances were standardised to 16S rRNA gene abundance as all bacteria carry at least one copy of the gene. Conserved regions of the gene could be targeted so most bacteria were quantified, thus 16S gene counts provided a surrogate measure of the total bacterial population size. By standardising the resistance gene abundances to total population, i.e. the proportion of the bacterial population carrying a specific resistance gene, data can be directly compared without differences in population size being an issue.

As mentioned bacteria carry at least one copy of the gene, meaning many bacteria can carry multiple copies. For example, actinobacteria have on average 3 copies of 16S rRNA in their genome, while bacteriodes have approximately 4 (14). Therefore standardising to 16S rRNA may not be the most appropriate way to account for the bacterial population size. Despite the flaw in this data normalisation method, there is currently no superior alternative and it has therefore become the accepted method of standardising absolute resistance gene abundances within the research field (references (15-19) are but a few examples of other antibiotic resistance environmental studies that normalise resistance gene abundances to 16S rRNA).

Anderson-Darling tests were used to check the normality of data sets. The null hypothesis represents data that is normally distributed; therefore, a significant P value (less than 5%) indicates data did not follow a normal distribution.

Occasionally, data required transformation to improve its distribution, and allow parametric statistical tests to be used robustly. For example, log transformation was the most appropriate way to analyse and present gene abundance, which has now become a discipline norm. However, for any other data, where the P values obtained by the Anderson-Darling test were lower for the log-transformed data than the original data, then transformed data were used for further statistical analyses.

The standard error and means were calculated using Microsoft Excel (2007). Statistical tests were performed using Minitab (Version 16) software. Levels of significance were pre-determined at $\alpha = 0.05$ (or $P < 0.05$), except in long-term mesocosm experiments were decreased level of control and natural variability justified $\alpha = 0.10$ (16).

2.9 References

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Chapter 3: Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils[3]

3.1 Statement of Co-authorship

The research in this chapter formed the basis of a co-written manuscript, which was published in an international, peer-reviewed journal. Each author's contribution is outlined in the manuscript itself (see Appendix 2), but the contribution of Seánín McCluskey is further elaborated here for the purpose of justifying the inclusion of this chapter in the thesis. The DNA extractions and qPCR analysis of the supplied samples were carried out by McCluskey. McCluskey also performed statistical analyses and interpretation of the results, as well as contributing to writing the manuscript.

3.2 Background

3.2.1 General Introduction

Many resistance genes carried by pathogens originate in the natural environment, and as a result, the role of the environment in propagating and maintaining antibiotic resistance has been the subject of much study and speculation. In recent years, studies have examined various locales (streams (1, 2), seawater (3-5) and soils (6, 7)) to determine the antibiotic resistance profile of the indigenous bacteria, then tried to link the presence of resistance to environmental conditions, for example, comparing resistance levels of a control stream to one, which has received industrial run off or wastewater discharge (8). Many studies were not quantitative and did not determine concentrations of contaminants, such as antibiotics or other factors that could provide selection pressure for antibiotic resistance, e.g., heavy metals or quaternary ammonium compounds (9, 10). Additionally, they fail to generate specific correlations between resistance phenotypes and possible selection pressures. Instead, many simply stated the amount of antibiotic resistant bacteria in a sample. The

[3] This chapter forms the basis of the manuscript "Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils". 2011. Charles W. Knapp*, Seánín M. McCluskey*, Brajesh K. Singh, Colin D. Campbell, Gordon Hudson.Published in *PLoS One* 6(11):e27300.
(*These authors contributed equally to writing the manuscript)

analyses were not carried out to a standard method, with set resistance breakpoints or limits (like clinical samples with MIC determinations), rather researchers often used their own method and definition of resistance. This means they yield limited comparable information, and different studies cannot be compared to look for patterns/correlations of resistance. Additionally, the focus of many studies has been the effect of anthropogenic or industrially impacted sites; therefore the natural impact of the environment remains unknown.

Environmental conditions influence community composition and function of bacteria. For example, geochemical conditions, such as pH and metal content, select for bacteria based on their tolerance to such conditions. Trace metals, such as copper and iron, are essential (at low concentrations) for all living organisms to function. They play a vital role in various biochemical processes such as redox enzymes in the electron transport chain (11); however, at higher concentrations, these metals are toxic (12). The potential of these essential, ubiquitous elements to select and propagate antibiotic resistance needs to be investigated. Metals have been linked to antibiotic resistance (as previously discussed in Chapter 1); however, the specific details of this relationship remain undetermined.

Two specific mechanisms have been implicated in linking antibiotic and metal resistance: co-resistance and cross-resistance. Co-resistance occurs when separate resistance genes for both an antibiotic and a metal are carried on the same piece of genetic material, while cross-resistance is when one gene confers resistance to both an antibiotic and a metal (9). In both cases, metals can act as a selection pressure for antibiotic resistance even in the absence of the antibiotics.

The role of the natural environment in the problem of antibiotic resistance is an obvious concern. It represents the original source of many antibiotics and in turn resistance genes, possibly acting as a reservoir for clinically-relevant resistance genes. Therefore there is a basal level of resistance genes. Not knowing baseline conditions, it is difficult to ascertain the human contribution to the problem of elevated resistance.

3.2.2 Rationale for Experiment

It was hypothesised that the geochemical conditions of soil (with particular focus on metal content) would directly correlate to the abundance of antibiotic resistance genes present in the soil. The James Hutton Institute in Aberdeen maintains an archive of soil samples, which

have been collected from various locations in Scotland over 70 years. These soil samples have originally been collected and analysed for another purpose, but the basic soil characterisation and chemistry have already been determined. For this study, samples collected between 1940 and 1970, relatively near the beginning of the antibiotic era, were selected. Since DNA could be extracted from dried archived soil (13), the samples presented a unique opportunity to compare soil samples of different geochemical character in terms of antibiotic resistance gene abundances. Essentially the effect of natural baseline geochemical conditions on antibiotic resistance could be observed, without the bias caused by contemporary antibiotic use.

3.3 Methods

3.3.1 Sample Selection

An array of 46 soil samples was selected at random; the samples had been collected from various sites throughout Scotland. Original sampling had been performed as part of a national survey to determine soil conditions. None of the soils were known to be influenced by any human or industrial activity. This allowed for the impact "background" or natural conditions have on antibiotic resistance to be observed.

A second set of samples originally collected in 2008 was also analysed as a comparison to the background samples. These samples were from sites in Hartwood, North Lanarkshire and Auchincruive, South Ayrshire were sewage sludge amended with various concentrations of copper had been applied— 0, 50, 100 and 200mg-Cu/kg between 1994 and 1998. Further details of these samples can be found in Macdonald et al. (14). Sewage sludge was routinely applied to fields for disposal and recycling of nutrients such as phosphorus. These industrially impacted samples allowed for an insightful comparison to the natural samples, particularly because sewage sludge acts as a sink for heavy metals and copper is a trace element, which has been extensively used as an antimicrobial for many years.

The locations of all sample sites can be seen in Figure 1 in the manuscript (Appendix 2).

3.3.2 Sample Collection and Storage

The samples had been stored in climate-controlled rooms at the James Hutton Institute. When sampling originally occurred, soil was collected at depths of 0-25cm and combined to provide a composite sample for that specific site. Soil was dried at 30°C and classified by sieve analysis (see section 2.1 in the methods chapter) giving particle size and an indication of silt, sand and clay content of the samples. Total metals were extracted using aqua regia (see section 2.6.4 in the methods chapter) and analysed using ICP (inductively coupled plasma) analysis. As the samples were valuable (long-term storage is expensive) and were available only in small quantities, it was decided to use the metal content data recorded in the historical records, rather than reanalysing. The records also provided information on pH and organic carbon content.

3.3.3 DNA Extraction

It has been shown long term storage does not bias DNA information; i.e., certain genes do not persist preferentially over others (13, 15). DNA was extracted from samples using a FastDNA Spin Kit (MP Bio, USA) according to manufacturer's instructions. As soils had been dried and archived, samples were left to sit in Binding Matrix E tubes for 15 minutes to allow for rehydration, then processed as normal (see section 2.5.1 in the methods chapter), and eluted in 100µl buffer.

3.3.4 qPCR analysis

Based on a previous study (16), which examined metal contaminated sediments, and the fact antibiotic resistance genes are extensively present in the environment (8, 17-19), it was decided to target eleven specific antibiotic resistance genes. These assays included genes for ribosomal protection proteins (*tet*) against tetracyclines, beta-lactam resistance (*bla*) and erythromycin resistant methylases (*erm*) (see Table 3-1).

Table 3-1 Genes and determinants assayed by qPCR

Tet resistance genes	Bla resistance genes	Erm resistance genes
tet(M)	bla_{TEM}	*erm*(B)
tet(Q)	bla_{SHV}	*erm*(C)
tet(W)	bla_{CTX}	*erm*(E)
-	bla_{OXA}	*erm*(F)

For the first set of samples (those from the national survey) all eleven genes were assayed; however, for the second set of samples (sewage sludge amendments), only *tet*(M), *tet* (W), *erm*(F), bla_{TEM}, bla_{SHV} and bla_{CTX-M} were assayed. The decision to selectively reduce the number of assays was based on the results from the first set of samples.

The qPCR assays were carried out using a methodology described fully in Chapter 2. All primers can be found in Table 2-5, while qPCR conditions are in Table 2-10.

The 16S-rRNA gene was quantified to standardise resistance gene abundance to account for minor differences in sample size and extraction efficiency.

3.4 Results

3.4.1 Baseline Conditions Samples

Bivariate analysis

For the initial set of samples (samples collected from national survey), bi-variate correlations were used to look at the correlations between the log-transformed values of resistance genes/16S and various geochemical conditions – carbon, phosphorus, sand, silt, clay, ash, pH, cobalt, chromium, copper, nickel, lead, zinc and iron.

A full table of the P values for these correlations are in Table 2 of Knapp, McCluskey et al. (2010; see Appendix 2). Statistically significant correlations are listed in Table 3-2 below.

Table 3-2 Significant bi-variate correlations between geochemical condition and gene abundance: P<0.05 and P<0.01

Geochemical condition	**Associated resistance gene/16S abundance**	
	P<0.05	**P<0.01**
Carbon	*erm*(C)	-
Phosphorus	*erm*(F)	-
Clay	bla_{SHV}	-
Ash	bla_{CTX}	-
	erm(E)	-
pH	bla_{OXA}	bla_{CTX}
	erm(B)	*erm*(E)
Chromium	bla_{CTX}	bla_{OXA}
	tet(M)	-
Copper	*tet*(M)	bla_{OXA}
	tet(W)	-
	erm(B)	-
	erm(F)	-
Nickel	*tet*(W)	-
Sand	-	bla_{SHV}
Silt	-	bla_{CTX}
	-	*erm*(E)
Iron	-	*tet*(M)

Overall, copper had the biggest influence on resistance gene abundance as it significantly correlated to 5 of the 11 genes assayed (in total, copper was associated with 8 of the 11 resistance genes). *tet*(M) was the resistance gene most often associated with the metal content of the soil samples - chromium, copper and iron all significantly correlated to *tet*(M) abundance.

Multi-linear regressions

Some of the geochemical conditions may synergistically influence resistance levels (also, many metal values are collinear; e.g., cobalt, chromium, nickel and copper all correlated to each other with a statistical significance of $p<0.01$, Table S5 of Knapp, McCluskey, et al. (2010) in Appendix 3). Multi-linear regressions (MLR) were performed to investigate the additive effect that metals and geochemical conditions may have on each gene simultaneously (Charles Knapp, personal communication). MLR took into consideration several factors – total chromium, copper, nickel, lead, extractable iron and pH – and used these factors in relation to abundance of each resistance gene to generate a prediction model. Full results are in the Appendix (see supplementary information Table S5 in Knapp, McCluskey, et al. (2010) in Appendix 3), but it should be noted the patterns of correlations obtained were consistent with those listed in Table 3-2. In fact, the associations between the metals and resistance gene abundances improved, i.e. $R>0.70$.

Low r^2 values (coefficient of determinants) were obtained using the bi-variate statistical approaches. When MLR were used, the patterns between metal and resistance gene abundance were consistent (as previously mentioned), however the correlations between genes and geochemical conditions became more evident, and the r^2 values improved, meaning more variations in gene abundances could be explained by multiple geochemical factors. The slightly low r^2 values, that is, predicting only around 50% of the variability of resistance gene abundance, were not unexpected as soils are comprised of a complicated matrix of substances, and therefore complicates prediction based on few factors. Additionally, the samples were from an archive and limited data were available, so the models themselves were not particularly complicated when taking the complexity of soil into consideration.

The improved correlation strength with MLR implies that a single geochemical condition (or metal) is perhaps not enough to impact resistance on its own. Metals and other geochemical factors could be synergistically impacting resistance. With this in mind, as well

as the fact that clay and pH also correlated to gene abundance, further analyses not included in the published manuscript were carried out.

Clay and pH related effects

Clay and pH are known to affect the bioavailability of metals (20, 21), and it is possible these factors contribute to the metals effect on resistance.

Pearson's bivariate correlations looked at the relationship between resistance gene abundance and metals, pH and clay. The results for copper are shown in Table 3-3 (also Table 2 in the published paper, in Appendix 2), as this metal was most commonly associated with resistance gene abundance. Additionally, the resistance genes investigated – *tet*(M), *tet*(W), bla_{TEM}, bla_{SHV} - were selected as *tet*(M) and *tet*(W) correlated to copper, while bla_{TEM} and bla_{SHV} did not. Comparisons of the impact of pH and clay had on copper ability to affect resistance were carried out this way to avoid bias.

Table 3-3 Pearson's bivariate correlations between copper and gene abundances, clay and pH. A correlation is weak if r<0.3, medium if 0.3< r <0.5, and strong if r>0.5.

Gene	**Pearson's correlation value**	**Correlation type**	**Significance p value**
tet(M)	0.439	Medium	0.047
tet(W)	0.296	Weak	0.054
bla_{TEM}	0.276	Weak	0.114
bla_{SHV}	0.280	Weak	0.088
Clay	0.102	Weak	0.509
pH	0.033	None	0.834

Partial correlations were then carried out using SPSS (Charles Knapp, personal communication). Partial correlations enable the control of certain parameters (i.e. remove their effect) by comparing the patterns of residual values between two sets of bivariate

correlations. For example, in this case, pH and clay content have a potential impact on gene-soil binding and metal bioavailability. Partial correlations of metals and resistance genes were generated controlling for clay, pH, and both clay and pH. The results for copper are shown below in Table 3-4, Table 3-5 and Table 3-6, while the full table of results is in Appendix 2.

Table 3-4 Copper partial correlations controlling for clay

Gene	**Pearsons correlation value**	**Correlation type**	**Significance p value**
tet(M)	0.331	Medium	0.195
tet(W)	0.588	Strong	0.013
bla_{TEM}	0.521	Strong	0.032
bla_{SHV}	0.398	Medium	0.113

Table 3-5 Copper partial correlations controlling for pH

Gene	**Pearsons correlation value**	**Correlation type**	**Significance p value**
tet(M)	0.421	Medium	0.100
tet(W)	0.614	Strong	0.009
bla_{TEM}	0.530	Strong	0.029
bla_{SHV}	0.201	Weak	0.438

Table 3-6 Copper partial correlations controlling for clay and pH

Gene	Pearsons correlation value	Correlation type	Significance p value
tet(M)	0.298	Weak	0.262
tet(W)	0.528	Strong	0.018
bla_{TEM}	0.497	Medium	0.050
bla_{SHV}	0.367	Medium	0.162

When controlling for clay, the correlations between *tet*(W), bla_{TEM}, bla_{SHV} and copper went from being weak and not statistically significant, to medium (bla_{SHV}) and strong (*tet*(W), bla_{TEM}) correlations, that for *tet*(W) and bla_{TEM} were also statistically significant. Interestingly for *tet*(M), the opposite was the case, the correlation to copper went from being medium and significant, to not significant with a lower Pearson's correlation value (still medium).

When pH was controlled, the correlation of *tet*(W) to copper stayed strong but became statistically significant, bla_{TEM} became strong and significant, while bla_{SHV} had a marginally lower correlation, but stayed small and not significant. Again like when clay was controlled for, the correlation of *tet*(M) to copper decreased and stopped being significant.

When both pH and clay were controlled for, the same pattern occurred, *tet*(W) and bla_{TEM} both became significantly correlated to copper, with the correlations themselves becoming strong and medium respectively. The bla_{SHV} and copper correlation strengthened to medium but remained not statistically significant and the copper/*tet*(M) correlation decreased from medium to weak and stopped being significant.

For other metals this mixed pattern was also observed, i.e. controlled for clay, pH or clay and pH, improved correlations to some resistance genes but weakened correlations to others. This implies that the cooperation of pH and clay with metals to increase resistance is not as clear cut as anticipated. It would have been anticipated clay and pH impacted

metals and gene interactions in a similar manner, i.e. all correlations would improve, however this was not the case.

3.4.2 Amended Samples

As a result of the association between copper and the abundance of several resistance genes, it was decided the relationship warranted further investigation. While the previous experiment represented basal gene abundances based on "natural" geochemical conditions, this experiment represents a "polluted" scenario with elevated concentrations of copper.

Samples from two separate sites amended with sewage sludge and different concentrations of copper were analysed for *tet*(M), *tet*(W), bla_{TEM}, bla_{SHV}, bla_{CTX} and *erm*(F); *tet*(M), *tet*(W), and *erm*(F) had correlations with copper in the previous set of samples. *ANOVA* (analysis of variance) was used to examine differences in resistance gene abundance between plots with different concentrations of copper. At the Hartwood site, levels of bla_{CTX} were found to be elevated in the plots amended with copper when compared to control plots. Additionally, plots amended with more than 150mg/kg of copper had statistically significant increased abundances of both bla_{TEM} and bla_{SHV} genes (*tet*(M) and *tet*(W) displayed similar trends; however the results were not significant). Strangely samples obtained from the Auchincruive site displayed no trends among copper amendments. Full results are highlighted in Table 3-7.

The lack of correlation between resistance genes and copper at the Auchincruive site could be explained by several reasons. As previously discussed, geochemical factors do not act in isolation. The Auchincruive site had a higher soil pH with lower clay and higher silt levels than the Hartwood site. It is possible that the conditions at Auchincruive were not conducive for the propagation of resistance genes, meaning the clay and pH conditions potentially reduced the impact of the copper. Additionally different activated sludge may have been applied than at the Hartwood site. Treatment strategies and waste sources have an impact on the bacteria (and gene) content of the waste material; therefore the chances of having copper resistance genes may have been different. For example, Burch et al. (22) found that aerobic digestion of activated sludge decreased the levels of antibiotic resistance genes.

Table 3-7 *ANOVA* analysis for amended samples

Hartwood Site		Sum of squares	d.f.	Mean squares	F	Sig.
$\log\left(\frac{\boldsymbol{tet}(\mathbf{M})}{\mathbf{16SrRNA}}\right)$	Between groups	2.789	4	0.697	1.442	0.327
	Within groups	2.901	6	0.484		
	Total	5.691	10			
$\log\left(\frac{\boldsymbol{tet}(\mathbf{W})}{\mathbf{16SrRNA}}\right)$	Between groups	1.760	4	0.440	0.451	0.770
	Within groups	6.830	7	0.976		
	Total	8.590	11			
$\log\left(\frac{\boldsymbol{bla}_{\mathbf{TEM}}}{\mathbf{16SrRNA}}\right)$	Between groups	0.954	4	0.238	3.411	0.075
	Within groups	0.489	7	0.070		
	Total	1.443	11			
$\log\left(\frac{\boldsymbol{bla}_{\mathbf{SHV}}}{\mathbf{16SrRNA}}\right)$	Between groups	1.238	4	0.310	4.535	0.040
	Within groups	0.478	7	0.068		

	Total	1.716	11			
$\log\left(\frac{bla_{CTX}}{16SrRNA}\right)$	Between groups Within groups Total	1.576 0.746 2.322	4 6 10	0.394 0.124	3.168	0.100
$\log\left(\frac{erm(F)}{16SrRNA}\right)$	Between groups Within groups Total	0.406 1.466 1.872	4 6 10	0.102 0.244	0.416	0.792

Auchincruive Site		Sum of squares	d.f.	Mean squares	F	Sig.
$\log\left(\frac{tet(\mathrm{M})}{16\mathrm{SrRNA}}\right)$	Between groups Within groups Total	2.252 3.855 6.106	4 8 12	0.563 0.482	1.168	0.393
$\log\left(\frac{tet(\mathrm{W})}{16\mathrm{SrRNA}}\right)$	Between groups Within groups Total	1.232 3.323 4.555	4 8 12	0.308 0.415	0.742	0.590
$\log\left(\frac{bla_{\mathrm{TEM}}}{16\mathrm{SrRNA}}\right)$	Between groups Within groups Total	0.087 0.153 0.240	4 8 12	0.022 0.019	1.147	0.401
$\log\left(\frac{bla_{\mathrm{SHV}}}{16\mathrm{SrRNA}}\right)$	Between groups Within groups Total	0.150 0.144 0.294	4 8 12	0.038 0.018	2.078	0.176
$\log\left(\frac{bla_{\mathrm{CTX}}}{16\mathrm{SrRNA}}\right)$	Between groups	0.716	4	0.179	1.259	0.361

	Within groups Total	1.138 1.854	8 12	0.142		
$\log\left(\frac{erm(\mathrm{F})}{16\mathrm{SrRNA}}\right)$	Between groups Within groups Total	0.170 1.554 1.724	4 8 12	0.043 0.194	0.219	0.920

Taken from Knapp, McCluskey et al. (23) supplementary information.

3.5 Discussion

The geochemical conditions of soil clearly had an impact on antibiotic resistance levels. Background metal conditions (particularly copper) correlated to antibiotic-resistance gene abundance in soil samples from various sites in Scotland.

The association between metals and antibiotic resistance was expected, as it has been known for some time that the two are linked (24). What was surprising was that relatively low concentrations of metals had a significant impact on resistance gene abundance in the soil. Background concentrations detected in soils were 1-100mg/kg for copper, 10-1000mg/kg for nickel and <1-1000 for chromium (25), while soil quality standards for Europe allow 10-10,000mg/kg of copper, 50-10,000mg/kg of nickel and >10->10,000mg/kg of chromium in soil (25). Up until now, most work investigated areas impacted by industrial or anthropogenic pollution. This study indicated the background effect of metals on antibiotic resistance was more innate than previously believed.

This may be analogous to exposure to sub-inhibitory levels of pharmaceutical antibiotics. Low levels of antibiotics do not kill bacteria and instead cause the exposed population to become increasingly tolerant and resistant. It is possible background metal levels cause bacteria to evolve in a similar manner, and the bacteria have simultaneously acquired antibiotic resistance genes from the selection pressure of metals.

The consequence of this interaction can have long term implications. Metals do not degrade from the environment and are ubiquitously present; therefore, it is possible that if antibiotic use was reduced, resistance levels would not necessarily follow a similar trend. This may explain why sulphonamide resistance levels did not decline when use of the drug was decreased significantly in the UK (26). Sulphonamides were derived from fabric dyes used at the start of the 20th century, and like antibiotics, they are naturally present in the environment as a result of industrial pollution and could have linked resistance mechanisms with metal resistance in the environment.

Multi-linear regression models improved associations between metals and antibiotic resistance gene abundances (in comparison with the bi-variate analysis). This may not be a result of metals working in collaboration to increase antibiotic resistance, instead this could have been simply due to each metal impacting resistance gene abundance individually and the MLR combining these interactions and treating the effects as being additive. As

previously mentioned, the levels of metals in the samples correlated to each other (see supplement S4 for paper in Appendix 3), likely due to natural geological depositions of various minerals and ores. The bacterial populations are not exposed to a single type of metal. Metals exist in combination, and so could their effects. Therefore, a simple bi-variate comparison does not give a view of the associations/relationships that exist between the metals and resistance.

Additionally, controlling for pH and clay content improved the interactions between copper and resistance gene abundance (same for other metals). This implies that geochemical conditions can impact the bioavailability and toxicity of the metal, which indirectly affect resistance levels. Concentrations of metals used for MLR analysis were "total" metals and not just dissolved. However, the distribution and speciation of metals, which are related to geological and chemical conditions—including pH and clay content, are important information to know when making comparisons between exposure and biological responses. Here, sample quantities were limited and had to rely on available information.

The overall negative role of clay in metal correlation to resistance gene abundance is slightly surprising. Clay particles bind and accumulate DNA, (27); it also protects the molecule from nuclease attack and DNA degradation (28). Additionally, the presence of divalent metals improves DNA-soil binding, so there are many geochemical factors that affect DNA presence. There are several naturally competent bacteria in the environment, such as *Pseudomonas*; and bacteria which naturally produce extracellular DNA e.g. *E. coli* (27). If clay binds and protects DNA, it would have been anticipated it preserved it, allowing for other bacterial cells to sequester the DNA. In the case of resistance genes on plasmids, it would have been anticipated clay held the plasmids in the soil, protected. As such, several things could explain the overall negative association of clay with metal/antibiotic resistance associations. Clay could be binding DNA but not releasing it, meaning the DNA is not available for uptake by bacteria through transformation. Additionally metals such as copper could be aiding the binding of DNA to clay, negatively impacting the metals natural ability to influence antibiotic resistance abundance.

pH effects the bioavailability of metals (21); additionally it affects DNA binding to clay particles. This needs to be explored further as it was only determined pH had an effect and not whether there was a preference for high or low pH conditions. The interplay between

metals/pH/clay should also be considered. Areas of particular clay content and certain pH's could be "hot spots" for natural resistance gene retention.

The soil at the Auchincruive site had different geochemical properties than the Hartwood site (slightly higher pH, lower clay and higher silt content at the Auchincruive site (29)) . As previously discussed, pH and clay content can negatively impact the effect metals have on antibiotic resistance. The differences between the two sites may mean that one was better suited to the maintenance of antibiotic resistance due to copper exposure than the other.

Studies other than this one (30, 31) have linked copper to the presence of antibiotic resistance in soil environments. Copper has traditionally been linked to ampicillin resistance (30), while tetracycline resistance is normally associated with mercury resistance (32), making the association between tetracycline resistance genes and copper particularly interesting. Mercury resistance is carried by the transposon Tn21, while tetracycline resistance is found on Tn10 (33). Both transposons have been found located together on plasmids (33) and other transposons (34). Copper and tetracycline could be linked in a similar manner. Relatively low concentrations of copper correlated to resistance gene abundance indicated that it is not just areas of metal pollution we need to worry about in terms of antibiotic resistance. Conclusively proving the link between copper and tetracycline and/or ampicillin resistance and the mechanism by which these resistance types are linked, is paramount.

Overall, the influence of geochemical conditions in the environment on antibiotic resistance should be investigated further, with particular emphasis on the role of clay in contributing to the effect metals have on antibiotic resistance. The current guidelines regarding acceptable levels of heavy metals in soil and what is defined as polluted may need revised. Could human populations be more at risk of antibiotic resistance infections, as a result of living in an area with high soil metal content and subsequent exposure to the metals? This could potentially be epidemiologically significant particularly when predicting or mapping infection outbreaks.

3.6 References

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Chapter 4: Does Copper Increase the Abundance of Tetracycline and Ampicillin Resistance Genes in Long-Term Soil Microcosms?

4.1 Introduction

Geochemical conditions were found to correlate to antibiotic resistance gene abundances in soil samples from various locations in Scotland (Chapter 3 and ref. (1)). Copper, in particular, was found to have one of the strongest correlations to the abundance of tetracycline, ampicillin, and erythromycin resistance genes. Samples were obtained from an archive at the James Hutton Institute that had been collected prior to the mainstream use of antibiotics (1940-1970). In these samples, relatively low metal concentrations correlated to the presence of resistance genes. As part of the previous experiment, an additional set of samples was obtained from sites supplemented with sewage sludge that had been amended with various concentrations of copper. These amended samples had statistically significant higher abundances of ampicillin resistance genes at copper concentrations over 150µg/g. Tetracycline resistance genes had a similar trend; however, it was not statistically significant (Chapter 3).

While the link between copper and ampicillin has been observed previously (2), the association between copper and tetracycline is novel and, therefore, of particular interest. Copper has long been used as an antimicrobial compound, particularly in agriculture as a growth promoter; additionally ampicillin and tetracycline are among the most highly used antibiotics. Therefore, a link between antibiotic resistance and copper would be of interest, particularly when trying to address the antibiotic resistance problem; copper could already be impacting resistance levels. According to the coefficient of determination (r^2) values based on the background geochemical samples, the models did not account for all sources of variability, and they did not accurately predict the results obtained from the copper amended samples. Factors other than the presence of metals in soil must be affecting resistance gene levels. As discussed in Chapter 3, clay and pH values, which have an influence on the bioavailability of metals, had a negative impact on the relationship between metals and resistance gene abundance. Statistically controlling for clay, pH, or clay and pH, improved the correlation between many metals and resistance genes (except for

tet(M)). As such, the relationship between copper and tetracycline resistance should be investigated further to determine whether the results obtained using the Scottish soil samples can be recreated and confirm the role of copper in increasing antibiotic resistance gene levels without the influence of variable geochemical conditions. Additionally, does copper only exert a selection pressure when present at low levels or does it have a direct relationship with resistance gene abundance over a wider range of exposures?

4.1.1 Hypothesis/Rationale for Experiment

To investigate whether the addition of copper had an effect on tetracycline and ampicillin resistance gene abundances, soil microcosms amended with various concentrations of copper (0, 10, 100, 1000 μg/g) were used. Sampling was carried out over 64 weeks, and resistance gene abundance was determined. It was hypothesised copper would display a direct effect on antibiotic resistance gene abundance up to a certain concentration, after which it may be too high to allow for community survival.

4.2 Methodology

4.2.1 Experimental Setup

To investigate the impact that copper concentration of a soil has on the abundance of genes conferring resistance to the antibiotics tetracycline and ampicillin, a series of soil microcosms was used. The design of a horizontal shallow trough was decided over traditional vertical columns, as this would minimise water-saturation and the formation of anoxic zones; additionally all samples could be taken from the same depth by just moving along the length of the microcosm for each time point, ensuring consistency.

Eight 1m lengths of PVC guttering were sealed with end caps (both from B&Q, Doncaster, UK) and cleaned with a 1% solution of Virkon™ (antibacterial, antifungal agent; Day-Impex, Colchester, UK), rinsed three times with distilled water, then sprayed with 70% ethanol and air dried.

2.7kg of topsoil was used per microcosm (see Figure 4-1). As clay has been shown to have a negative influence on the relationship between copper and resistance gene abundance (Chapter 3), a low clay content soil was used to maximise the effect of copper to select for

resistance. However, the clay was not completely excluded, nor its fraction varied, as the influence of clay was not under investigation. According to store (B&Q) product certification, all in accordance to British Standards, the soil was graded as sterilised sandy-clay loam. Commercial topsoil was used for reproducibility, and as it was sterilised, it was decided to add 0.3kg of "inoculum soil" to each microcosm to provide a diverse bacterial community. Raeburn Drilling (Hamilton, UK), kindly provided a low clay content soil sample, which was taken from an uncontaminated site in Montrose, Scotland, to be used as an inoculum for the microcosms. Due to confidentially agreements Raeburn have in place with all their clients, further information regarding the site was not available. The soil provided was classified using a sieve analysis method described in Chapter 2, according to the established method (3). It was determined to be a slightly silty sand (according to the standard method (4)), meaning sand was the main component, with any fines present being silt. 0.3kg of this soil was mixed with 2.7kg of topsoil and added to each microcosm (see Figure 4-2). The created soil blend was re-analysed (3, 4) and was found to be a "very sandy silt".

Four copper concentrations: 0µg/g, 10µg/g, 100µg/g and 1000µg/g (Table 4-1) were used as amendments in the microcosms, based on copper concentrations detected in the "background" Scottish soil samples (0-140µg/g) and the amended plots where copper had an effect (150µg/g and higher). The 1000µg/g treatment was significantly higher than previous observations, but represented a contaminated site—in the upper range of what soil quality standards allow (5).

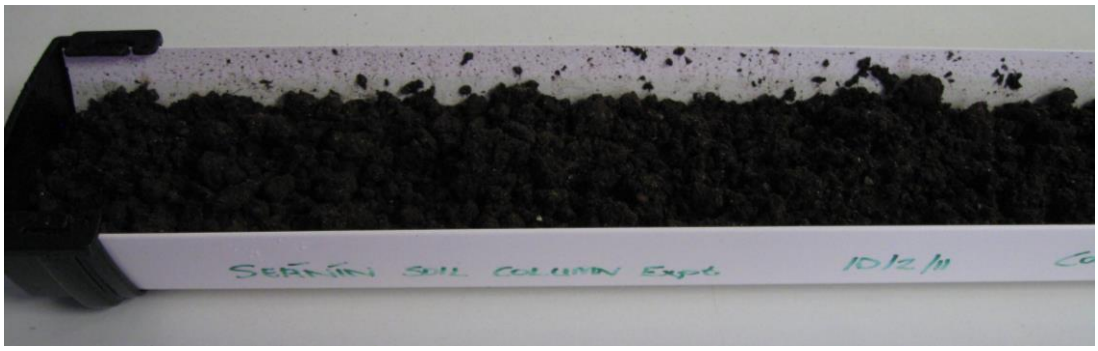

Figure 4-1 Soil Microcosm

Based on the fact there were 3kg of soil per microcosm (3000g), 30mg, 300mg, and 3g of copper (as copper sulphate) were weighed out and added to separate spray bottles containing 50ml of distilled water. The copper was allowed to dissolve, and the solution was mixed into the microcosms as an aerosol; 50ml of distilled water was applied to the control microcosm.

Table 4-1 Copper Amendments

Microcosms	**[Cu] (µg/g soil)**
1 + 2	0
3 + 4	10
5 + 6	100
7 + 8	1000

Each microcosm was then covered in cling film (Saran wrap) to minimise water evaporation and to maintain the moisture content of the soil. This was then covered over with tin foil to prevent light interference with the experiment (see Figure 4-2).

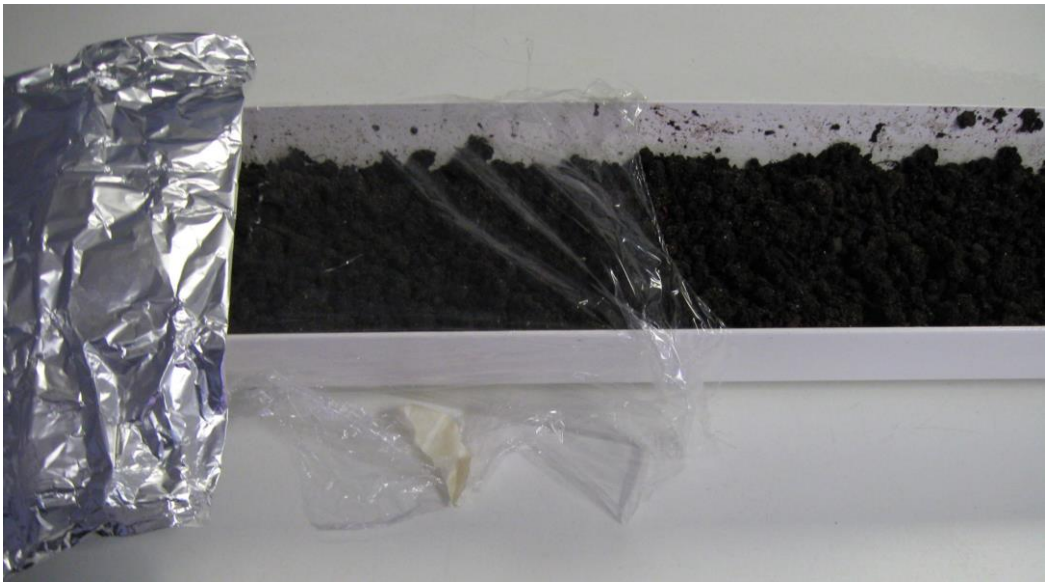

Figure 4-2 Soil microcosm with cling film and tin foil covering

4.2.2 Sampling

Sampling was carried out over a 64 week period (t=time): t_0, t_1, t_2, t_4, t_8, t_{16}, t_{32} and t_{64}. Soil was collected from the microcosms using sterile spatulas and centrifuge tubes; they were then stored at -80°C prior to molecular analysis. Sampling started at the left hand side of the microcosm and then moved along at 10cm intervals for each consecutive time point.

4.2.3 Molecular Analysis

It was decided, due to costs, only samples collected at t_0, t_{16}, t_{32} and t_{64} would be analysed initially. The time points between t_0 and t_{16} were unlikely to have had sufficient time for apparent changes in resistance gene abundances; however, if results obtained were significant, the additional samples could have been analysed later.

DNA was extracted from approximately 300mg of thawed sample using a FastDNA spin kit for soil (MP Biomedicals Europe, Illkirch, France), following the manufacturer's instructions and eluted using 100µl of elution buffer. The samples were then stored at -20°C (see Chapter 2 for further details).

qPCR was used to quantify tetracycline and ampicillin resistance genes, as well as the 16S rRNA gene (a surrogate for quantifying total bacteria), as described in Chapter 2. As the link between tetracyline and copper was of particular interest, a wide range of tetracycline resistance genes was assayed. To screen as many tetracycline genes as possible, multiplex

qPCR assays were used (i.e. multiple genes quantified in the one reaction mix). Three groups of *tet* genes were assayed Ng et al. (6). Groups 1 (*tet*(B),*tet*(C), *tet*(D)) and 2 (*tet*(A), *tet*(E), *tet*(G)) were all efflux genes, while group 3 (*tet*(K),*tet*(L),*tet*(M),*tet*(O),*tet*(S)) was a mix of efflux and ribosomal protection genes. Primers and PCR conditions were used directly from the paper (described in Chapter 2). *tet*(W) was carried out as a separate assay on a select number of samples, but due to low abundances being detected, it was not quantified in all samples. bla_{TEM}, bla_{CTX}, bla_{SHV} and bla_{OXA} assays were carried out using primers and PCR conditions described in Knapp et al.(7). All ampicillin resistance genes (bla_{TEM}, bla_{CTX}, bla_{SHV} and bla_{OXA}) were initially assayed in a select number of samples (one reactor from each pair, t_0, t_{32}, t_{64}, all in duplicate) to determine whether it was worth analysing the entire sample set. Only bla_{TEM} and bla_{CTX} were quantified in all samples.

4.3 Results

As discussed in Chapter 2, resistance gene abundances standardised to 16S were normalised to t_0 (values in Appendix 4). Normalised data (t/t_0) sets are visualised in Figure 4-3 to Figure 4-7. To make the charts easier to interpret, the values for each pair of soil microcosms has been averaged and displayed as a treatment. The range is displayed as error bars to indicate how much the values differed within each treatment set.

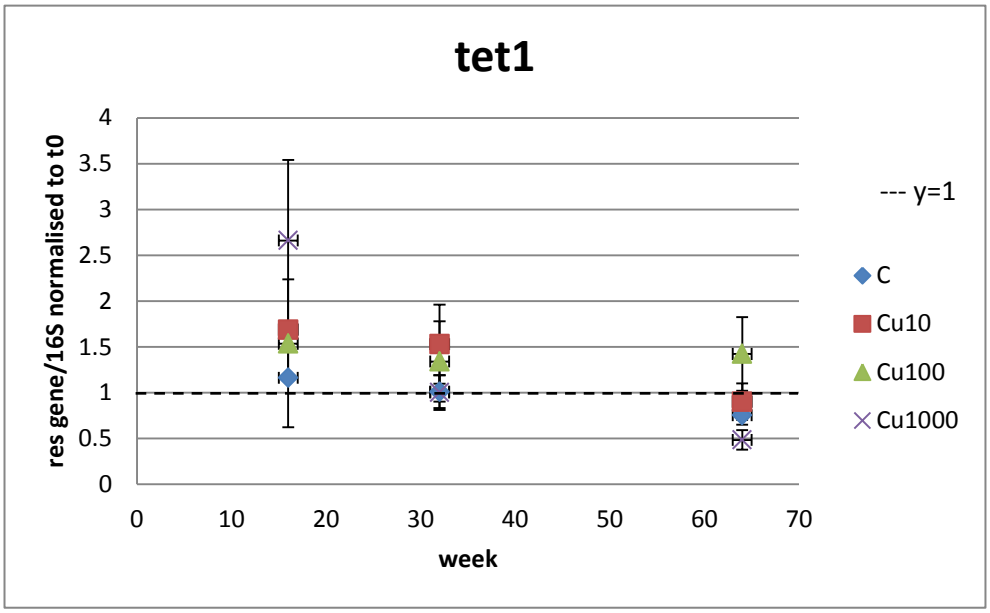

Figure 4-3 tet1/16S relative genes abundances as compared to initial (t_0) value (indicated by y=1 line).

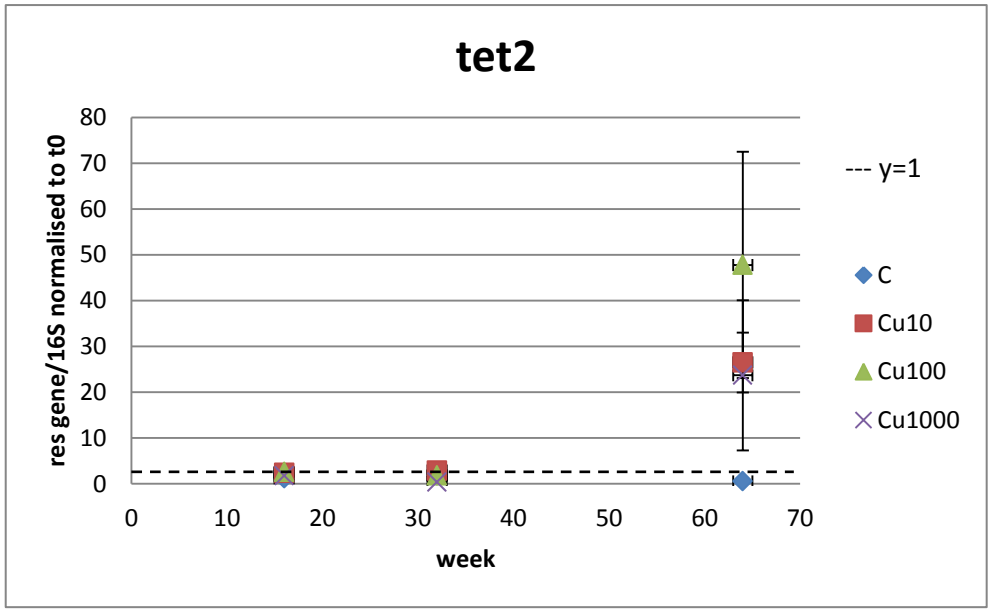

Figure 4-4 tet2/16S relative genes abundances as compared to initial (t_0) value (indicated by y=1 line).

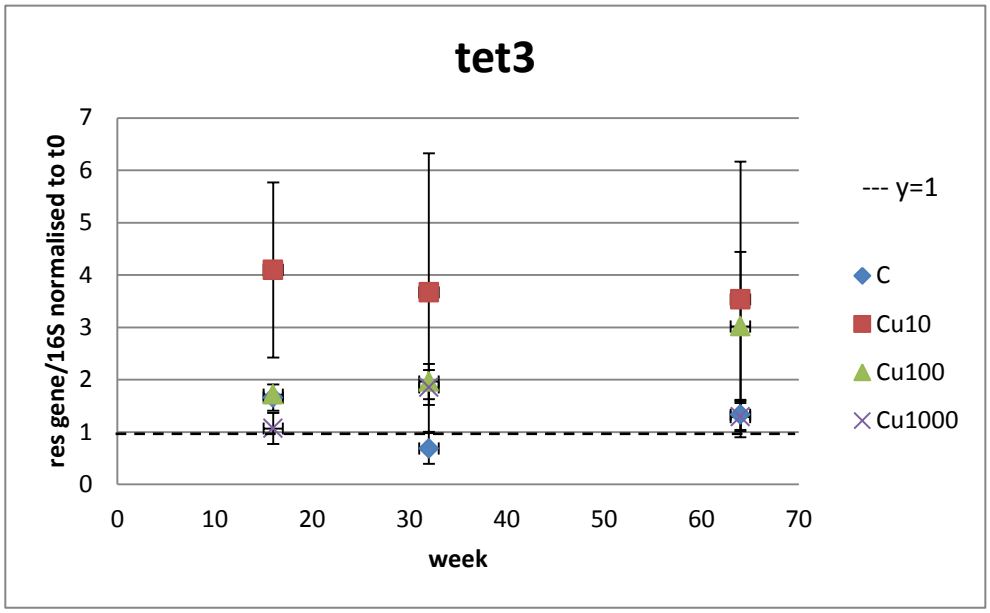

Figure 4-5 tet3/16S relative genes abundances as compared to initial (t_0) value (indicated by y=1 line).

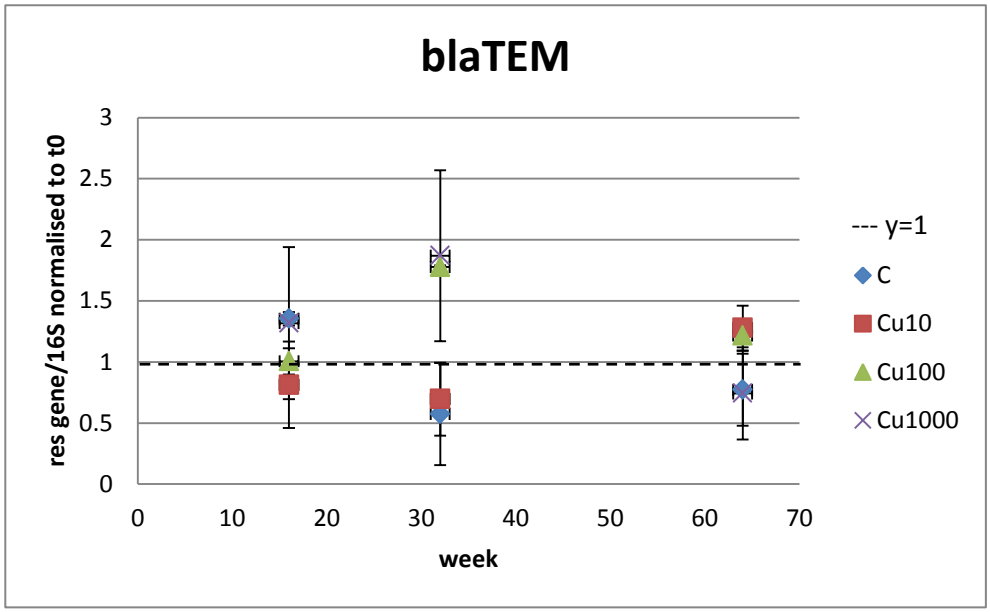

Figure 4-6 *bla*$_{TEM}$/16S relative genes abundances as compared to initial (t_0) value (indicated by y=1 line).

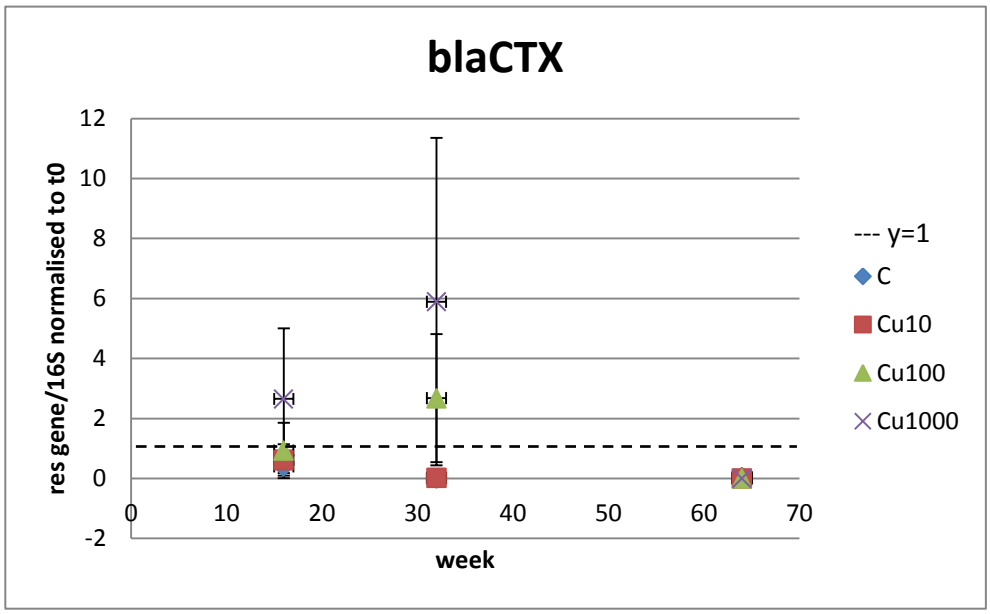

Figure 4-7 *bla*$_{CTX}$/16S relative genes abundances as compared to initial (t_0) value (indicated by y=1 line).

For Tet1 genes, higher copper concentrations increased resistance gene levels at t_{16}; however, this trend did not continue to the end of the experiment and treatment results were clustered at t_{32} and t_{64}. Tet2 results remained clustered at t_{16} and t_{32}, but copper increased tet2 genes by the end of the experiment. Cu10 microcosms had higher levels of tet3 resistance genes than control microcosms for the duration of the experiment, while Cu100 and Cu1000 had similar or slightly higher levels of tet3 relative to controls. While the Cu100 and Cu1000 microcosms had more *bla*$_{TEM}$ genes than controls and Cu10 at t_{32}, the differences were less pronounced at other time periods. Cu1000 microcosms had visibly higher levels of *bla*$_{CTX}$ than the rest of the microcosms at t_{16} and t_{32}, while Cu100 had higher abundances than other treatments and control microcosms at t_{32}. At t_{64} all reactors had similar levels of *bla*$_{CTX.}$ The concentration of copper required to maintain resistance gene levels appears to vary for different genes.

To determine whether differences between treatments in terms of resistance gene development were significant, the slope of each treatment - res/16S (t/t_0) values – was calculated, as was the standard error of the slope. Values obtained are below in Table 4-2, *ANOVA* analysis was then used to compare treatments based on these values Table 4-3.

Table 4-2 Slope and standard error of slope values for resistance gene/16S relative genes abundances as compared to initial (t0) value. Standard errors are denoted in parenthesis below the respective slope values.

	tet1	**tet2**	**tet3**	***bla*$_{TEM}$**	***bla*$_{CTX}$**
Control 1	-0.010 (0.004)	-0.015 (0.011)	-0.004 (0.021)	0.000 (0.025)	-0.001 (0.004)
Control 2	-0.007 (0.003)	-0.020 (0.027)	-0.002 (0.019)	-0.019 (0.002)	-0.011 (0.009)
Cu10 1	-0.010 (0.002)	0.684 (0.209)	-0.028 (0.021)	0.011 (0.001)	-0.002 (0.002)
Cu10 2	-0.024 (0.002)	0.389 (0.140)	0.006 (0.010)	0.010 (0.013)	-0.020 (0.016)
Cu100 1	0.011 (0.016)	0.479 (0.170)	0.061 (0.023)	-0.004 (0.016)	-0.055 (0.083)
Cu100 2	-0.014 (0.008)	1.546 (0.564)	-0.005 (0.015)	0.006 (0.017)	-0.002 (0.012)
Cu1000 1	-0.060 (0.030)	0.145 (0.063)	-0.001 (0.031)	-0.009 (0.014)	-0.007 (0.005)
Cu1000 2	-0.022 (0.013)	0.848 (0.340)	0.004 (0.002)	-0.021 (0.021)	-0.140 (0.186)

Table 4-3 *ANOVA* comparison of slopes, based on linear regression slopes and standard error of slopes. Statistically significant values ($p<0.10$) are indicated by asterisk (*).

	tet1	**tet2**	**tet3**	***bla*$_{TEM}$**	***bla*$_{CTX}$**
F value	2.3584	4.1397	1.6783	0.6464	0.4364
P value	0.0348*	0.001*	0.1332	0.7157	0.8751

Based on the *ANOVA* analysis, it is evident that there are statistically significant differences in rates of development for both tet1 and tet2 resistance gene abundances (however for tet1, the trend was negative i.e. genes declined). By looking at the graphs themselves, it would have been expected that tet3 also had significant treatment based differences in terms of gene abundance; however, this was not the case. Additionally both *bla*$_{TEM}$ and *bla*$_{CTX}$ seem to have a clear difference between control and Cu10 microcosms and the Cu100 and Cu1000 microcosms at t_{32}; those amended with the high copper concentrations appear to have higher resistance gene abundances. As the analysis looks at the trend of each treatment as a whole (based on slope measurements) these single time points had no impact on the overall significance of the treatment; however, as the data for some of the gene sets is not completely linear, this may have influenced/effected the results obtained.

It cannot be determined whether the gene abundances over time have significantly differed from t_0 based on slopes and its standard error of the slope; therefore, a one-tailed T test compared the values for each treatment (see Figure 4-3 to Figure 4-7) to a hypothesised mean of 1, the value of t_0. Mean treatment values significantly higher than 1 indicate increasing gene abundances. Statistical descriptors and test values obtained are below in Table 4-4.

Table 4-4 P values for 1 population T test. Statistically significant values (p<0.1) are indicated by *. T values are displayed below in brackets.

	Tet1	**Tet2**	**Tet3**	***bla*$_{TEM}$**	***bla*$_{CTX}$**
C	0.074* (-2.26)	0.229 (-1.37)	0.870 (-0.17)	0.210 (-1.44)	0.000* (-8.04)
Cu10	0.419 (0.88)	0.198 (1.48)	0.051* (2.55)	0.330 (-1.08)	0.003* (-5.37)
Cu100	0.004* (4.92)	0.276 (1.22)	0.002* (5.69)	0.051* (2.56)	0.492 (0.74)
Cu1000	0.145 (1.72)	0.257 (1.28)	0.065* (2.36)	0.223 (1.39)	0.376 (0.97)

For tet1, the Cu100 microcosms had significantly higher mean gene abundances in comparison to t_0, as do copper concentrations > 10ug/g for tet3. For *bla*$_{TEM}$ Cu100 microcosms had significantly higher mean gene abundances. Therefore, these treatments showed an increase in gene abundance from t_0 across the time series. Although control microcosms for tet1 and control and Cu10 microcosms for *bla*$_{CTX}$ have significant p values, their mean values are not all >1.

When this is taken into consideration with the *ANOVA* analysis of the slopes, amendment with 100µg Cu/g led to a significant increase in tet1 resistance gene abundances. For the other treatments and resistance genes, while it appeared that there was a difference in resistance gene abundances based on copper amendment (as discussed above), results were not statistically significant. This may have been due to data not being strictly linear in some cases, thus making the analysis less effective.

As previously discussed, both *bla*$_{TEM}$ and *bla*$_{CTX}$ appear to have treatment based differences at week 32, additionally while tet3 appears to have treatment based differences for all time points, it is most apparent at week 32. As the previous statistical analyses examined gene abundances as a trend over the time series, and tet3 treatments appear to have similar slopes but there is an obvious difference between treatments, it was decided to analyse t_{32} in isolation to determine whether there were any significant treatment based differences, i.e. determine whether higher copper concentrations had higher resistance gene abundances.

ANOVA with post-hoc Tukey analysis was used to compare treatments at t_{32} as this is where differences in resistance gene levels was most apparent in the resistance gene trend charts above. P values obtained are by Tukey analyses are listed in Table 4-5 below.

Table 4-5 *ANOVA* with post hoc Tukey analysis for log transformed resistance gene/16S at t_{32}. Statistically significant values ($p<0.10$) are indicated by *.

	tet1	**tet2**	**tet3**	**blaTEM**	**blaCTX**
F value	0.36	3.33	7.03	1.72	16.75
P value	0.788	0.138	0.045*	0.301	0.010*

tet1, tet2 and *bla*$_{TEM}$ showed no significant treatment based difference in terms of gene abundance at t_{32}. While this was anticipated for tet1 and tet2, it is unexpected for *bla*$_{TEM}$ as it appears that at week 32, the control and Cu10 data points are clustered together lower than the points for Cu100 and Cu1000. Tet3 and *bla*$_{CTX}$ did display a statistically significant treatment based difference in gene abundance at week 32.

While tet1 displayed a significant difference in terms of gene abundance as an overall trend, tet3 and *bla*$_{CTX}$ only do so for an isolated time point (t_{32}), indicating the trend does not continue at t_{64}. From the absolute 16S rRNA gene abundances (shown below in Figure

4-8) one can see that for Cu100 and Cu1000 treatments, there was an overall decline in 16S rRNA gene abundances from t_{32} to t_{64}. The opposite was the case for the control and Cu10 microcosms, which actually increased from t_{32} to t_{64}, but the abundances were still lower than at the start of the experiment for t_0.

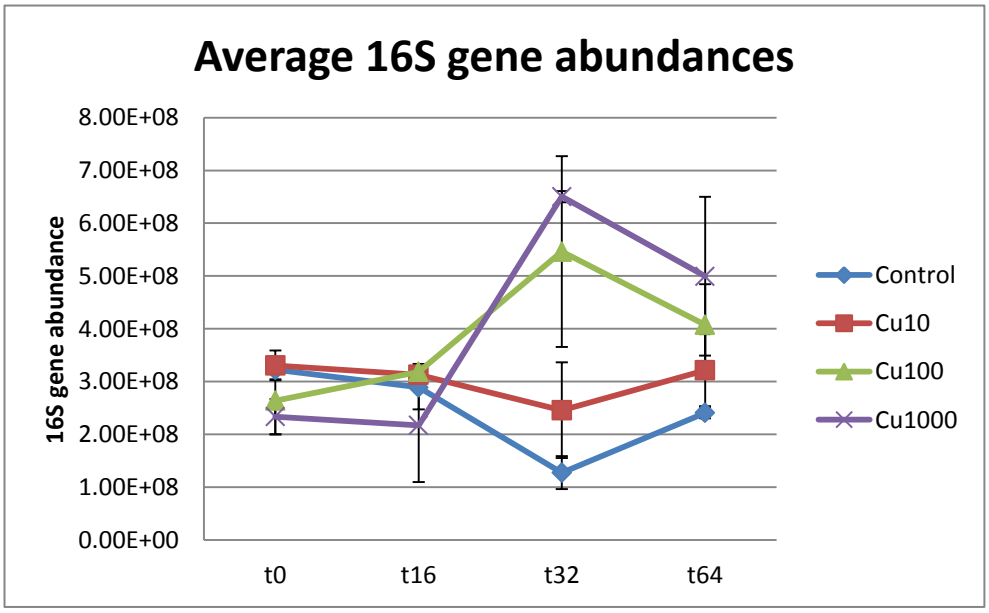

Figure 4-8 16S rRNA gene abundances over time

4.4 Discussion

The original hypothesis for the experiment was that copper exposure would directly affect antibiotic resistance gene abundances, while high concentrations of copper (1000µg/g) would perhaps inhibit many susceptible bacteria and prevent resistance development.

Both tet1 and tet2 gene sets had significant treatment based differences in terms of resistance gene abundances; however, tet1 only had significantly higher mean resistance gene abundances for the Cu100 treatment. In the previous chapter (Chapter 3), copper levels present in "background" (0-140µg/g) and "amended" (>150µg/g) samples were significantly related to the abundances of both tetracycline and β lactam resistance genes. The addition of 100µg/g copper resulted in significantly higher abundances of tet1 genes, which is consistent with the results in Chapter 3. Rather than being a correlated

observation from a highly varied environment as in the previous experiment, here the sole manipulation was the addition of copper.

When t_{32} was analysed in isolation, both tet3 and *bla*$_{CTX}$ had significant treatment based differences in gene abundance. In the previous study (Chapter 3), *tet*(M) (quantified as part of tet3 in this study) was associated with background levels (0-140µg/g) of copper, while *bla*$_{CTX}$ was associated with amended copper samples (>150µg/g). The treatment based differences observed at t_{32} for tet3 and *bla*$_{CTX}$ are consistent with the results obtained in the previous experiment; however the overall trend for these genes were not significant. The microcosms were set up as a controlled system and minimal nutrients were added to the soil; therefore, they also lacked the cycling of allochthonous carbon and nitrogen; as a result, the bacterial community likely faced stress in the form of decreased nutrient availability. Previous work has shown the physiological state of a donor cell impacts the rate of plasmid transfer, however it is independent of the growth rate of recipient cells (8).

The individual genes quantified in the tet1 multiplex assay – *tet*(B), *tet*(C) and *tet*(D) are all plasmid associated and therefore easily transferrable between bacteria. *Tet*(B) is associated with the transposon Tn10 and has an extremely broad host range (9). *bla*$_{CTX}$ is also plasmid bound (10), therefore plasmid transfer likely played a significant role in copper mediated increases in resistance gene abundance. As copper amendment had resulted in the selection of plasmid associated antibiotic resistance genes, it is probable genes for copper resistance are also carried on these plasmids and copper is linked to tetracycline and β lactam resistance by a co-resistance mechanism. Further investigation would be required to confirm this; however, this is outside the remit of the original experimental aim, which was to confirm whether copper increased resistance gene abundances and not to determine the mechanism by which the selection effect may occur.

As the tetracycline resistance genes linked to copper are plasmid bound or associated with transposons, it may have been beneficial to look at integrons such as Int1B to investigate whether these gene acquisition systems (11) have played a role in the association between copper and tetracycline resistance; and whether these mobile elements have become integrated into bacterial genomes. However as this is more focused on the mechanism linking the two and the experimental aim was simply to confirm the two are in fact linked, it was decided this line of investigation was unnecessary but should potentially be incorporated into future work.

It has been suggested that multiplex qPCR less sensitive than regular qPCR i.e. quantifying individual targets. Additionally the resistance gene abundances are standardised to 16S rRNA abundances. Bacteria can harbour several copies of this gene (12) meaning it may not be the most appropriate method for estimating population size. Despite these potential analysis flaws, it was decided that it would be more beneficial to look at a wider range of resistance genes using a multiplex assay format and risk issues with sensitivity, than to target the analysis towards a few specific genes and potentially miss uncovering a link between resistance types. Additionally as discussed in chapter 2, it is becoming standard within this field to normalise resistance gene abundances to 16S rRNA. It is hoped continued development and improvement in experimental practice and design drive the research area forward, improving quality and relevance of data obtained.

Overall, the correlations observed between copper, tetracycline and ampicillin resistance gene abundances in natural soil systems, were recreated in low clay content soil microcosms. As the microcosms differed only in copper content, it adds strength to the observed associations and strongly infers copper is the component responsible for the increased resistance gene abundance. The exact mechanisms underpinning the link between copper and resistance genes needs further investigation, but the role of the environment and the presence of heavy metals should, in future, be taken into consideration when planning strategies to combat the current antibiotic resistance problem currently presented.

4.4.1 Future Work

One aspect of the experiment that should be further investigated is the role of soil type on copper exposure and transfer of resistance genes. The soil used in this experiment was a "very sandy silt", therefore it had a low clay content and a high sand fraction. Sand confers a protective effect to chromosomal DNA (in comparison to plasmid DNA (13)) and therefore transfer of chromosomal DNA occurs more frequently than plasmid DNA in a sand matrix (13). Additionally, DNA is degraded more in sand soils than in clay (14). As the resistance genes associated with copper in the experiment were all plasmid bound, the effect of soil type on the relationship between copper and antibiotic resistance should be investigated. Could the soil type have negatively impacted the influence of copper on resistance gene abundance?

As discussed in Chapter 3, clay may have a negative influence on the effect copper has on resistance gene abundances, however sand may also negatively select against plasmid DNA. In future work, a sterile artificial soil should still be used with a bacterial inoculum for consistency, but varying amounts of clay could be added to a low clay content soil like the one used in this experiment, therefore the role soil type plays in the influence copper has on antibiotic resistance could be further investigated. Additionally the gene sets that yielded significant results in this study should perhaps be assayed individually to determine which genes were associated with copper, with the eventual aim of elucidating the mechanism linking the two.

4.5 References

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Chapter 5: Copper-Related Effects on Tetracycline- and Ampicillin-Resistance in Wastewater Microcosms

5.1 Background

Antibiotic resistance is a substantial clinical problem; with the prospect of a future without antibiotics, a "post-antibiotic era" becoming a real concern. Increasing numbers of infections once effectively treated with antibiotic therapy are becoming difficult to treat. Multidrug resistance is becoming more common, and if resistance continues to spread at its current rate, patients will become defenceless against bacterial infections. The discovery of new antibiotics has slowed considerably since the 1950s (1); as a result we cannot rely on the introduction of new drugs to replace those that have become obsolete. The issue of antibiotic resistance, therefore, needs to be addressed. As the overuse of antibiotics has been implicated in the propagation of resistance genes, measures taken so far to mitigate the resistance problem relate to their use. However, despite an EU-wide ban of the use of antibiotics as animal feed additives (2) and a campaign to reduce clinical prescriptions (3), resistance levels have not declined (Chapter 1).

The environment (4, 5) and heavy metals (6, 7) have long been implicated in increasing and maintaining antibiotic resistance. Heavy metals are linked to antibiotic resistance through co- and-cross resistance mechanisms. Resistance genes for both an antibiotic and a heavy metal can be carried on the same piece of genetic material (co-resistance), or one gene may able to confer resistance to both an antibiotic and a metal (cross resistance). This means only the metal or the antibiotic needs to be present to maintain resistance to both (8). The environment is the natural source of both antibiotics and antibiotic resistance genes (9, 10), and resistance genes have been shown to move from the environment to the clinical setting (11-13), and *vice versa*, with resistance genes first appearing in the environment (14).

The wastewater treatment (WWT) process acts as an interface between the clinical setting and natural environment and, as such, may contribute to the cycling of resistance genes. WWT receives wastes from a diversity of sources – households, hospitals, surface water and agricultural runoff. Subsequently, they contain resistant bacteria, antibiotic residues, heavy metals, and selection pressures that contribute to resistance-gene maintenance.

WWT is thought to select for antibiotic resistance (15-18), but the exact mechanisms by which this occurs remain unknown. WWT plants contain high densities of bacteria as well as the selection pressures to possibly maintain high levels of resistance (e.g., activated sludge is known to act as a heavy metal sink). The water is discharged to the environment, and the biosolids from the activated sludge process are often spread on soils to recycle phosphorus (19); however, this has been shown to increase the metal concentration of the soil (19). WWT could be contributing to the selection and maintenance of antibiotic resistance via different avenues.

Copper concentrations in a soil environment influence the abundance of antibiotic resistance genes, with particular reference to tetracycline resistance genes and, to a lesser extent, β-lactam (ampicillin) resistance genes (Chapter 3, Chapter 4, (20)). Copper is the third most commonly used metal globally (19) and has been used as a growth promoter in agriculture, while tetracycline and ampicillin are both from the most commonly used classes of antibiotics (21). Additionally copper (22-25), penicillins (including ampicillin) (26, 27) and tetracyclines (26-29) are all commonly detected in WWT plants. Ampicillin and tetracycline are likely to select for their own resistance genes, but the impact of copper and its potential role maintaining resistance gene abundances within the WWT process, thereby contributing to the cycling of resistance genes, warrants investigation.

5.1.1 Justification for the Experiment

The potential role of WWT in selecting for antibiotic resistance needs to be determined. Areas with naturally higher metal deposits or increased levels of industrial activity may be at an elevated risk of antibiotic resistance gene selection during the WWT process. Subsequently, there may be increased levels of genetic pollution discharged to the environment. If this is the case, current practices in WWT, effluent discharge and the amendment of activated sludge to agricultural land may need to be improved.

Using a controlled microcosm to help minimise extraneous variables, the effect copper on β lactam (ampicillin) and tetracycline resistance was investigated, following a modified version of the methodology by Peltier et al (30), who examined zinc-related effects. Peltier et al (30) showed zinc amendments caused a significant increase in tylosin resistance, while reactors amended with zinc and antibiotics (oxytetracycline, ciprofloxacin, and tylosin) had

developed significantly higher levels of resistance to those antibiotics (in comparison to controls or those amended with zinc or antibiotics only).

5.1.2 Hypothesis

The following hypotheses are investigated:

1. Does copper increase the abundance of antibiotic resistance genes? Relative (per 16S-rRNA) gene levels will be compared between treatments, as well as making before-after treatment (i.e., when antibiotics and/or copper is added) comparisons.
2. Are there any synergistic effects when microbial communities are exposed to both copper and antibiotics (i.e. tetracycline and ampicillin)? Treatments will be combined to investigate any enhanced changes.

5.2 Methodology

5.2.1 Experimental Set Up

To investigate whether copper or antibiotic levels increases tetracycline and β-lactam (ampicillin) resistance, an activated-sludge system was used as the experimental system. Eight bioreactors were created using 2-litre borosilicate glass bottles. Initially 1350ml of synthetic wastewater (recipe from Knapp and Graham 2007 (31), as described in Chapter 2) was added along with 150ml of wastewater treatment activated sludge from Shieldhall WWTP, Glasgow (Scottish Water). The activated sludge sample was homogenised, divided into 50ml aliquots, and added to the reactors. The remaining portions of activated sludge were quickly frozen at -80°C for periodical additions to the reactors to maintain community and genetic diversity (mentioned later). Previous bioreactor experiments have resulted in population extinctions due to competitive exclusion and wash-out (31), which is not representative of wastewater treatment plants, where there is a continuous replenishment of exogenous bacteria. One sample from the treatment plant was taken and used for the duration of the experiment to provide consistency and minimise effects resulting from chemical and community changes over time.

The microcosm bottles were covered in tinfoil to keep out light. The lids of the bioreactors had plastic septa with holes drilled in them to allow tubing to pass into the reactor (see

Figure 5-1). The reactors were aerated using fish tank pumps (Hailea Fish Tank Pump, amazon.com) via silicon tubing attached to a plastic Pasteur pipette with a fish tank aeration diffusor stone (Aquarium Fish Tank Cylinder Bubble Air Stones Blue, amazon.com) at the very bottom. The airflow rate supplied by the pumps was 0.90L/min (calculated based on air displacement in a submerged graduated cylinder). The rate for the pumps was pre-set and could not be adjusted. Aeration stones were used to create smaller air bubbles, allowing for better aeration and mixing of the bioreactor contents. An exhaust port for each bioreactor fed into a 50ml Falcon tube filled with 70% ethanol to inactivate any bio-aerosols.

The reactors were operated as semi-batch mode with particle settling to maintain biomass. Real WWT systems are continuous flow; however, these systems are fully automated and are operated on a much larger scale, therefore it was decided a semi batch approach was more appropriate for a bench-scale microcosm. During each run, a third of the reactor contents were replaced every three days, yielding a 9-day hydraulic retention time; the solids-retention time would be much longer, but it was not quantified. Routinely, 550ml were removed from each reactor and allowed to settle for 10mins; 500mls were poured off (and retained for later analysis) with the remaining 50ml of settled solids being returned to the reactor. The reactor was brought back to volume (1500ml) by adding 500ml of fresh synthetic wastewater. Weekly, 15ml of the cryogenically frozen activated-sludge sample was added to each bioreactor to help maintain bacterial populations and genetic diversity.

To monitor biomass levels within the bioreactors, the reactors were sampled bi-weekly to determine the suspended solids (MLSS) and volatile suspended solids (MLVSS) of the mixed liquor. 50ml aliquots were used for the analyses. Samples were filtered using sterile Buchner systems and fibreglass filters that had been heat-treated and pre-weighed. The filters were weighed after heating at 105°C for the MLSS analysis, and again after 500°C for MLVSS analysis. Filters were left in a desiccator to ensure filters remained dry. These methods were carried out according to methods described in “Suspended, Settleable and Total Dissolved Solids in Waters and Effluents, 1980” from Methods for the Examination of Waters and Associated Materials (56). These analyses continued for the duration of the experiment to monitor biomass conditions.

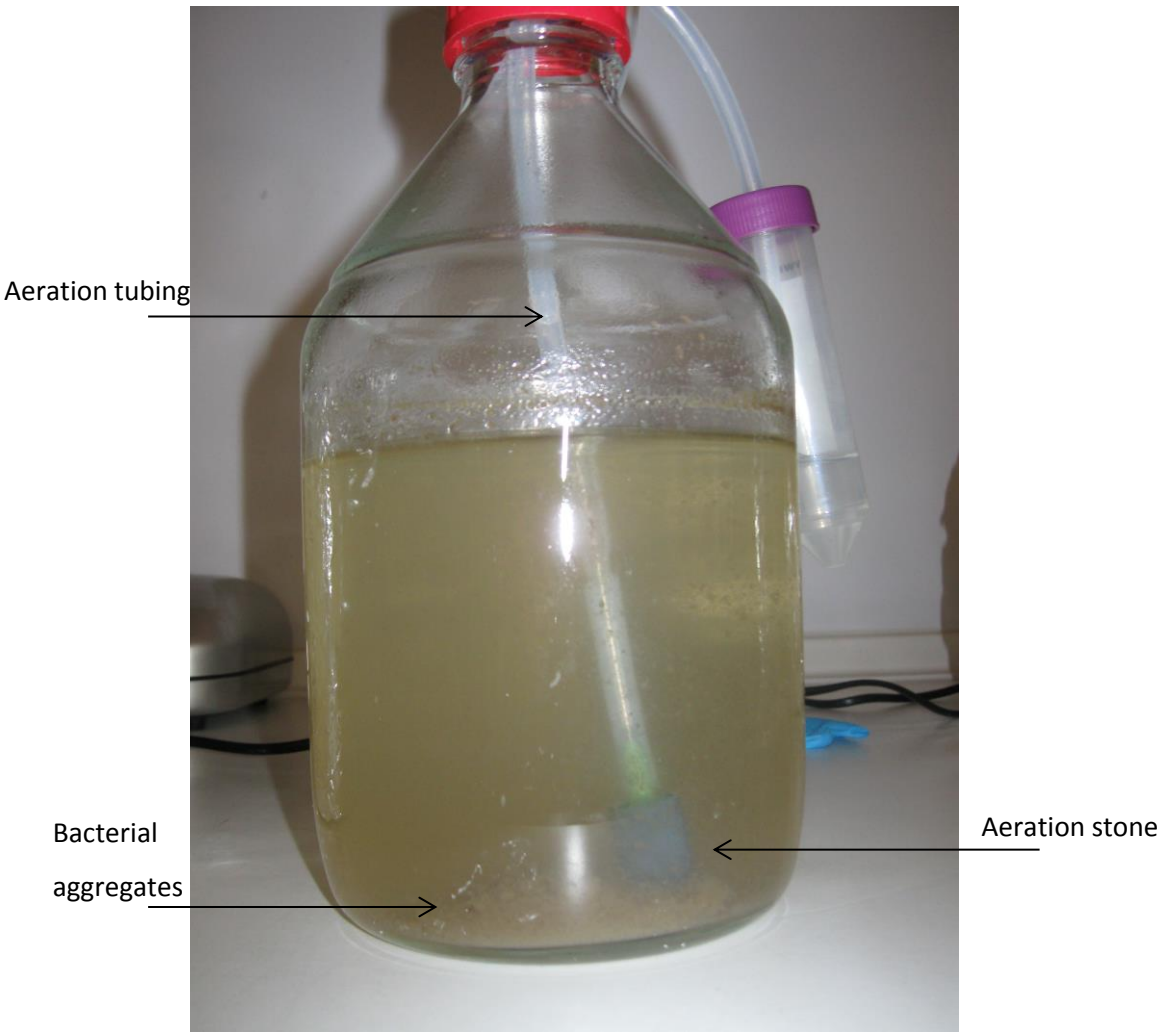

Figure 5-1 Bioreactor Setup

The experiment was divided into three separate treatment phases (see Table 5-1). During phase 1, bacterial communities were allowed to reach stable densities. For 6 weeks, all reactors were fed synthetic wastewater and a weekly dose of sludge inoculum. Phase 2 lasted 8 weeks; four of the reactors (reactors 1-4) had their feed amended with 3 mg-Cu/L copper (added as 7.5mg-$CuSO_4$/L). Phase 3 also lasted 8 weeks; reactors 1-4 continued to be amended with 3 mg/L copper; additionally, four of the reactors were amended with 0.2 mg/L tetracycline and ampicillin (reactors 1, 2, 5, and 6).

A concentration of 3 mg/L was decided to be enough to stress the bacterial populations, without being too high and inhibit entire populations. Peltier et al. (32) managed to achieve a change in antibiotic resistance with 5.0 mg/L of zinc. According to Fjallborg et al (19), zinc concentrations in sewage works are approximately 33% higher than copper concentrations.

The same concentrations of antibiotics were used as Peltier et al. (32) (0.2mg/L), as they were effective for their bioreactor experiments.

Table 5-1 Feed amendment schedule.The concentrations of the amendments added to the synthetic wastewater media were 3mg/L for copper (Cu) and 0.2mg/L for both tetracycline (Tet) and ampicillin (Amp).

	Phase 1 (Weeks 0-6)	**Phase 2 (Weeks 6-14)**	**Phase 3 (Weeks 14-22)**
Reactor 1	-	Cu	Cu, Tet, Amp
Reactor 2	-	Cu	Cu, Tet, Amp
Reactor 3	-	Cu	Cu
Reactor 4	-	Cu	Cu
Reactor 5	-	-	Tet, Amp
Reactor 6	-	-	Tet, Amp
Reactor 7	-	-	-
Reactor 8	-	-	-

5.2.2 Plate Count Analysis

To determine the resistance levels among the culturable bacteria, a traditional plate count method was used. Samples were collected bi-weekly from reactors as part of the draw-and-fill feeding process (seven days following last wastewater addition and re-inoculation). This yielded three time-series samples that reflect conditions within each treatment phase; a fourth could be considered as a “time-zero.” Samples were serially diluted and spread on agar plates using an L-shaped spreader, colonies were counted after being incubated and values were adjusted to consider dilutions, thus yielding colony-forming units (CFU) per ml as a result. Plate-Count Agar (Oxoid; Basingstoke, UK) was used, as it determined bacterial

levels from previous environmental studies (33-37). To enumerate resistance levels, Plate-Count Agar had been amended with copper, ampicillin, or tetracycline.

The concentrations of the copper and antibiotic amendments were determined by examining inhibitory threshold concentrations used in previous studies, rather than carrying out MIC dilution tests on isolates to determine a resistance breakpoint for the reactors. Guardabassi et al. (38) used 16μg/ml ampicillin and 8μg/ml tetracycline to screen for resistant bacteria, while Luczkiewicz et al. (39) found the MICs for environmental isolates to be 2-16μg/ml for ampicillin and 0.5-8μg/ml for tetracycline. It was decided to use 8μg/ml tetracycline and 16μg/ml ampicillin to count resistant bacteria in the bioreactors. However, when the first analysis was initially carried out, it became apparent (due to high proportions of the population being tetracycline resistant) that of tetracycline concentration (8μg/ml) was too low; therefore concentrations were increased to 16μg/ml, and previous plate counts were repeated.

Copper concentration was also based on previous studies. Sabry (40) found that 0.05mM (3.18mg-Cu/L) copper inhibited 21% of environmental isolates, 1mM (63.5 mg-Cu/L) of copper inhibited 78%, and 10mM (635 mg-Cu/L) inhibited 100% of environmental bacteria tested. Calomiris (41) used 400μg/ml (approximately 6mM) of copper to determine bacterial resistance levels in drinking water. It was decided to add 1mM of copper to plates to determine resistance levels in the bioreactor samples.

The plates were covered in tinfoil to prevent photo-degradation of the tetracycline, and cultures were left to grow for 7 days at room temperature (20 ^{o}C) prior to enumeration. Each sample was processed in duplicate, except for weeks 12 and 22, which were processed in triplicate to estimate the standard error of the plate-count methodology.

5.2.3 Molecular Analysis

Samples were collected bi-weekly from the bioreactor during the feeding process. 1.5ml aliquots were frozen at -80°C to preserve DNA for future molecular analysis. DNA was extracted using a FastDNA spin kit for soil (MP Biomedicals-Europe, Illkirch, France), following the manufacturer's instructions, and eluted with 100 μl of elution buffer (see Chapter 2 for further details). Samples were stored short-term at -20°C and long-term at -80°C.

qPCR was used to quantify antibiotic-resistance and 16S-rRNA gene abundances. For all qPCR reactions, 3μl of DNA template, 1μl of appropriate primers (500 nM final concentration), 10μl of BioRad iQ Supermix and 6μl nuclease-free water were combined to give a total reaction volume of 20μl. Every sample was processed in triplicate (see Chapter 2 for further details). All assays were initially carried out on a few random samples in duplicate to determine sample dilution factors, as samples could contain PCR inhibitors (see Chapter 2 for further details).

16S-rRNA genes were assayed as a surrogate method for quantifying total bacteria in the sample, and genes conferring resistance to ampicillin and tetracycline were assayed to detect potential resistance levels. To save cost of reagents and quantify as many tetracycline genes as possible, a multiplex qPCR was used (42) to screen the samples. All samples were assayed for Group 1 [*tet*(B), *tet*(C), and *tet*(D)], Group 2 [*tet*(A), *tet*(E), and *tet*(G)] and Group 3 [*tet*(K), *tet*(L), *tet*(M), *tet*(O), and *tet*(S)]. Groups 1 and 2 are all efflux genes, and group 3 is a combination of efflux and ribosomal protection genes. Group 4 [*tet*A(P), *tet(*Q), *tet*(X)] represented genes for efflux, ribosomal protection and enzymatic degradation respectively; unfortunately this group did not yield any results in initial dilution tests, so this assay was not performed on all samples. qPCR standards were created using one gene from each group. Primers and PCR conditions, described by Ng et al. (42), were used (see Tables 2-4 and 2-10). Dilution checks were carried out for of β-lactam (ampicillin) resistance genes bla_{TEM}, bla_{SHV} and bla_{CTX}. Only bla_{TEM} and bla_{CTX} were detected so qPCR assays for both genes were carried out using primers and PCR conditions described by Knapp et al. (43) (see Tables 2-4 and 2-10). Full details are in Chapter 2.

DGGE (denatured gradient gel electrophoresis) examined changes in reactor community diversity (44). All bacteria carry at least one copy of the 16S-rRNA gene. Within this gene, there are conservative regions that are similar among many bacteria, while other regions are phylogenetically variable. By running a PCR, with primers flanking a variable region of the gene and then passing the products on a DGGE gel, a banding pattern is obtained. Separate bands represent separate groups of bacteria based on GC content, and the disappearance/appearance of a band would indicate a change in diversity.

Based on diversity changes in the bioreactors, it may suggest whether any increase in resistance can be attributed to either vertical or horizontal selection. Increased resistance, with little change in diversity, could indicate that one (or more) group of bacteria

supposedly with a resistance trait gained dominance. Increased resistance with consistent or increased diversity could indicate that the resistance gene is being transferred horizontally between the bacteria. This information is important to gain a better idea of community dynamics.

The PCR used 16S-rRNA primers (see Table 2-4) and conditions described by Muyzer et al. (45) (see Table 2-7), specifically for looking at bacterial diversity using DGGE. The acrylamide gel (25% v/v 40% BIs/acrylamide) was cast with a chemical gradient of 30% to 60%; it was run using a BioRad DCode system (BioRad, Hertfordshire, UK) at 200V for 4.5 hours. The gel was stained using ethidium bromide and visualised using a 2UV transilluminator (UVP, California, US). A marker was included in the middle and at both ends of each gel to ensure the gel gradient had been cast level. The marker was DNA extracted from a single colony picked at random off a bioreactor plate count plate (later identified as *Stenotrophomonas maltophilia*). The same marker was used for all DGGE.

5.3 Results and Discussion

5.3.1 MLVSS

Mixed liquor volatile suspended solids (MLVSS) are often used to determine the amount of organic matter in an activated sludge process, and it was used here to monitor biomass levels and ensure treatments did not have a detrimental effect. MLVSS were measured bi-weekly for each bioreactor. Mean values with 95% confidence limits are shown below in Table 5-2. From the mean values it can be determined that biomass levels increased as the experiment progressed. Values with a high corresponding 95% confidence interval were due to the increase in MLVSS values within each phase, as well as differences in MLVSS values between the reactors within the pair.

Table 5-2 MLVSS mean values with 95% confidence intervals. Values are mg/L and were determined for reactor pairs in each phase of the experiment

	R1+R2		R3+R4		R5+R6		R7+R8	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Phase 1	157.7	16.5	156.3	5.5	156.0	6.0	160.3	7.6
Phase 2	213.4	31.4	206.5	17.1	164.8	8.7	165.3	10.4
Phase 3	268.4	65.0	222.5	47.5	232.3	73.7	206.5	47.0

5.3.2 Copper Content

Copper concentration for each reactor was analysed every four weeks using ICP-OES (Scottish Environmental Technology Network (SETN) at University of Strathclyde). Total copper content is in Table 5-3, while dissolved copper content is listed in Table 5-4.

Table 5-3 Total copper content in the reactors, mg/L

	week 6	**week 10**	**week 14**	**week 18**	**week 22**
R1	<0.01	1.14	1.61	2.06	1.29
R2	<0.01	1.21	1.99	1.61	1.91
R3	<0.01	0.83	0.81	1.53	1.10
R4	<0.01	0.85	1.22	1.63	0.67
R5	<0.01	<0.01	0.13	0.05	-
R6	<0.01	<0.01	0.13	0.05	-
R7	<0.01	<0.01	0.02	0.02	-
R8	<0.01	<0.01	0.03	0.03	-

Table 5-4 Dissolved copper content in the reactors, mg/L

	week 14	**week 22**
R1	1.06	1.27
R3	0.49	0.70
R5	0.05	0.01
R7	< 0.002	0.008

There should have been 3mg/L of copper in reactors 1 to 4 for week 10 onwards (nominal concentration in the synthetic wastewater media). While there is less copper than expected in these reactors (for both total and dissolved copper), there is clearly significantly higher concentrations of copper present than in the amended reactors (R1-R4) than those not supplemented with copper (R5-R8).

The difference between total and dissolved copper indicates that copper is either present inside or bound to the bacterial cells; therefore, the unaccounted for copper in the amended reactors must be present in the floc. As biomass were allowed to settle before sampling, most of the floc were not sampled. The upper aqueous supernatant was removed and stored for analysis. The results confirmed reactors 1 – 4 had higher concentrations of copper than reactors 5-8; therefore, any differences in resistance to tetracycline or ampicillin was likely due to increased copper concentrations.

5.3.3 Plate Count Analysis

Samples were spread onto Plate Count Agar to determine the total CFU/ml of the sample, as well as agar amended with Cu, Amp, or Tet to get values for corresponding resistance levels. Resistant CFU values were divided by the total CFU values for each sample, so it represents the proportion of the population resistant to copper, tetracycline or ampicillin, rather than absolute values. Normalised CFU values are displayed in Figure 5-2, Figure 5-3 and Figure 5-4. Raw abundances and standard error values for weeks 12 and 22 are below in Table 5-5 and Table 5-6. All reactors display the same overall trends in terms of both

copper and ampicillin resistance; i.e., there were no differences in resistance levels. This implies the treatments had no effect on increasing either resistance phenotype (see Figure 5-2 and Figure 5-4). However, for tetracycline resistance, there was a clear difference between the reactors amended with copper (Reactors 1-4) and those not (Reactors 5-8) (see Figure 5-3).

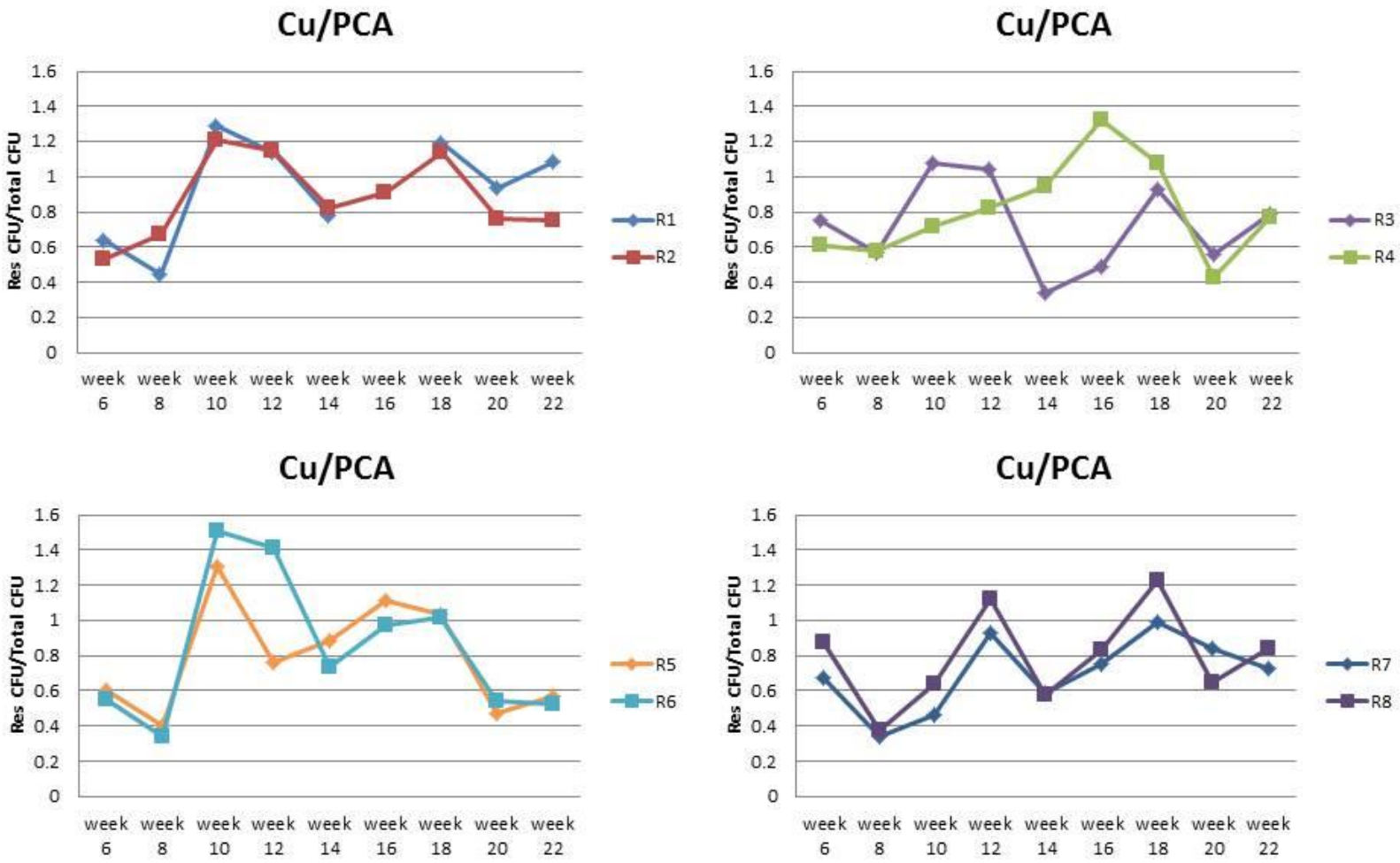

Figure 5-2 Relative copper-resistant CFU for each reactor over time. Aberrant values (R1 week 16) not shown.

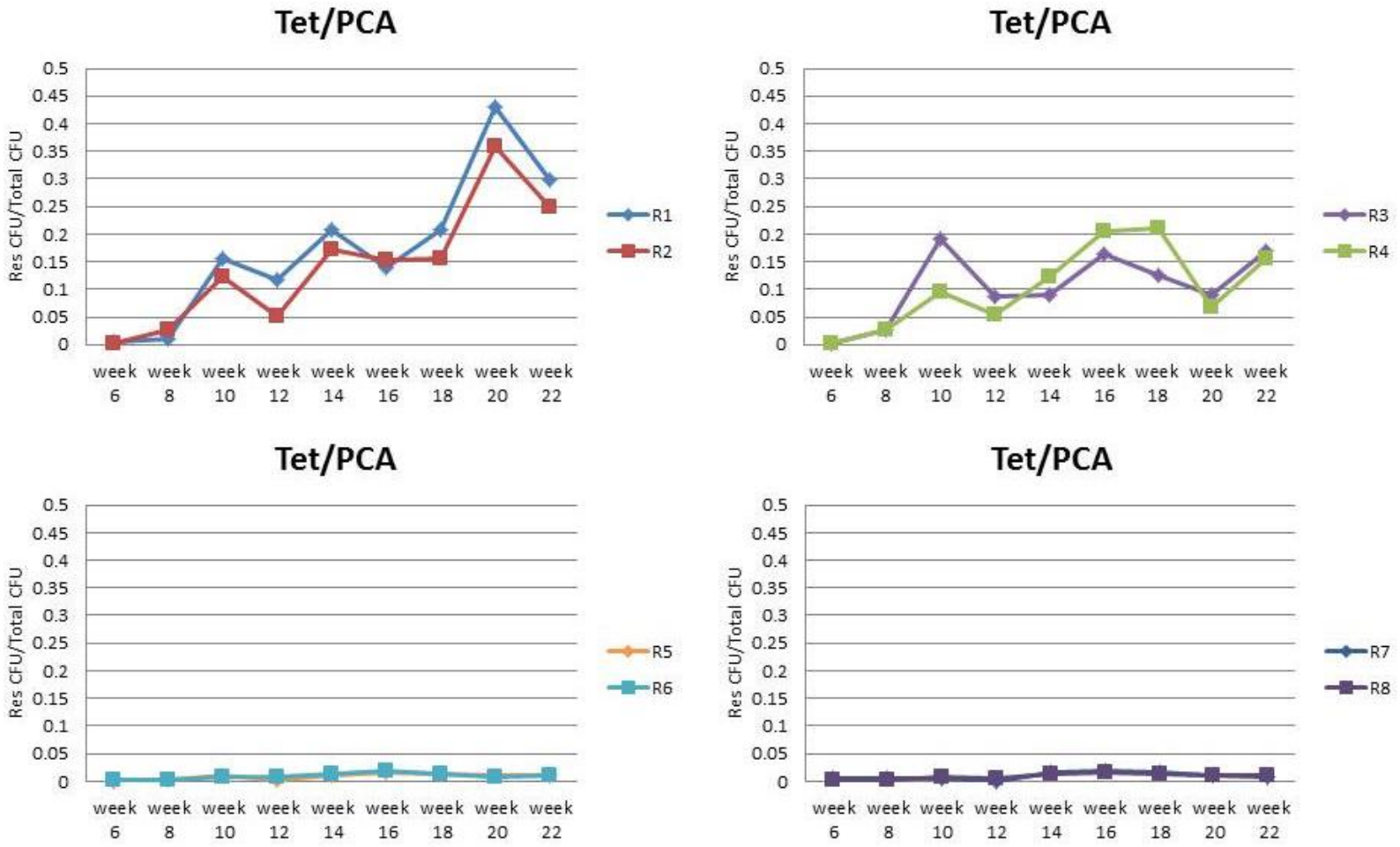

Figure 5-3 Relative tetracycline-resistant CFU for each reactor over time.

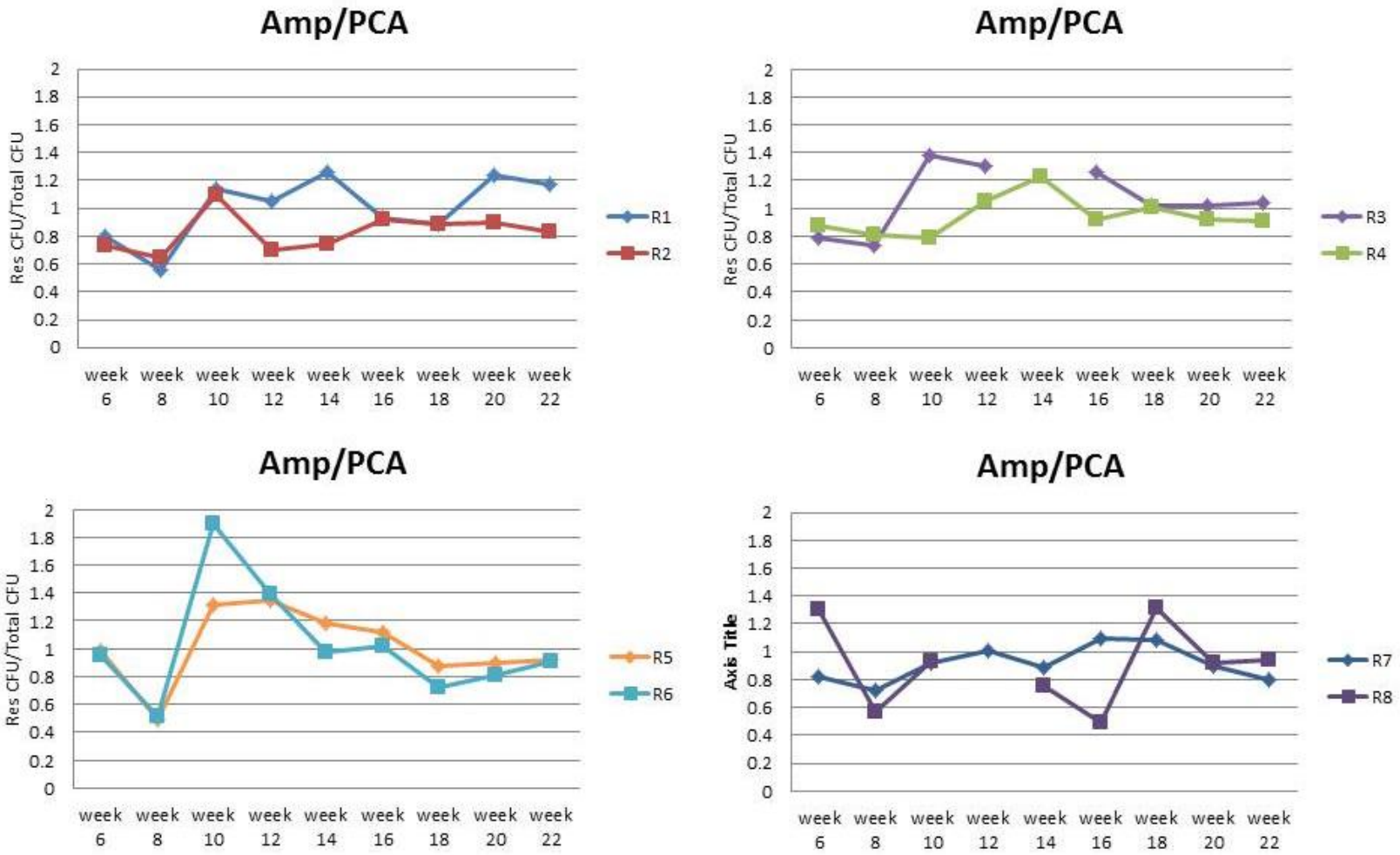

Figure 5-4 Relative ampicillin-resistant CFU for each reactor over time. Aberrant values (R3 week 14 and R8 week 12) not shown.

Table 5-5 Means and standard errors of resistance gene CFU/total CFU from week 12.

Reactor	Cu		Amp		Tet	
	Mean relative CFU	**Standard Error**	**Mean relative CFU**	**Standard Error**	**Mean relative CFU**	**Standard Error**
1	1.143	0.259	1.054	0.192	0.118	0.332
2	1.152	0.056	0.697	0.056	0.053	0.455
3	1.041	0.135	1.300	0.141	0.087	0.241
4	0.823	0.275	1.049	0.239	0.056	0.453
5	0.764	0.433	1.343	0.308	0.001	0.552
6	1.416	0.109	1.387	0.067	0.007	0.120
7	0.930	0.083	1.007	0.062	0.000	0.155
8	1.121	0.062	3.569	1.109	0.005	0.309

Table 5-6 Means and standard errors of resistance gene CFU/total CFU from week 22 (n=3).

Reactor	Cu		Amp		Tet	
	Mean relative CFU	**Standard Error**	**Mean relative CFU**	**Standard Error**	**Mean relative CFU**	**Standard Error**
1	1.085	0.182	1.169	0.202	0.298	0.163
2	0.753	0.127	0.833	0.225	0.250	0.167
3	0.790	0.216	1.038	0.152	0.169	0.169
4	0.767	0.078	0.909	0.143	0.156	0.083
5	0.568	0.138	0.925	0.136	0.010	0.238
6	0.521	0.088	0.913	0.040	0.011	0.152
7	0.730	0.153	0.794	0.138	0.008	0.250
8	0.841	0.342	0.941	0.081	0.010	0.096

ANCOVA was used to compare resistance levels between reactors for each of the data sets. *ANCOVA,* much like a traditional *ANOVA*, allows for the comparison of more than two treatments, but allows a covariant to be taken into consideration, in this case, time. The reactors (treatment type) were compared in terms of resistance levels over time. The F and P values obtained are in Table 5-7.

Table 5-7 F and P values for plate count *ANCOVA* comparisons by treatment type. Data were analysed for an entire phase to look at treatment based differences (n=11), but also as early and late phase (3 week blocks, n=6) to determine if/when changes in levels of resistance CFU occurred. Statistically significant values (P<0.05) are indicated by *.

Weeks	**Cu/PCA**		**Tet/PCA**		**Amp/PCA**	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**
6,8,10 (early phase 2)	0.86	0.559	0.81	0.596	0.91	0.527
10,12,14 (late phase 2)	1.54	0.229	8.84	0.000*	0.66	0.704
6-14 (entire phase 2)	0.95	0.481	2.94	0.018*	1.38	0.250
14,16,18 (early phase 3)	2.87	0.041*	0.82	0.589	0.50	0.817
18,20,22 (late phase 3)	0.23	0.972	0.53	0.800	0.25	0.963
14-22 (entire phase 3)	0.57	0.771	1.12	0.374	0.32	0.940

Reactors were compared either as three time points (i.e. early phase or late phase), or as an entire phase. There were no statistically significant differences between reactors in terms of ampicillin resistant CFU at any stage of the experiment. Copper resistant CFU abundance was significantly different between reactors for weeks 14, 16 and 18, but by looking at the chart, it is obvious this difference was due to week 16. CFU abundances between reactors in each pair (same treatment) were not consistent; therefore, the significant differences could not be attributed to a treatment, rather inconsistency between reactors.

During weeks 10 to 14, there was a statistical difference in tetracycline resistant CFU between reactors (P<0.05). There was also significant difference from week 6-14 (P<0.05, likely due to the difference already mentioned from weeks 10-14). All reactors had equivalent levels of tetracycline resistance before week 10. The only treatment difference at this point was the addition of copper, so it could be the cause of increased tetracycline resistance. The trend charts indicate the difference between copper amended and un-amended continues until the end of the experiment (week 20), but the *ANCOVA* did not support this.

Suspecting that the statistical power of the *ANCOVA* was not adequate to distinguish differences, a two-tailed *t* test was used to compare tetracycline resistant values between R1-R4 and R5-R8 (copper amended and control reactors). This was only carried out for specific time points previously found to be significant by the *ANCOVA* (weeks 6-14; see Table 5-7), as well as weeks 14-22, which were not significant according to *ANCOVA* (Table 5-9).

Table 5-8 P values for *t* tests comparing tetracycline resistant CFU abundances in Phase 2 (weeks 6-14). Statistically significant values (p<0.05) are indicated by *.

Weeks 6,8,10	**R1-4**	**R5-8**
R1-4	~	0.084
R5-8	0.084	~
Weeks 10,12,14	R1-4	R5-8
R1-4	~	0.000*
R5-8	0.000*	~
Weeks 6-14	R1-4	R5-8
R1-4	~	0.000*
R5-8	0.000*	~

During late phase 2 (weeks 10 to weeks 14), there was a statistically significant difference between copper amended (R1-4) and control reactors (R5-R8). This backs up what can be interpreted from the data plotted in Figure 5-3 and the *ANCOVA* analysis of the data sets, there are higher proportions of tetracycline resistant bacteria in reactors amended with copper. The difference in tetracycline CFU between reactors amended with copper (R1-R4) and those without (R5-R8), continued to be significant for the duration of phase 3 (weeks 14-22, see Table 5-9).

For weeks 18-22 (late phase 3), there is a significant difference between R1+R2 (amended with copper and antibiotics) and R3+R4 (amended with copper only). This is also the case for R5+R6 (amended with antibiotics) and R7+R8 (controls). R1+R2 had higher levels of tetracycline resistance than R3+R4; therefore, the presence of tetracycline helped generate increased tetracycline tolerance, potentially working in synergy with copper. R5+R6 and R7+R8 have approximately equivalent levels of resistant CFU.

Table 5-9 P values for T tests comparing tetracycline resistant CFU abundances for Phase 3 (weeks 14-22). Significant values (P<0.05) indicated by *.

14,16,18	**R 1+2**	**R3+4**	**R5+6**	**R7+8**
R 1+2	~	0.404	0.000*	0.000*
R3+4	0.404	~	0.001*	0.001*
R5+6	0.000*	0.001*	~	0.232
R7+8	0.000*	0.001*	0.232	~
18,20,22	**R 1+2**	**R3+4**	**R5+6**	**R7+8**
R 1+2	~	0.014*	0.001*	0.001*
R3+4	0.014*	~	0.002*	0.002*
R5+6	0.001*	0.002*	~	0.635
R7+8	0.001*	0.002*	0.635	~
14-22	**R 1+2**	**R3+4**	**R5+6**	**R7+8**
R 1+2	~	0.014*	0.000*	0.000*
R3+4	0.014*	~	0.000*	0.000*
R5+6	0.000*	0.000*	~	0.474
R7+8	0.000*	0.000*	0.474	~

Overall, the reactors amended with copper had higher levels of tetracycline CFU. Copper somehow selected for, or increased, resistance to tetracycline during weeks 10-22 of the experiment.

Cross-resistance plate count analysis was attempted. Resistant colonies were challenged with a stressor then patched on another plate with a different chemical exposure. For various reasons discussed previously (in Chapter 2), they proved unsuccessful. The few successful results were from tetracycline-resistant colonies re-plated on copper during weeks 6 and 8, and *vice versa*. This represented the start of the copper amendment phase. A total of 150 individual colonies were selected from the appropriate amended agar, and

patched out as 3 batches of 50 colonies. After enumeration, results were averaged and then converted to give percentage of cross resistance. Values for all reactors were then averaged and the 95% confidence intervals determined. Results are shown below in Table 5-10. Results are reasonably consistent between all reactors, which as previously mentioned, was to be expected. What was unexpected, however, was the difference between the percentage of copper resistant colonies, also resistant to tetracycline (45-48%) and the percentage of tetracycline resistant colonies, also resistant to copper (82-88%). The limited results suggest a tetracycline resistance phenotype is frequently displayed by cells that are also copper resistant, however a copper resistant phenotype is just as often found in isolation as it is with a tetracycline resistance phenotype. However, with limited data available, it is impossible to determine whether this would have been consistent for the duration of the experiment.

Table 5-10 Copper- and tetracycline cross-resistance during weeks 6 and 8 (copper resistant colonies re-plated on tetracycline and *vice versa*). Values represent means and 95% confidence intervals for reactors.

Reactor	Cu resistant on tet		Tet resistant on Cu	
	Week 6	Week 8	Week 6	Week 8
mean	48%	45%	82%	88%
95% CI	5%	4%	10%	6%

5.3.4 DGGE Analysis of Population Diversity

As previously discussed (section 7.2.3), DGGE was used to examine changes in bioreactor population diversity. Banding patterns for all reactors at week 6 (end of phase one), week 14 (end of phase two/start of phase three) and week 22 (end of phase three) were obtained and are shown below – Reactors 1-4 in Figure 5-5 and Reactors 5-8 in Figure 5-5.

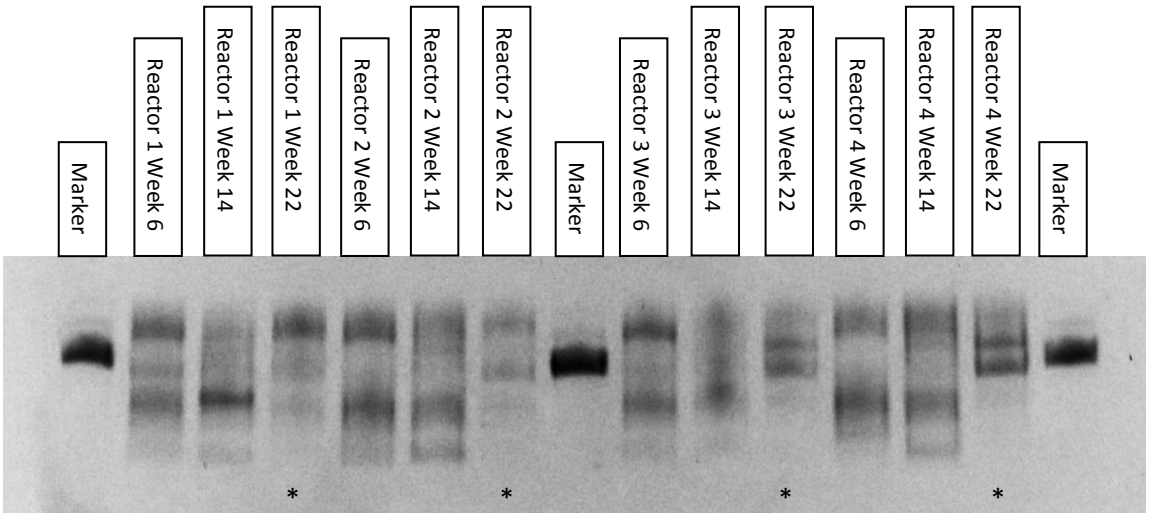

Figure 5-5 DGGE banding patterns obtained for Reactors 1-4 at both the start and end (week 6 and week 14) of Phase 2 (copper amendment), and both the start and end (week 14 and week 22) of Phase 3 (copper and antibiotic amendment). Lanes showing a decrease in diversity due to disappearance of a band are indicated by *.

Bacterial population diversity was consistent throughout phase two of the experiment (during copper amendment); however during phase three, diversity declined as indicated by the disappearance of a band from week 14 to week 22. This decrease in diversity was observed for reactors 1-4, despite the fact that during phase three, the reactors were experiencing different amendments – reactors 1 and 2 had copper, ampicillin, and tetracycline added to their feed, while reactors 3 and 4 were only amended with copper. Therefore, the decline in diversity was not a result of the antibiotic amendment. The moment when diversity declined needs to be better determined to help elucidate the cause.

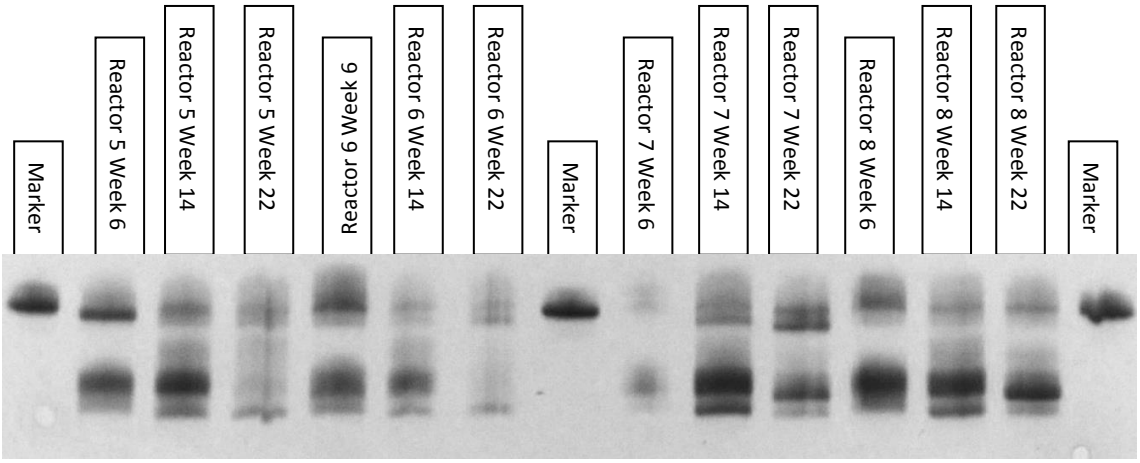

Figure 5-6 DGGE banding patterns obtained for Reactors 5-8 at both the start and end (week 6 and week 14) of Phase 2 (copper amendment), and both the start and end (week 14 and week 22) of Phase 3 (copper and antibiotic amendment).

The banding pattern displayed by reactors 5-8 was consistent for all reactors, for the duration of phase two and three, confirming antibiotics had no effect on decreasing bacterial diversity (reactors 5 and 6 were amended with tetracycline and ampicillin during phase 3, but diversity remained consistent). Additionally reactors 1-4 shared the pattern displayed by reactors 5-8 during phase two. Therefore, all reactors had the same bacterial community diversity (prior to the decline in diversity shown by reactors 1-4). The use of a single sample of activated sludge to inoculate the reactors weekly was successful at ensuring experimental consistency.

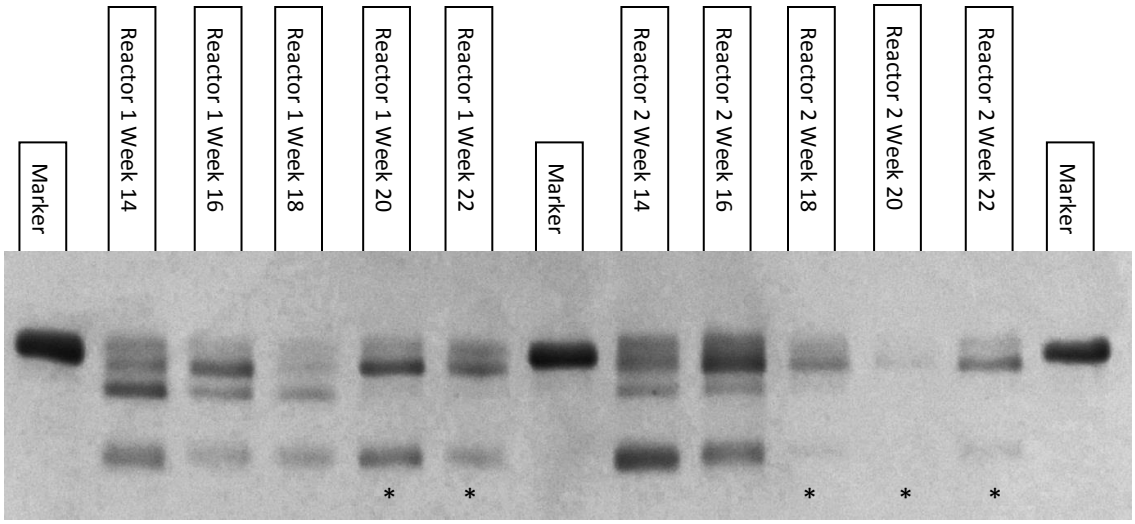

Figure 5-7 DGGE banding patterns obtained for Reactors 1 and 2 during phase three (weeks 14, 16, 18, 20 and 22) when both reactors were amended with copper, ampicillin and tetracycline. Lanes showing a decrease in diversity due to disappearance of a band are indicated by *.

For reactor 1 (Figure 5-7), the third band disappeared at week 20 and this continued for week 22. For reactor 2, the same band got lighter at week 18 before disappearing completely for weeks 20 and 22. Additionally for reactor 2, the bottom band became lighter at week 18, and it remained this way until the end of the experiment. Overall, there was a decrease in bacterial diversity in the last few weeks of the phase 3 for both reactors 1 and 2.

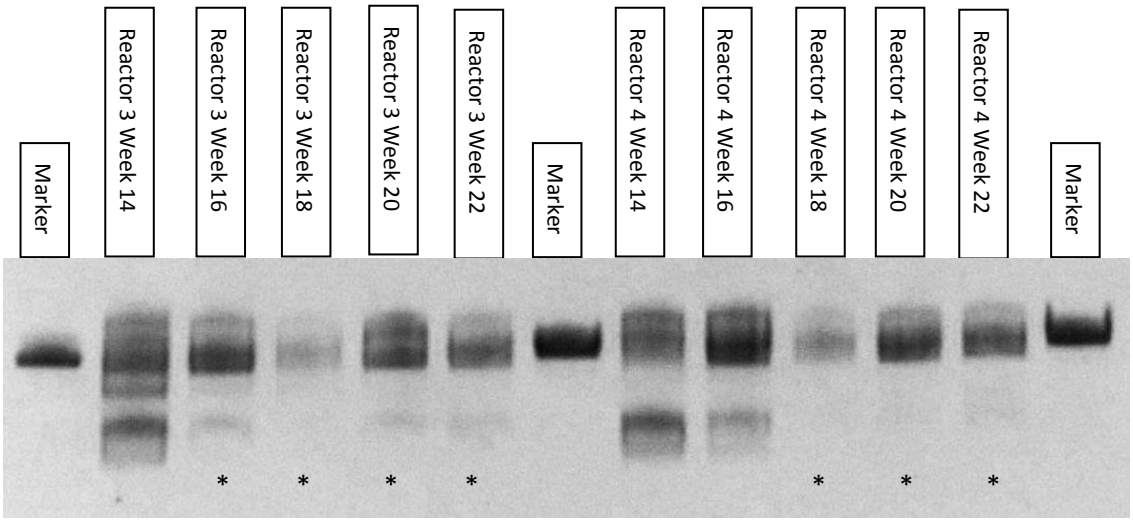

Figure 5-8 DGGE banding patterns obtained for Reactors 2 and 3 during phase three (weeks 14, 16, 18, 20 and 22) when both reactors were amended with copper. Lanes showing a decrease in diversity due to disappearance of a band are indicated by *.

For reactor 3 (Figure 5-8), the third band down became lighter at week 16, before disappearing completely for the rest of the experiment, while the bottom band became significantly lighter at week 16 and remained so for the rest of the experiment. For reactor 4, the bottom band disappeared in week 18. Like with reactors 1 and 2, the bacterial population diversity decreased in reactors 3 and 4 during the final weeks of the experiment.

The decrease in bacterial diversity was conserved across reactors 1-4, with the same bands getting lighter or disappearing for both reactor pairs. This indicated the shift in diversity was likely due to a common causitive agent; all four reactors were exposed to copper. Reactors 5-8 were not amended with copper and did not display any decrease in population diversity. Other than the addition of copper to the feed for reactors 1-4, all reactors were identical.

Copper addition began in week 6; however, a decrease in diversity did not become apparent until week 16. DGGE looks at DNA and could detect dead, or inactive, cells. It takes three times the hydraulic retention time to effectively flush out the system—in this case, about 27 days. Based on this, it may be weeks 10-12 before any declining populations resulting from the presence of copperare able to be detected.

The marker used in all DGGE gels was created using DNA extracted from a colony isolated from one of the control bioreactors. The colony was sequenced and identified as *Stenotrophomonas maltophilia* (see Chapter 2 for further details). *S. maltophilia* has a GC content of 66.7% and has a "remarkable capacity for drug and heavy metal resistance" (46). All bands that got lighter or disappeared from reactors 1-4 banding patterns had relative mobilities greater than the marker; therefore, they had a higher GC-content than *S. maltophilia*. There are few genera of bacteria that have higher GC contents than *S. maltophilia*. Actinomycetes, such as *Streptomyces*, have high GC content, e.g., *Str. coelicolor* having a GC content of 72% (47). *Streptomyces* are responsible for the production for many antibiotics and naturally carry resistance genes to protect themselves. They are ubiquitous in the environment and are often resistant to heavy metals. It is, therefore, surprising that they would decline in the system.

Attempts were made to excise bands that disappeared from the acrylamide gels and send them to be sequenced, to confirm the species of bacteria; however, the PCR products were too short and resulted in insufficient reads to make an identification using BLAST. PCR products chould have been cloned into plasmids and then sequenced, but it was too costly. While knowing the identity of the bacteria that disappeared is interesting, it is not essential to understand what has happened in the system.

The increase in tetracyline resistant CFU displayed by reactors 1-4, was observed after week 10. The increase in resistance may be linked to the decrease in diversity – resistant bacteria could be out competing non-resistant bacteria causing an increased proportion of the population to be resistant to tetracycline. As previously discussed, a high percentage of tetracycline bacteria were also found to be resistant to copper in weeks 6 and 8.

5.3.4 qPCR Analysis

qPCR was used to quantify the 16S rRNA gene, select tetracycline resistance genes, and β lactam resistance genes bla_{CTX} and bla_{TEM}. Attempts to quantify the copper resistance gene *copA* proved unsuccessful. Values for resistance gene/16S have been averaged for each pair of reactors and are listed below in Table 5-11 to Table 5-15, with the range values in brackets below the mean.

Table 5-11 Tet1/16S qPCR resistance gene abundances (log transformed) averaged for each reactor pair. Range values displayed in brackets.

Tet1/16S	week 6	week 8	week 10	week 12	week 14	week 16	week 18	week 20	week 22
R1+R2	-0.343 (0.071)	-0.200 (0.417)	0.011 (0.440)	-0.327 (1.109)	-1.956 (0.448)	-2.026 (0.413)	-2.246 (0.427)	-1.338 (1.622)	-1.641 (1.016)
R3+R4	0.239 (0.777)	0.277 (0.111)	-0.384 (1.043)	-1.561 (0.783)	-1.595 (2.019)	-2.030 (0.155)	-1.360 (0.076)	-1.099 (2.367)	-1.773 (1.524)
R5+R6	0.329 (0.169)	0.064 (0.387)	-0.556 (0.787)	-1.780 (0.490)	-2.991 (0.210)	-3.010 (0.254)	-2.228 (0.005)	-2.498 (0.122)	-2.848 (0.221)
R7+R8	-0.093 (0.150)	-0.006 (0.238)	-0.887 (0.124)	-2.334 (0.448)	-2.639 (0.199)	-2.577 (0.088)	-2.101 (0.486)	-1.646 (0.278)	-1.933 (0.273)

Table 5-12 Tet2/16S qPCR resistance gene abundances (log transformed) averaged for each reactor pair. Range values displayed in brackets.

Tet2/16S	week 6	week 8	week 10	week 12	week 14	week 16	week 18	week 20	week 22
R1+R2	-0.963 (0.443)	-1.070 (0.819)	-1.230 (0.425)	-1.130 (0.304)	-1.625 (0.175)	-1.689 (0.262)	-1.837 (0.669)	-1.482 (1.944)	-1.911 (0.805)
R3+R4	-0.738 (0.893)	-0.761 (0.144)	-1.546 (0.913)	-1.862 (0.787)	-1.680 (1.804)	-2.324 (0.158)	-1.273 (0.237)	-1.223 (2.013)	-1.791 (1.267)
R5+R6	-0.663 (0.172)	-0.991 (0.175)	-1.484 (0.658)	-1.731 (0.622)	-2.624 (0.195)	-2.550 (0.328)	-2.366 (0.760)	-2.697 (0.077)	-2.364 (0.278)
R7+R8	-1.083 (0.019)	-1.037 (0.256)	-1.842 (0.068)	-2.265 (0.484)	-2.115 (0.009)	-2.159 (0.022)	-2.192 (0.213)	-1.572 (0.845)	-1.846 (0.244)

Table 5-13 Tet3/16S qPCR resistance gene abundances (log transformed) averaged for each reactor pair. Range values displayed in brackets.

Tet3/16S	week 6	week 8	week 10	week 12	week 14	week 16	week 18	week 20	week 22
R1+R2	-0.983 (0.226)	-0.897 (0.319)	-1.228 (0.104)	-1.010 (0.103)	-2.938 (0.277)	-2.653 (0.168)	-1.854 (0.434)	-1.399 (1.136)	-1.974 (0.831)
R3+R4	-0.802 (0.448)	-0.630 (0.539)	-2.075 (1.936)	-3.414 (0.726)	-3.239 (1.390)	-2.370 (0.086)	-0.893 (0.331)	-0.653 (2.515)	-1.691 (1.226)
R5+R6	-0.805 (0.025)	-0.739 (0.143)	-1.245 (0.862)	-2.582 (0.548)	-2.968 (0.047)	-2.878 (0.314)	-1.817 (0.629)	-1.858 (0.284)	-1.874 (0.489)
R7+R8	-0.994 (0.062)	-0.497 (0.104)	-1.706 (0.194)	-3.116 (0.544)	-2.840 (0.530)	-2.489 (0.025)	-1.353 (1.589)	-1.302 (0.131)	-1.748 (0.285)

Table 5-14 *bla*$_{TEM}$/16S qPCR resistance gene abundances (log transformed) averaged for each reactor pair. Range values displayed in brackets.

bla$_{TEM}$/16S	week 6	week 8	week 10	week 12	week 14	week 16	week 18	week 20	week 22
R1+R2	-2.645 (0.101)	-2.640 (0.386)	-3.222 (0.159)	-3.045 (0.387)	-3.162 (0.517)	-4.046 (0.088)	-3.713 (0.281)	-3.395 (0.933)	-3.868 (1.467)
R3+R4	-2.404 (0.540)	-2.778 (0.097)	-3.651 (0.759)	-4.003 (0.865)	-3.627 (1.264)	-3.756 (0.293)	-2.552 (0.354)	-3.459 (0.350)	-3.514 (1.155)
R5+R6	-2.034 (0.326)	-2.999 (0.541)	-3.381 (0.505)	-3.894 (0.323)	-4.960 (0.474)	-5.019 (0.514)	-4.023 (0.670)	-3.835 (0.288)	-4.128 (0.161)
R7+R8	-2.878 (0.272)	-2.887 (0.298)	-3.687 (0.301)	-4.309 (0.634)	-4.793 (0.324)	-4.727 (0.130)	-4.530 (0.757)	-3.461 (0.117)	-4.769 (1.282)

Table 5-15 *bla*$_{CTX}$/16S qPCR resistance gene abundances (log transformed) averaged for each reactor pair. Range values displayed in brackets.

bla$_{CTX}$/16S	week 6	week 8	week 10	week 12	week 14	week 16	week 18	week 20	week 22
R1+R2	-1.410 (0.327)	-1.392 (0.735)	-1.787 (0.813)	-1.845 (0.488)	-2.350 (0.340)	-3.259 (0.118)	-3.991 (0.532)	-0.687 (1.829)	-1.206 (1.010)
R3+R4	-1.498 (0.033)	-1.181 (0.168)	-2.101 (0.821)	-2.947 (0.773)	-2.610 (1.469)	-3.265 (0.543)	-2.305 (0.891)	-0.410 (1.454)	-0.928 (1.434)
R5+R6	-0.958 (0.215)	-1.650 (0.536)	-1.687 (0.679)	-2.123 (0.103)	-3.417 (0.707)	-3.798 (0.404)	-1.486 (0.005)	-1.612 (0.289)	-1.909 (0.658)
R7+R8	-1.460 (0.528)	-1.922 (0.610)	-1.963 (0.380)	-2.470 (0.971)	-3.078 (0.720)	-3.498 (0.137)	-1.392 (0.300)	-0.876 (0.104)	-0.309 (0.840)

In order to compare differences between reactors in terms of resistance gene abundance, *ANCOVA* analysis of variance was used to analyse each data set, with time as the covariant, (same as with the plate count analysis). P values for all resistance genes are found in

Table 5-16 below.

Table 5-16 qPCR ANCOVA results: F and P values. Data was analysed an entire phase to look at treatment based differences, but also as early and late phase (3 week blocks) to determine if/when changes in levels of resistance gene abundance occurred. Statistically significant values ($P \leq 0.05$) are indicated by *

Weeks	Tet1		Tet2		Tet3		TEM		CTX	
	F value	P value	F value	P value	F value	P value	F value	P value	F value	P value
6,8,10 (early phase 2)	1.05	0.44	1.32	0.31	0.45	0.85	1.11	0.41	0.72	0.66
10,12,14 (late phase 2)	3.00	0.04*	1.62	0.21	1.82	0.16	2.50	0.06	1.25	0.33
6-14 (entire phase 2)	1.60	0.17	1.80	0.12	0.93	0.50	2.16	0.07	1.12	0.37
14,16,18 (early phase 3)	2.77	0.05*	2.35	0.08	1.14	0.39	4.49	0.02*	0.21	0.98
18,20,22 (late phase 3)	2.82	0.04*	3.01	0.04*	2.71	0.05*	1.49	0.25	0.97	0.49
14-22 (entire phase 3)	3.05	0.02*	2.39	0.05*	0.91	0.52	3.29	0.03*	0.53	0.80

The P values (≤0.05) indicate there was statistically significant difference in Tet1 [*tet*(B), *tet*(C) and *tet*(D)] resistance gene abundances between reactors from week 10 to week 22. While for both Tet2 [*tet*(A), *tet*(E) and *tet*(G)] and Tet3 [*tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S)] resistance gene groups, there were statistically significant differences in gene abundances between the reactors for weeks 18-22. bla_{TEM} abundances varied significantly between the reactors for weeks 14-18 only.

While *ANCOVA* tells us that there is a difference between the reactors in terms of resistance gene abundance, it is unclear which reactors had increased levels of resistance genes; therefore, it cannot be determined whether the results are due to a treatment-based effect; i.e. amended reactors had higher gene abundances than controls. Students *t* test was used to compare each pair of reactors for weeks/genes that yielded statistically significant results when analysed by *ANCOVA* (see Table 5-17 for genes/weeks of interest). T-test P values are found below in Table 5-18 to Table 5-21.

Table 5-17 qPCR gene abundances and corresponding weeks that were statistically significant based on *ANCOVA* analysis

Gene	Weeks	Weeks	Weeks	Weeks
tet1	10,12,14	14,16,18	18,20,22	14-22
Tet2	14,16,18	18,20,22	14-22	-
Tet3	18,20,22	-	-	-
$\boldsymbol{bla_{TEM}}$	14,16,18	14-22	-	-

Table 5-18 P values for *t*-test comparison of reactor pairs for Tet1 gene abundances. Significant values ($p<0.05$) are indicated by *.

Weeks		**R1-4**	**R5-8**		
10,12,14					
	R1-4	~	0.040*		
	R5-8	0.040*	~		
14,16,18		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.494	0.007*	0.046*
	R3+4	0.494	~	0.015*	0.058
	R5+6	0.007*	0.015*	~	0.121
	R7+8	0.046*	0.058	0.121	~
18,20,22		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.515	0.050*	0.657
	R3+4	0.515	~	0.033*	0.275
	R5+6	0.050*	0.033*	~	0.003*
	R7+8	0.657	0.275	0.003*	~
14-22		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.612	0.000*	0.080
	R3+4	0.612	~	0.003*	0.076
	R5+6	0.000*	0.003*	~	0.003*
	R7+8	0.080	0.076	0.003*	~

Table 5-19 P values for *t*-test comparison of reactor pairs for Tet2 gene abundances. Significant values (p<0.05) are indicated by *.

Weeks 14,16,18		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.900	0.001*	0.007*
	R3+4	0.900	~	0.058	0.251
	R5+6	0.001*	0.058	~	0.030*
	R7+8	0.007*	0.251	0.030*	~
18,20,22		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.495	0.059	0.718
	R3+4	0.495	~	0.023*	0.267
	R5+6	0.059	0.023*	~	0.016*
	R7+8	0.718	0.267	0.016*	~

Table 5-20 P values for *t*-test comparison of reactor pairs for Tet3 gene abundances.

weeks 18,20,22		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.197	0.675	0.406
	R3+4	0.197	~	0.125	0.435
	R5+6	0.675	0.125	~	0.173
	R7+8	0.406	0.435	0.173	~

Table 5-21 P values for *t*-test comparison of reactor pairs for bla_{TEM} gene abundances. Significant values ($p<0.05$) are indicated by *.

weeks		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
14,16,18					
	R 1+2	~	0.371	0.007*	0.018*
	R3+4	0.371	~	0.006*	0.013*
	R5+6	0.007*	0.006*	~	0.514
	R7+8	0.018*	0.013*	0.514	~
14-22		**R 1+2**	**R3+4**	**R5+6**	**R7+8**
	R 1+2	~	0.168	0.008*	0.077
	R3+4	0.168	~	0.001*	0.009*
	R5+6	0.008*	0.001*	~	0.325
	R7+8	0.077	0.009*	0.325	~

Based on the above P values obtained by the T test analysis (Table 5-18) and the standardised resistance values themselves (Appendix 5), copper amendment had a significant effect on tet1 abundance - Reactors 1-4 (copper amended) had significantly higher levels of tet1 resistance genes than reactors 5-8 (no copper amendment) for weeks 10-18. Additionally, copper-amended reactors had higher tet1 gene abundances, than reactors amended with tetracycline itself – reactors 1-4 had higher abundances of tet1 than R5+R6 (ampicillin and tetracycline amended weeks 14-22) from week 10 until the end of the experiment (week 22). Furthermore, tetracycline did not amplify the copper-related effect; i.e. R1+R2 (copper, ampicillin and tetracycline amended) did not have higher levels of tet1 in comparison to R3+R4 (copper amended) during phase 3 of the experiment (weeks 14-22).

Copper, ampicillin and tetracycline amendment (R1+R2) displayed increased tet2 gene abundances (P values listed in Table 5-19) in comparison to controls (R7+R8) and reactors

amended with ampicillin and tetracycline (R5+R6) during early phase 3. While copper-amended (R3+R4) reactors had higher tet2 abundances than antibiotic amended reactors (R5+R6) during late phase 3. It remains unclear whether copper and tetracycline is more effective at maintaining tet2 genes than copper alone; however, it is clear that copper has had a more significant effect on tet2 gene levels than tetracycline itself.

There were no significant differences in Tet3 abundances between reactor pairs (treatment type) (see Table 5-20). While there were overall, differences in abundances between reactors (based on *ANCOVA* analysis), both reactors in each pair did not exhibit the same trend, and therefore, the difference was not treatment based.

Copper amended reactors (R1-4) had significantly higher abundances of bla_{TEM} than reactors not amended with copper (R5-8) during phase 3 of the experiment (see Table 5-21) suggesting that copper had a more significant effect on bla_{TEM} abundances than ampicillin. Additionally, ampicillin did not seem to increase copper-related effects on bla_{TEM} abundances; i.e. R1+R2 did not have higher abundances of bla_{TEM} than R3+R4.

In summary, copper-amended reactors had significantly higher abundances of tetracycline-resistance (tet1 and tet2) and bla_{TEM} genes, than those not exposed to copper. Additionally copper had more of an effect on the abundances of these antibiotic resistance genes than the antibiotics the genes confer resistance to.

The experimental aim was to determine whether a stressor such as copper, could increase antibiotic resistance. WWT plants act as the interface between the clinical and natural environments; therefore, they may play a role in the cycling of antibiotic resistance genes and contribute to the clinical resistance problem. WWT currently aims to reduce the overall bacterial load prior to releasing effluent to the environment, there is however no consideration given to genetic pollution, or the antibiotic resistance profile of the effluent. WWT has been shown to potentially select for or maintain antibiotic resistance- higher proportions of resistance bacteria have been detected leaving the WWT plants than what originally entered (15-17, 48). While the mechanisms by which this selection of antibiotic resistance occurs are largely unknown, the fact that activated sludge acts as a heavy-metal sink, it is not too far a stretch to suggest metals in WWT could be playing a role in selecting and maintaining antibiotic resistance.

Copper increased the proportion of tetracycline resistant bacteria in the bioreactors. This was most evident from late phase 2 (week 10) and continued for the duration of the experiment. Reactors amended with both copper and antibiotics had significantly higher proportions of tetracycline resistant bacteria than reactors amended with copper alone, suggesting a possible synergistic, or additive, effect between copper and tetracycline.

Cross-resistance analysis suggest that, although a tetracycline resistance phenotype is usually present in conjunction with a copper resistance phenotype, copper resistance is less frequently found with tetracycline resistance. While copper may be associated with multiple tetracycline resistance determinants, a smaller overall proportion of copper resistance mechanisms may be linked to tetracycline resistance.

The increase in tetracycline CFU within copper amended reactors (in comparison to those not amended with copper) as previously mentioned became statistically significant in week 10. As this corresponds to the time tet1 resistance gene abundances also increased, it is reasonable to suggest that the two are linked, i.e. the increased tetracycline resistance phenotype is due to the dissemination of tet1 resistance genes. Both the plate count and qPCR analysis are standardised and refer to the proportion of the population displaying the phenotype or carrying a gene, so when a greater proportion of the population had copies of tet1 genes, a greater proportion of the population displayed a tetracycline resistance phenotype.

The DGGE banding patterns indicated that the population diversity within copper amended reactors (R1-R4) displaying increased tetracycline resistant CFU and tet1 resistance gene abundances was consistent from week 10 until week 16. Taking into consideration any lag between a decrease in diversity occurring and the time it would take to become apparent by DGGE analysis, the earliest diversity in copper amended reactors began to decline is week 12. Therefore, it is unlikely that the increase of both the tetracycline resistant phenotype and tet1 resistance gene abundances in copper amended reactors, which became apparent during week 10, are linked to the decreased population diversity. While DGGE gives an idea of shifts in population dynamics it is not sensitive enough to detect and display more discrete changes, so the assumption that a decrease in diversity is not responsible for increased resistance levels may not be correct. Newer more sensitive methods such as metagenomics and next generation sequencing would allow for a more

detailed analysis of the changes in community dynamics. However, these methods are expensive and the data analysis can be tricky, therefore due to lack of funding and the time it would take to acquire competency in bioinformatics it was decided that DGGE was a more appropriate method of analysis. Copper is somehow maintaining tet1 resistance genes abundances, leading to a corresponding increase in tetracycline resistant CFU. Copper could be linked to tet1 resistance genes by either a cross- resistance (one gene conferring resistance to both) or co-resistance mechanism, i.e. two genes on same genetic material.

The effect copper has on tet2 gene abundances is perhaps aided by the presence of tetracycline and or ampicillin. Reactors amended with both copper and antibiotics had higher abundances of resistance genes than controls or reactors amended with copper or antibiotics only. Copper is having a clear effect; however, the role antibiotics play themselves in resistance maintenance is less pronounced.

The difference in tet2 abundance between copper amended reactors and those not supplemented with copper became apparent in week 14. This coincides roughly with the decrease in diversity shown by copper amended reactors (likely sometime after week 12), and suggests those tet2 genes are being selected for/maintained somewhat.

In this study, copper exposure at sub-lethal concentrations, saw a corresponding increase in tetracycline resistance, however copper resistance levels did not significantly differ as a result of copper exposure (based on plate count analysis). This makes the link between copper and antibiotic resistance difficult to interpret, as it would have been expected copper resistance would have increased along with tetracycline resistance if both resistance determinants were being transferred in unison. As discussed in Chapter 4, genes that regulate copper resistance/homeostasis mechanisms may be linked to antibiotic resistance. CopY is a copper responsive repressor which controls transcription of copper resistance genes (49), however it may also activate β lactam resistance (50). Additionally, CueR, another transcriptional regulator of copper resistance genes (51), has binding sites other than copper resistance genes. In fact in *E. coli's* genome, there are 129 CueR box like sites with 54 of these sites being associated with genes of unknown function (52). Copper could be interacting with copper responsive repressors leading to the transcription of antibiotic resistance genes.

The tet1 group of tetracycline genes included the genes *tet*(B), *tet*(C) and *tet*(D). All three encode efflux pumps, which extrude tetracycline from the cell. Many efflux pumps are non-specific and deal with more than one substrate, therefore it is possible (but perhaps unlikely) one of them could also be involved with copper homeostasis. *tet*(B) has an extremely broad host range and is normally plasmid associated, but can also been found on the transposon *Tn*10 (53). Additionally *tet*(B) is linked with several antibiotic resistance genes including bla_{TEM}, but most interestingly *int1*, a class 1 integron, suggesting that it is highly transferable. *Tet*(C) and *tet*(D) are also plasmid associated, but it remains unknown whether they are linked to other resistance genes or genetic elements. The tet2 group of genes included *tet*(A), *tet*(E) and *tet*(G). As with tet1 genes, they are all efflux genes and normally associated with Gram-negative bacteria. Specifically *tet*(A) is plasmid-associated and has been linked to mercury resistance (53), while *tet*(G) is found both on plasmids and chromosomal DNA. *tet*(E) is associated with aquatic bacteria making it particularly relevant to this study; however, it is commonly found on non-conjugative plasmids (53). *tet*(A) is already associated with another metal, but this does not necessarily lead to the assumption it is also linked with copper.

The primary hypothesis for this experiment - whether elevated copper levels result in a corresponding increase in antibiotic resistance genes abundance has been shown to be correct. Whether subsequent hypotheses - i.e., does the rate of resistance development differ based on whether the bacterial population has been exposed to copper or an antibiotic; or whether copper and an antibiotic interact and enhance resistance development – are also correct, is more difficult to determine. The rate of resistance development is more difficult to compare. Copper increased tetracycline resistant CFU and tet1 resistance gene abundances from week 10 onwards. Increases in tet2 and bla_{TEM} became apparent from weeks 14 onwards therefore after antibiotic amendment began, however, as resistance levels were significantly higher in reactors amended with copper, the impact of antibiotics is overshadowed. Therefore, copper likely induces antibiotic resistance development at a faster rate than antibiotics themselves; however, further investigations would be required to confirm this.

5.4 Future Work

While the work here suggests a possible link between copper and antibiotic resistance within a WWT environment, considerable future work is needed if this is to translate to making a real world contribution to reducing the clinical resistance problem.

- The mechanism linking copper to tetracycline and ampicillin resistance needs to be elucidated.
- A robust plate count methodology for cross resistance analysis needs further development. It may prove useful in uncovering the link between copper and antibiotic resistance
- CopA multiplex primers for qPCR need to be designed. As designing a single set of primers to target a wide range of bacteria proved unsuccessful, perhaps a multiplex approach such as the one utilised to quantify tetracycline resistance genes would be of benefit.
- Investigate other combinations of metals and antibiotics - test metals in combination to determine whether there is a synergistic effect in a more real life situation.

5.5 Conclusions

Copper has been shown here to select for or maintain antibiotic resistance within a WWT microcosm. This work has implications in terms of how we treat our waste, and it has confirmed a suspected link between copper and antibiotic resistance. Previous work has shown a correlation between concentration of copper and gene abundance, however this provides experimental evidence that copper can select for and increase tetracycline- (and ampicillin-) resistance phenotype (20, 54, 55).

Elucidating the exact mechanism that links copper and tetracycline (and to a less extent ampicillin) resistance is of utmost importance. The results presented here, the impact copper had on increasing resistance phenotypes, and gene abundances is of real world interest and suggests a review of WWT practices needs to be revised.

5.6 References

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Chapter 6: Does the Presence of Copper and/or Tetracycline in Receiving Water Microcosms, Enhance Retention of Antibiotic Resistance Genes from Wastewater Treatment Discharge?

6.1 Background

It has been suggested the wastewater treatment (WWT) process can select for or enhance antibiotic resistance. This is based on comparisons between resistance levels in WWT influent and effluent, with greater proportions of resistance being present in effluent. Furthermore, zinc (1) and copper (see Chapter 5) have been shown to select for antibiotic resistance genes within WWT microcosms, implying metals are related to resistance selection effect mediated by the treatment process. Levels of antibiotic resistance genes have been found to be elevated downstream from WWT plants (2, 3), possibly indicating that the bacteria and resistance genes released after the treatment process persist in the environment. As metals may play a role in the selection and/or maintenance of antibiotic resistance, could the levels of metals present in receiving water influence environmental retention of resistance? Reducing the use of antibiotics themselves has had limited impact on reducing the levels of resistance (4), and the environmental gene reservoir has been implicated in maintaining resistance. Antibiotic runoff from agricultural land, or heavy metals (naturally present or because of industrial contamination) may be exacerbating the problem created by the WWT process in terms retaining the resistance genes the treatment process selected for.

Copper has been shown to select for/maintain tetracycline resistance genes in soil (Chapters 3 and 4); and wastewater treatment microcosms – Chapter 5. Could it also exhort the same effect on resistance levels in WWT receiving waters, meaning, areas with higher levels of copper are at increased risk of resistance gene retention in the environment?

6.1.1 Hypothesis

Increased (polluted) concentrations of copper in surface water microcosms will display elevated tetracycline resistance genes retention in comparison to control microcosms or those amended with background levels of copper and or tetracycline.

6.2 Methodology

6.2.1 Experimental Setup

To determine whether microcosms mimicking WWT receiving waters containing elevated concentrations of copper and/or tetracycline are able to maintain antibiotic resistance genes for a greater period of time, a modified version of the methodology described by Engemann et al (5) was used.

Twenty litres of water was collected from Loch Ard in Scotland, by the fill and withdraw method, and stored in a sterilised plastic drum for transport. Loch Ard is a freshwater lake downstream of Loch Chon in Queen Elizabeth forest park; and it should contain minimal industrial (metal) or agricultural (antibiotics, resistant bacteria or resistance genes) contamination.

Six microcosms were set up; 1.5 litres of the water from Loch Ard was added to each of the sterile 2-litre borosilicate beakers. Four of the beakers were then amended with copper in the form of copper sulphate. All six beakers were then covered in cling film (to reduce evaporation) and left to acclimatise at room temperature for 4 weeks. As tetracycline is known to degrade over time only copper was added initially for the acclimatization process. Copper concentrations of 0.14-10.6 μg/L have been detected in various streams throughout the UK (6), therefore 3μg-Cu/L was added to two beakers to simulate normal copper levels, and 30μg-Cu/L added to two others to simulate heavy copper pollution. Therefore a range of copper conditions found in aquatic systems was used.

After the four-week acclimatization period, three of the reactors were amended with 0.2mg/L of tetracycline: one of the controls and one of each pair of copper amended beakers. 0.2mg/L tetracycline was used in the previous study (Chapter 5) which provided the synthetic sewage discharge for this experiment; therefore, tetracycline concentrations were kept consistent (0.2mg/L was used to generate the resistance levels so it would likely be required to maintain the gene abundances).

All six beakers were then spiked with an activated sludge and synthetic wastewater mix, which was provided by a previous study (Chapter 5). While activated sludge is not the same as WWT effluent, as it contains more organic matter and a higher bacterial load than would be released from a plant, it was known to contain elevated levels of tetracycline resistance genes which was desirable for monitoring resistance gene decline. Engemann et al (5) used

1:50 dilution of inoculum to total volume, however a 1:100 dilution of sewage sludge was thought to be more appropriate for this experiment as real WWT effluent is likely to be more dilute.

Table 6-1 Surface water microcosm amendments

Microcosm Amendment Type
Control
0.2mg/L tetracycline
3μg/L of copper
3μg/L of copper+ 0.2mg/L tetracycline
30μg/L copper
30μg/L copper + 0.2mg/L tetracycline

All beakers were stirred continuously at 200rpm using mechanical stirrers to mimic the natural movement of water and minimise biofilm formation. Additionally they were exposed to 12+12 hour light-dark cycle (7am to 7pm) to correspond to the sunrise and sunset times for Scotland at the time the experiment was conducted, using a bank of grow-lamps Experimental setup can be seen in Figure 6-1.

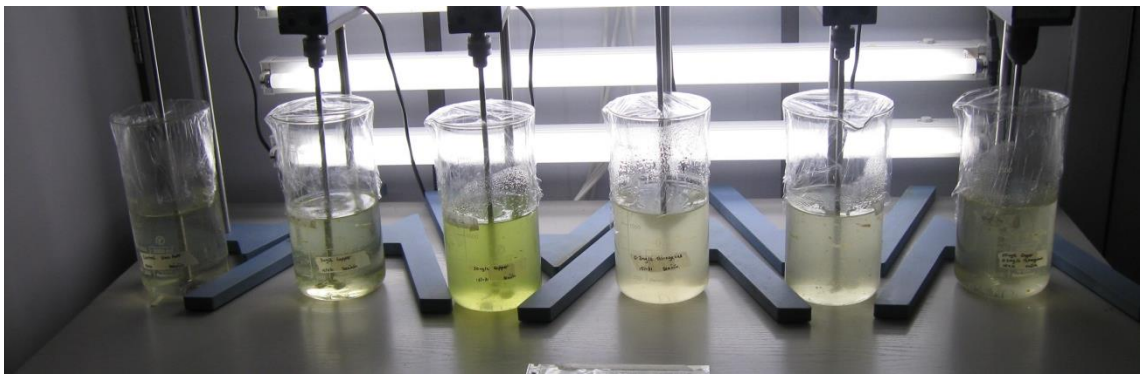

Figure 6-1 Experimental Setup

6.2.2 Sampling

Sampling occurred on days 0, 1, 4, 7, 14, 21 and 29 of the experiment. 100ml was poured out of each beaker and then sterile filtered with 0.2µm pore size, 47mm diameter cellulose nitrate membrane filters (Whatman, US) to capture bacterial cells. The filters were retained and stored at -80°C for later DNA extraction. The flow through was then passed through a DNA extraction filter using a vacuum manifold to capture and condense any "free DNA" contained in the samples. Free DNA was then eluted from the filters using 100µl of elution buffer. Samples were frozen short term at -20°C and long term at -80°C.

6.2.3 Molecular Analysis

DNA Extraction

DNA was extracted from bacterial cells on cellulose nitrate membrane filters using a FastDNA spin kit for soil (MP Bio, US). Filters were added to binding matrix E tubes and samples then processed following the manufacturer's instructions (further details in chapter 2). DNA was eluted using 100µl of elution buffer and samples were stored at -20°C short term and -80°C long term.

qPCR

qPCR was used to quantify antibiotic resistance genes as well as the 16S-rRNA gene (a surrogate method to quantify total bacteria). To save costs on reagents and to assay as many tetracycline genes as possible as they were of particular interest, multiplex qPCR was

used, meaning several tetracycline genes were quantified per reaction. Three groups of tetracycline genes were assayed (groups 1-3 from Ng et al. 2001 (7)). Groups 1 (*tet*(B), *tet*(C), *tet*(D)), 2 (*tet*(A), *tet*(E), *tet*(G)) both contain genes that encode efflux pumps, while group 3 (*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S)) contains both efflux pump and ribosomal protection genes.

bla_{TEM} and bla_{CTX} (8) β lactamase genes were also assayed as previous work (Chapter 3 and Chapter 5) had found an association between copper and the abundance of β lactamase resistance genes. Additionally both genes were present at detectable levels in the bioreactors that provided the initial wastewater inoculum.

3µl of sample DNA was added to 1µl of primer mix, 10µl of qPCR supermix and 6µl nuclease free water and 0.2µl of 100x SYBR green. Each assay was carried out in triplicate using a Biorad iCycler (USA). Full details of primers and reaction conditions used are found in Chapter 2. qPCR standards were created using one gene from each of the groups (again full details in Chapter 2).

6.3 Results

6.3.1 Copper Analysis

As four of the six microcosms were amended with copper, analysis was carried out to verify the concentrations of copper in all microcosms at t_{14} and t_{29} (middle and end of experiment). Samples were digested using aqua regia and the dissolved copper concentration was determined using an ICP-OES (Scottish Environmental Technology Network (SETN) at University of Strathclyde). Dissolved copper concentrations for all microcosms are shown below in Table 6-2.

Table 6-2 Dissolved copper concentration of microcosms mg/L

Dissolved Copper mg/L	T14	T29
Control	0.029	0.062
Cu3ug/L	0.023	0.091
Cu30ug/L	0.065	0.075
Tet	0.025	0.042
Cu3ug/L+Tet	0.033	0.039
Cu30ug/L+Tet	0.068	0.056

Copper was added to the microcosms four weeks prior to the commencement of the experiment to allow for acclimatisation. For t_{14}, microcosms amended with 30µg/ml of copper have as expected, higher concentrations of copper than the other microcosms. Copper concentrations for all microcosms increased from t_{14} to t_{29}. As it was the dissolved copper and not total copper that was measured, the amount of copper stored with in bacterial cells will affect the results. This could account for the increase in copper from t_{14} to t_{29}, i.e. cells lyse and release copper. The inoculum contained high levels of bacteria that had been previously exposed to copper (see Chapter 5) and, therefore, could have sequestered copper.

6.3.2 Molecular Analysis

qPCR was used to quantify the 16S rRNA gene, several tetracycline resistance genes, and β lactam resistance genes bla_{CTX} and bla_{TEM}. Graphs displaying the standardised resistance gene abundances with the standard errors displayed as error bars are below (See Figure 6-2 to Figure 6-6).

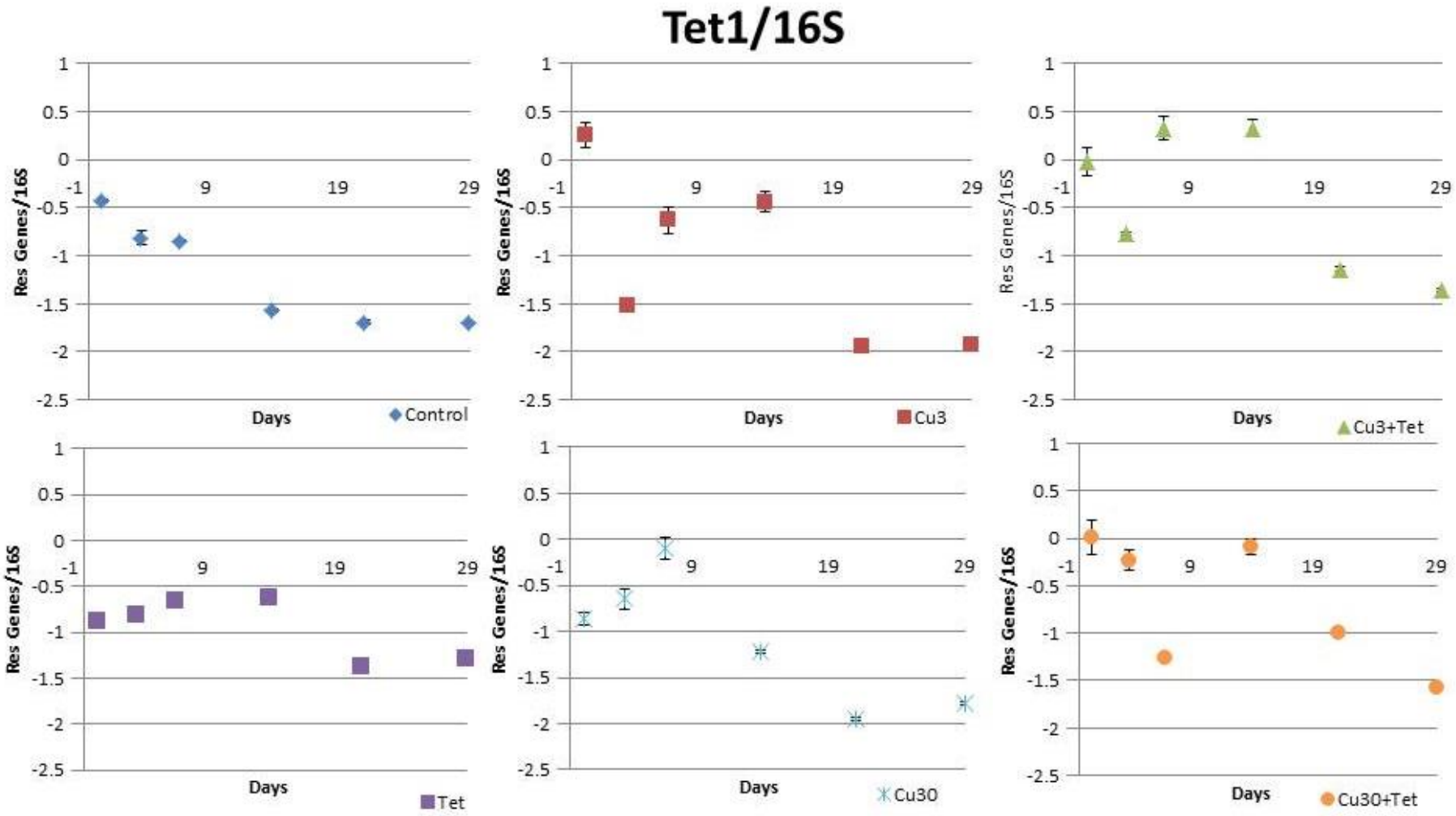

Figure 6-2 tet1/16S resistance gene abundances (log transformed)

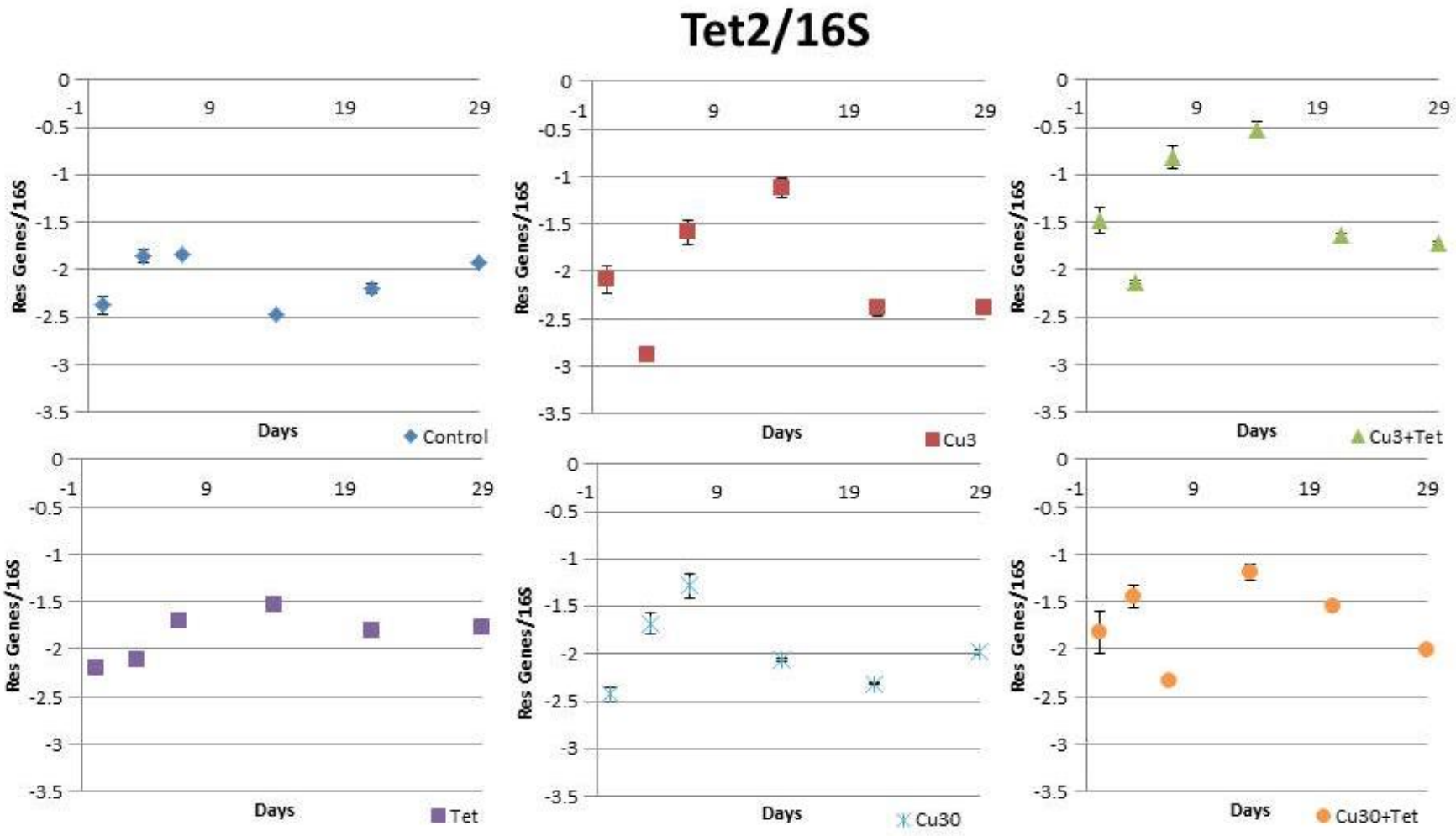

Figure 6-3 tet2/16S resistance gene abundances (log transformed).

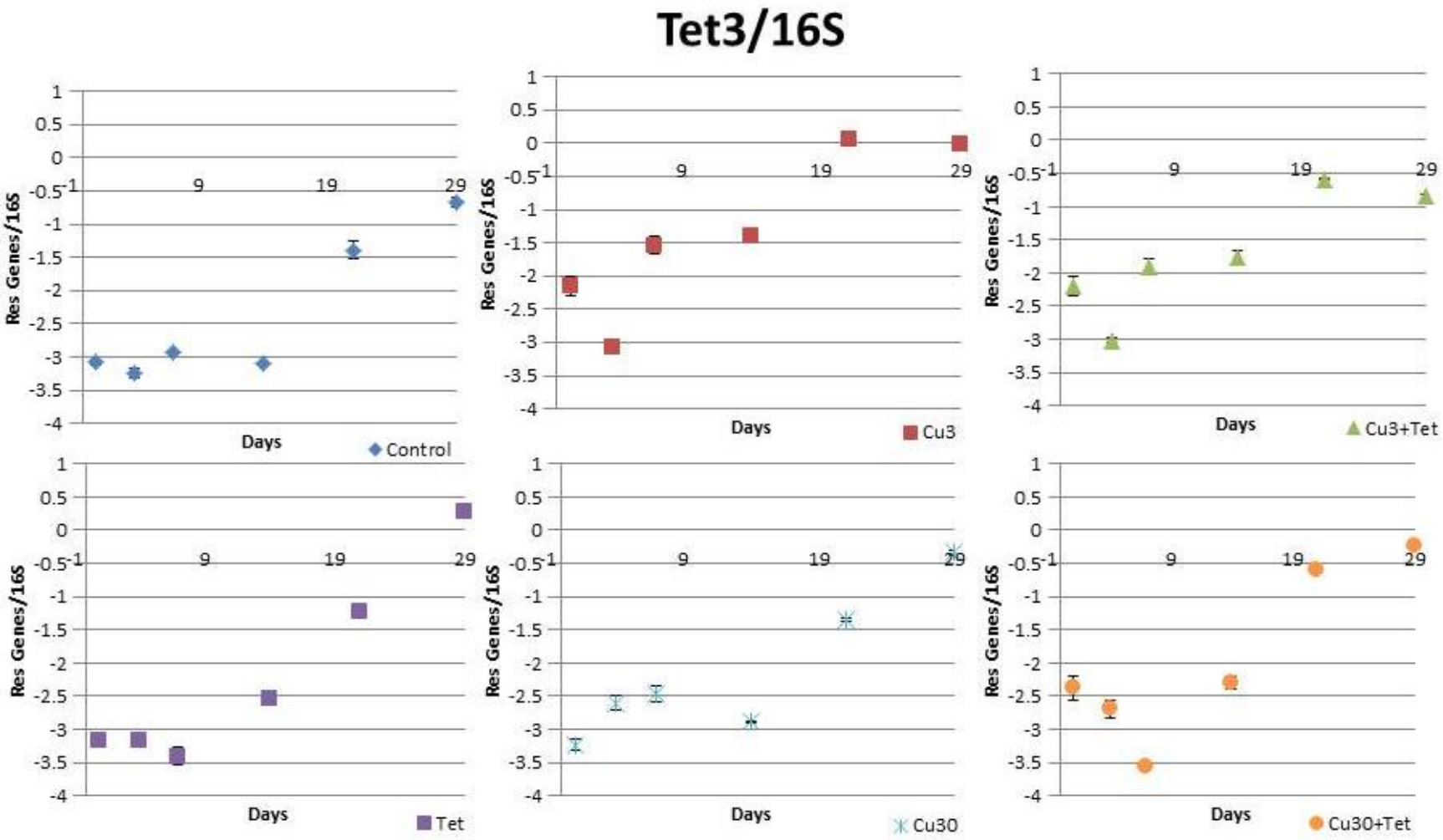

Figure 6-4 tet3/16S resistance gene abundances (log transformed).

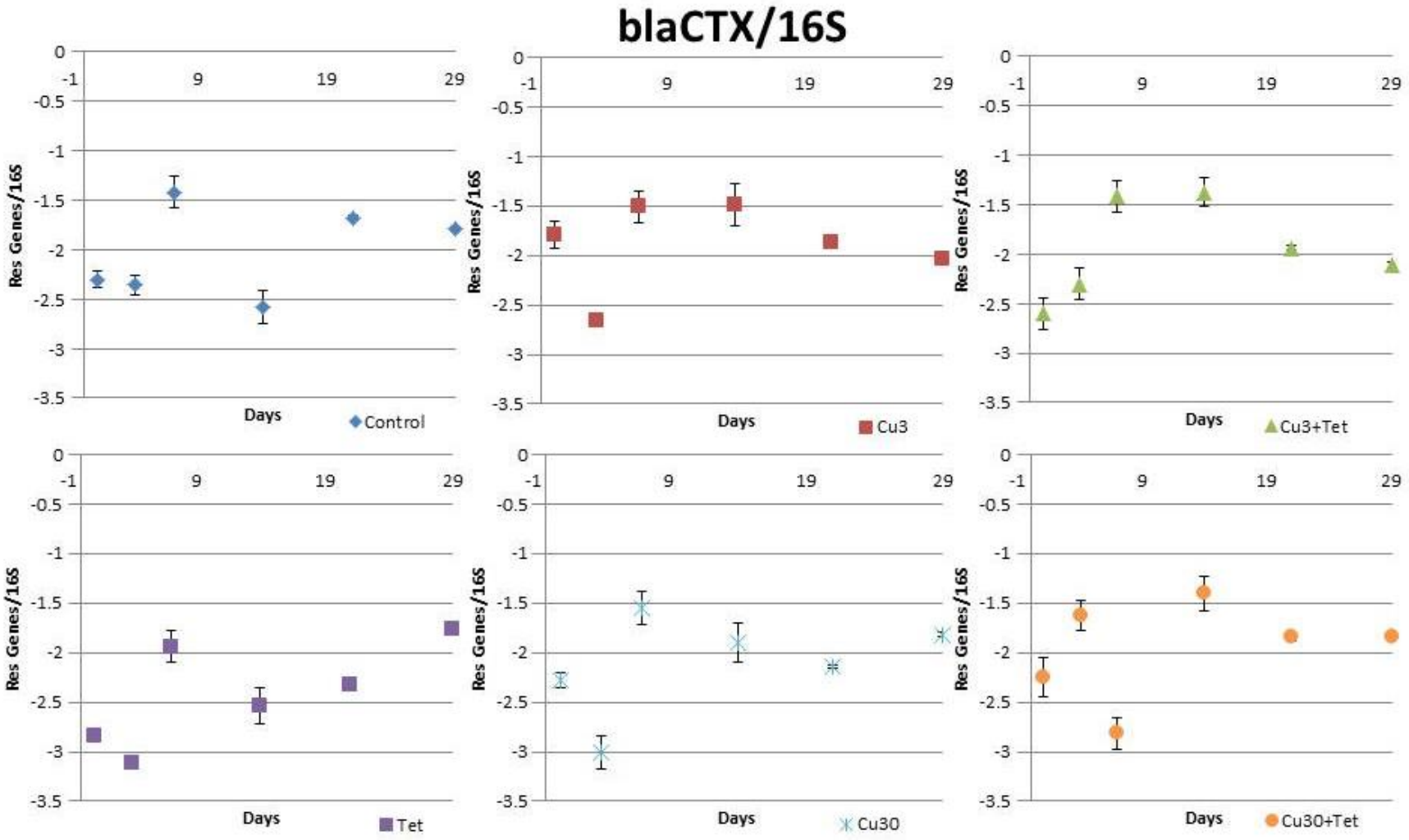

Figure 6-5 *bla*$_{CTX}$/16S resistance gene abundances (log transformed).

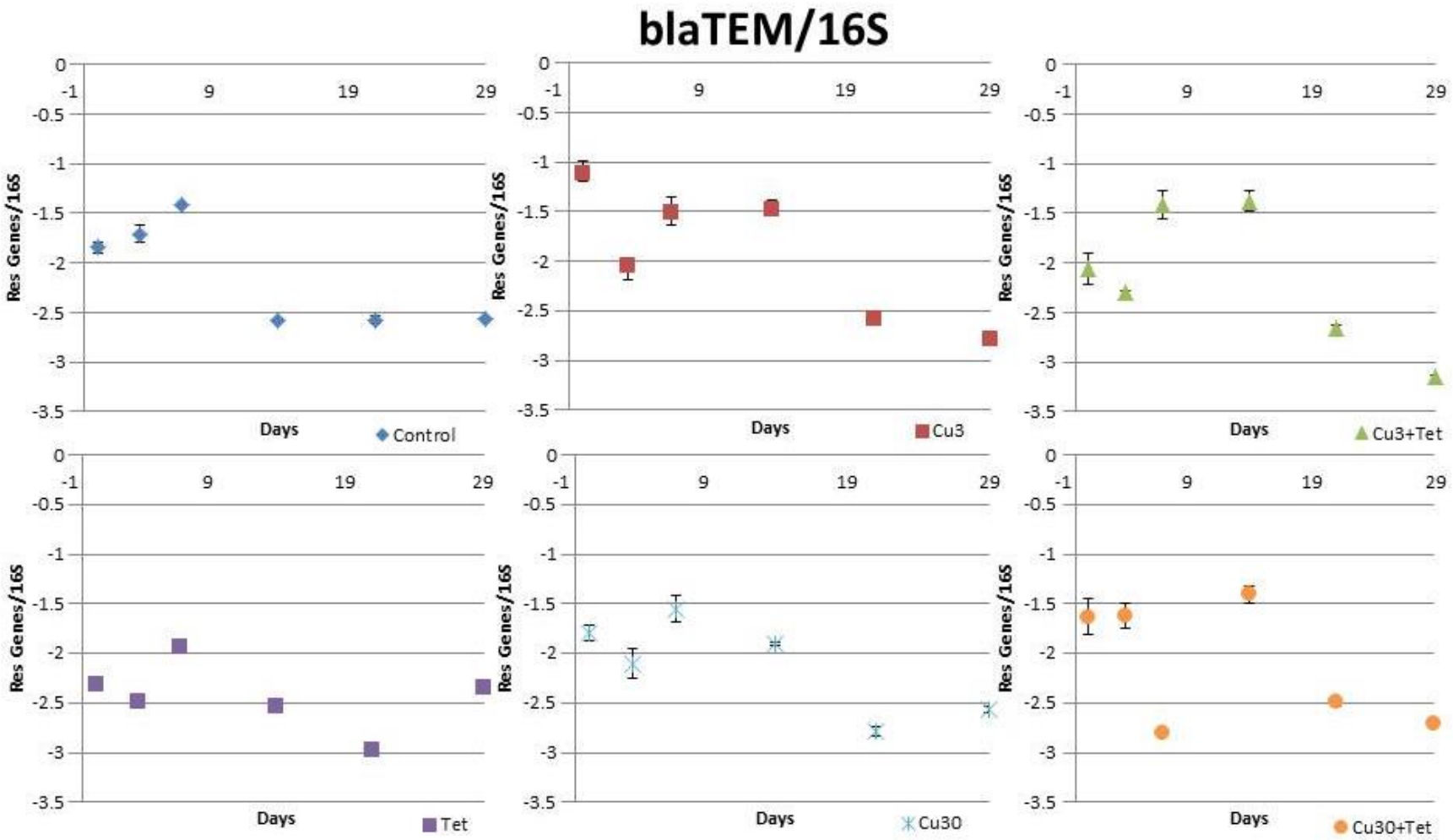

Figure 6-6 bla_{TEM}/16S resistance gene abundances (log transformed).

It had been anticipated that the trend lines for the above scatterplots would show an overall decline (of varying gradients based on treatment); however, this was not the case. For tet3 genes, abundances actually increase as the experiment progressed. Additionally, results are slightly variable until day 14 after which point abundances start to decrease. There were no obvious differences between microcosms for any of the resistance genes; however, further statistical analysis was required to confirm this.

To analyse whether there was any significant treatment based effects on resistance gene retention, various methods of statistical analysis were considered, such as using *ANCOVA* to compare the data sets with time as a covariant. However, it was decided that comparing the gradient of the trend lines for each treatment would be most appropriate. The slope and its standard error were determined (Table 6-3). Additionally, one-way *ANOVA* analysis (based on the mean and standard error of the slope) was used to compare treatment based effects on gene abundance. The F and P values obtained by the *ANOVA* analysis are below in Table 6-4.

Table 6-3 linear regression slopes with standard errors in parentheses

	Tet1	**Tet2**	**Tet3**	***bla*$_{TEM}$**	***bla*$_{CTX}$**
Control	-0.046 (0.010)	0.002 (0.013)	0.093 (0.020)	-0.039 (0.014)	0.015 (0.020)
3Cu	-0.060 (0.029)	-0.004 (0.029)	0.101 (0.025)	-0.051 (0.017)	0.003 (0.020)
30Cu	-0.053 (0.019)	-0.007 (0.019)	0.092 (0.021)	-0.034 (0.014)	0.019 (0.021)
Tet	-0.021 (0.010)	0.013 (0.010)	0.129 (0.020)	-0.012 (0.015)	0.034 (0.017)
3Cu+Tet	-0.045 (0.026)	-0.004 (0.028)	0.072 (0.021)	-0.040 (0.025)	0.010 (0.022)
30Cu+Tet	-0.043 (0.022)	-0.001 (0.019)	0.102 (0.030)	-0.031 (0.025)	0.016 (0.022)

Table 6-4 *ANOVA* comparison of slopes, based on linear regression slopes and standard error of slopes.

	tet1	**tet2**	**tet3**	***bla*$_{TEM}$**	***bla*$_{CTX}$**
F value	0.391	0.116	0.637	0.464	0.263
P value	0.851	0.988	0.673	0.800	0.930

Based on the *ANOVA* analysis, there was no statistically significant treatment based difference in terms of resistance gene retention. It was expected copper would have increased or enhanced resistance gene retention; however this was not the case for any of the resistance genes assayed.

6.4 Discussion

The aim of this experiment was to determine whether the presence of copper and or tetracycline in receiving waters, would increase the retention of antibiotic resistance genes released by the WWT process. It had been anticipated resistance gene abundances (standardised to total bacteria) would decline in a linear fashion over the course of the experiment based on results obtained by a similar previous study (5), however, this proved not to be the case. Furthermore, for tet3 genes, abundances actually increased and there was no significant treatment based effect on resistance gene abundances.

Baquero et al. (9) suggest surface water is one of four genetic reactors involved in antibiotic resistance. Water and sediments downstream of urban activity often have elevated levels of antibiotic resistance genes (2, 3, 10, 11). As discussed previously in Chapter 3, DNA and metals bind to clay and sand. It is possible that the sediment fraction is required for the interaction between metal and bacteria and contributes to the effect metals have on gene retention. Metals dissolve in water, however they also likely settle out at higher concentrations and accumulate in sediment (copper is permissible at much higher concentrations in sediment than in fresh water (12)). As mentioned resistance genes are found at increased levels downstream of wastewater treatment facilities, therefore it could be in the sediment fraction as opposed to the water column that enables metals to confer a selection or maintenance effect on antibiotic resistance genes. Whilst this experiment did not show increased tetracycline gene retention in the presence of copper, at either background or polluted concentrations, it is not to say that copper does not affect resistance gene retention.

The inoculum used itself was provided by a bioreactor that had been fed synthetic wastewater for over four months. The inoculum was used because it was known to have a

high density of tetracycline resistance genes and was thought to be ideal for monitoring for this gene fate experiment; however, it contained bacteria accustomed to copper exposure of 3mg/L therefore the concentrations utilised in this study, 3µg/ml or 30µg/ml, are considerably lower and may not have been high enough to influence gene retention.

Resistance gene abundances have been found to be elevated downstream of WWT plants, particularly in sediments which is likely due to the genes binding to sediment or clay particles, which offers protection from degradation. Future experiments should consider adding an element of organic matter or sediment as they also bind copper and could affect interactions between copper, DNA and bacteria. As discussed in chapter 3, the presence of clay seems to have a negative impact on how copper is able to affect antibiotic resistance; however, clay does protect DNA from degradation. This could be further impacted by pH, but we cannot yet tell which pH has preferential effect.

Copper may not have a linear relationship with resistance gene abundances. As previously mentioned the inoculum provided all microcosms with copper. It is possible that this baseline concentration of copper was sufficient to activate whatever mechanisms by which copper is able to select for or maintain resistance gene abundances. Any copper added above this would have been superfluous and had no effect on increasing the retention of antibiotic resistance genes. Cells have a threshold level of copper that must be detected to activate copper homeostasis mechanisms (see Chapter 4 for further details); regardless of the concentration of copper, once the threshold is reached the same mechanisms are activated. Some cells that carry additional systems to tolerate extreme concentrations of copper activate these systems when the initial ones become overwhelmed. The copper mechanisms may have been activated by the concentration within the inoculum. As there was an increase in copper concentration from t_{14} to t_{29} cells may have already been saturated to capacity with copper, meaning there would be no difference between the microcosms as the external milieu was having no effect on the cells or resistance gene retention or propagation. While the water column itself may not pose a risk in terms of metal concentration relating to resistance gene maintenance or retention, the sediment fraction and the role it plays requires further investigation.

6.5 Future Work

Rerun the surface water microcosm experiment with similar parameters however -

- Include a sediment fraction and compare to see if this increases gene retention
- Use real or fresh synthetic WWT effluent as opposed to activated sludge

WWT effluent would contain less copper and organic matter than was added in this experiment as activated sludge is removed and disposed of separately so for future work perhaps actual WWT effluent should be used.

6.6 References

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Chapter 7: Conclusions

The overall aim of this thesis was to address the research question, “do heavy metals enhance and maintain antibiotic resistance in the environment?” The intent was to increase the understanding of the role the environment and heavy metals as a selection pressure, in antibiotic resistance gene retention. This could in turn lead to better informed decision making in terms of combatting the clinical resistance issue. The research question was addressed using a series of experiments. Whilst each chapter has its own conclusions section with specific details, here the overall findings and impact of the research are discussed.

To investigate whether “background” levels of metals affect antibiotic resistance levels, metal concentrations from various uncontaminated sites in Scotland were compared to antibiotic resistance gene levels. While metal levels had been anticipated to have an effect on resistance levels due to previously observed relationships between both resistance types, it was previously thought that higher polluted levels of metals were necessary to exhort an effect on antibiotic resistance. It was, therefore, surprising when low background levels were found to significantly correlate with several specific resistance genes. How exactly this correlation occurs warrants further investigation, however the link between background conditions and resistance levels is significant.

Additionally, it was anticipated that other geochemical conditions would play a positive role in the relationship between metals and antibiotic resistance. For example, pH and clay are known to impact the bioavailability of metals and would, therefore, enhance the interaction between bacteria and metals. Surprisingly, both pH and clay had a negative impact on the relationship between metals and clays. Additional research is required to fully elucidate the effects that clay and pH may have as certain areas with specific geochemical conditions could harbour an increased reservoir of resistance genes. This could have implications with regards to exposure risks on a more local scale.

Overall, what has become apparent from this experiment is that metal exposure induces selection of antibiotic resistance, and it is clearly more intrinsic than anticipated. While it is unrealistic to think that this knowledge can be exploited to reduce the antibiotic resistance problem clinically, it reinforces the need to understand the full cycle and movement of

resistance between the clinical setting and the environment. Interrupting this cycle and reducing the amount of resistance genes entering the environment could also reduce the potential for resistance genes to be harboured in the environment.

The correlation of copper to ampicillin and tetracycline resistance genes was of particular interest, and it became the focus for the remainder of the thesis. Potential mechanisms linking copper to both antibiotic resistance types were investigated using a literature based approach. Based on the statistical association discussed above, as well as the literature based findings, it was decided to experimentally confirm that metals, specifically copper, are the causative agents for increased resistance gene abundances. As such, simulated polluted environments were used to explore whether there is a relationship between metals and antibiotic resistance. Soil microcosms amended with various concentrations of copper were utilised. The increased presence of copper was shown to have an association to both tetracycline and ampicillin resistance genes; however, it was not as pronounced as anticipated. This is possibly due to suboptimal geochemical conditions in terms of soil type or experimental design in the form of nutrient limitation, as there was no nutrient recycling within the microcosms. Further work is needed to fully determine the effect high levels of copper have on resistance in soil. However the results suggest that areas of high copper exposure may be, as discussed above, at increased risk of resistance gene retention and gene exposure.

The wastewater treatment process acts as the literal interface between the clinical and environmental setting as wastewater treatment plants receive domestic and hospital waste, which is then treated and discharged to the environment. Additionally activated sludge, a by-product from the treatment process is often applied to fields to recycle nutrients. A literature investigation identified that the treatment process may select and maintain antibiotic resistance genes. Metals within the treatment process may be involved in this selection process. Activated sludge microcosms were utilised to determine whether a cause and effect relationship between metals and antibiotic resistance gene abundances exists.

Copper was shown to maintain levels of resistance genes to both tetracycline and ampicillin within the microcosms. Similar associations between copper and antibiotic resistance were found as in the soils. While the specific mechanism linking copper and ampicillin and tetracycline resistance needs to be elucidated, the most imperative finding from this

research is that the wastewater treatment process should be re-evaluated and possibly modified to consider genetic pollution in the effluent, rather than just ensuring an overall decline in bacterial numbers.

Wastewater treatment may be selecting for antibiotic resistance and increasing the proportion of resistance genes released to the environment, where they can potentially be maintained by metals. Furthermore, areas of higher natural metal levels may be at increased risk of resistance gene selection due to metal accumulation in activated sludge within the treatment plant. Practices relating to how sludge is handled (i.e., whether it should be spread on fields, etc.) or whether hospitals should pre-treat their waste should be reviewed with particular focus on areas of high metal.

Having identified the potential of the wastewater treatment process in selecting for or maintaining antibiotic resistance, the effect of aqueous copper in maintaining resistance gene abundances became the next important question. Surface-water microcosms supplemented with copper and or tetracycline were utilised to monitor rate of antibiotic resistance gene decline. Surprisingly, there was a lack of association between copper concentration and resistance gene retention in this experiment. It was predicted copper would exhort a selection effect based on previous experimental results, however this was not the case.

Some of the methods employed throughout this thesis such as multiplex qPCR and DGGE are less sensitive than more up to date or expensive alternatives. Metagenomics or next generation sequencing , as well as running individual gene assays would have been preferential, however these relatively new molecular techniques are still expensive and as a result their use is not yet widespread throughout the research area. Achieving a balance between quality and quantity of data is difficult. In this thesis, the maximum data has been obtained from experimental setups, with minimal concessions on the quality of data obtained and presented. While it would have been preferential to utilise more of the newer molecular techniques, it is hoped that in future; the expense of such analyses decreases making them more accessible and widely used, thus allowing greater insight into antibiotic resistance being achieved.

Overall, the potential for metals to contribute to the antibiotic resistance problem has been strengthened by the research, however further work is required. The specific genetic

mechanisms linking copper with ampicillin and tetracycline resistance needs to be determined. Additionally, the microcosms do not reflect the complexity of real world systems. The identification of the potential for wastewater treatment to amplify antibiotic resistance gene abundances is significant. Modifications to the treatment process could lead to a disruption in the cycle of resistance and in turn eventually help reduce genetic potential for resistance.

Appendix

Appendix 1 -- Manuscript Knapp, McCluskey et al.

Appendix 2 – Supplementary information for manuscript Knapp, McCluskey et al.

Appendix 3 – Chapter 4 soil microcosm experiment qPCR data

Appendix 4 – Chapter 5 bioreactor experiment data

Appendix 5 – Chapter 6 gene fate experiment qPCR data

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PLoS one

Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils

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Abstract

The vast majority of antibiotic resistant genes (ARG) acquired by human pathogens have originated from the natural environment. Therefore, understanding factors that influence intrinsic levels of ARG in the environment could be epidemiologically significant. The selection for metal resistance often promotes AR in exposed organisms; however, the relationship between metal levels in nature and the intrinsic presence of ARG has not been fully assessed. Here, we quantified, using qPCR, the abundance of eleven ARG and compared their levels with geochemical conditions in randomly selected soils from a Scottish archive. Many ARG positively correlated with soil copper levels, with approximately half being highly significant ($p \gg 0.05$); whereas chromium, nickel, lead, and iron also significantly correlated with specific ARG. Results show that geochemical metal conditions innately influence the potential for AR in soil. We suggest soil geochemical data might be used to estimate baseline gene presence on local, regional and global scales within epidemiological risk studies related to AR transmission from the environment.

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- These authors contributed equally to this work.

Introduction

There is a growing concern about antibiotic resistance in natural and clinical settings. The overuse, or misuse, of antibiotics in medicine and agricultural operations are major suspects for increased antibiotic resistance. Areas of elevated resistance are often found in the environment, which is most evident in locations where environmental pollution has affected the local abundance of resistance traits [1–3]. In fact, there are various known mechanisms by which resistance traits may be retained or propagated in the presence of elevated chemical stressors (e.g., quaternary ammonium compounds [4] and metals [1,5,6]), which can locally influence resistance markers in exposed microbial populations. However, less attention has been paid to the intrinsic capacity for natural environments to retain and promote resistance. Relationships between geochemical conditions, micro-organisms and human health have been long documented in terms of altered mobility and toxicity of metals (e.g., mercury and arsenic) [7], but the influence of background geochemical conditions on intrinsic ARG abundance has not been considered relative to AR proliferation. The practical question is whether elevated metal levels in soils increase the prevalence of antibiotic genes in the environment.

As background, some trace metals are essential at low concentrations for enzymatic biochemical processes in bacteria, albeit they also can be toxic at higher levels, causing damage to DNA and membranes [8,9]. As an example, copper has been used as an antiseptic for many years [10] and, as such, metals like copper might act as environmental selectors that demand cell defence. Alternately, other metals like chromium and lead have no known function in bacterial cells, but can also cause oxidative stress [11]. In fact, bacterial resistance mechanisms exist to mitigate toxicity effects of excessive bio-available metals as part of their SOS (stress) response strategy [12]. Defence-associated metal resistance genes are often closely associated with those responsible for AR on mobile genetic elements. These genes can either encode for generic detoxifying mechanisms (e.g., efflux pumps), which non-specifically reduce intracellular concentrations of both metals and antibiotics (cross resistance), or may involve separate genes, which are integrated on the same genetic element (co-resistance).

In essence, the presence of one stressor is likely to select for the other; however, the extent to which soil-metal levels affect the selection of the resistant bacteria and AR gene levels is not known. To our knowledge, there is no experimental evidence or quantitative data that relate metal levels in the environment to ARG abundance, which we contend may be important to

understanding the capacity of the natural environment to retain and transmit AR. As such, by elucidating relationships between metals and AR in theenvironment, we might better define how environmental reservoirs might influence AR transmission to clinically important strains. For example, genes can be harboured among environmental microorganisms, but with horizontal gene transfer, these traits can be shared with other bacteria, including pathogens, which was apparent for extended spectrum beta-lactamase genes found in soils prior to their appearance in clinical settings [13]. Specifically, the aim of this experiment is to determine whether such relationships exist between metal content and ARG in soils.

Methods

We hypothesise that AR is directly related to geochemical conditions in the soils, and to test this hypothesis, we examined two sets of archived soils from The James Hutton Institute in Aberdeen in Scotland. Previous data had shown that DNA can be effectively obtained from dried archive soils [13], and the archive provided a collection of soils from throughout Scotland with good ancillary geochemical information. The first soil series comprised of 46 randomly selected archived soils originally collected across Scotland (see Fig. 1 and Supplemental Table S1), which were analysed for an array of ARG. This soil series was collected 1940 to early 1970s—very early in the antibiotic era, which provides a unique opportunity to view soils before human antibiotic use had likely much impact. The soil profiles sampled were each chosen to be typical of a soil series by the soil surveyor who mapped the soils in the area around the sampled profile. A single sample was generally taken around the mid-point of each soil horizon in the profile and we used the top sample from each profile for this study. While, there may be regional, non-point sources affecting sites (in a very broad sense; e.g., atmospheric deposition), we cannot attribute metal concentrations to any particular event or human activity in many locales. The soils were, in general, collected as part of a ''national survey'' to gain a representative sense of soil conditions.

As additional comparison, we also examined a second soil series comprised of soils collected in 2008 from experimental plots at Hartwood, North Lanarkshire and Auchincruive, South Ayrshire. These different plots had been provided sewage sludge amended with copper at 0, 50, 100 and 200 mg-copper/kg (between 1994 and 1998), respectively, and this series of impacted soils provided a valuable contrast to results from the first soil series. Details of the second set of soils can be found in Macdonald et al. [14], the experiment from which the archived soils were obtained. These soils were also archived at The James Hutton Institute.

Figure 1. Distribution of sample locations in Scotland (n = 46).
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Soil collection and analysis

Archived samples were stored in climate-controlled rooms within The James Hutton Institute. According to archive records, soils were originally collected from multiple locations (0–25 cm) and pooled to obtain composite samples representative of each site. Soils are dried (30uC) and sieved for storage and analysis. Total-metal concentrations were determined by ICP (inductively coupled plasma) analysis, following aqua regia extraction [15]. These soils were extremely valuable and had limited availability, therefore we chose to use historical metal records, maintained at The James Hutton Institute, than re-analysing the samples with contemporary technology. Furthermore, the historic data is more representative of conditions under which the samples had been originally collected. Other analyses included soil pH, organic carbon/ash content, total phosphate, and particle size; again, information was based on archived records and were based on standard analytical methods [e.g., 15].

DNA analysis

Long-term storage does not bias DNA results [16,17]. Once retrieved from the archive, soils were sterilely weighed into prepared centrifuge tubes containing buffer and extraction beads (by weighing tubes before and after soil addition); usually 200–300 mg (as dry weight) of soil was used. Cells incubated in the buffer 15–20 minutes for rehydration. DNA was extracted from soil using FastDNA Spin Kit (MP Bio) according to manufacturer's instructions. DNA was eluted with 100-mL elution buffer and temporarily stored at -20uC; long-term storage was at 280uC.

qPCR methods

Eleven determinants, targeting tetracycline resistance (tet), extended-spectrum beta-lactamases (bla), and erythromycin resistant methylases (erm), were chosen based on previous experience assessing metal-contaminated sediments [18] and their general ubiquity in the environment [16,19–22]. Specifically, assays included tet(M), tet(Q) and tet(W) [19], which are three highly promiscuous (common) resistance traits that encode for ribosomal protection proteins against tetracyclines. Tet(B) [20], an efflux gene, was also tested but did not generate many positive results. Primers for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{OXA} targeted conservative regions on the four common beta-lactamase genes (which encode for enzymes that inactivate penicillin and other

beta-lactam antibiotics) [19], and specific primer pairs targeting erm(B), erm(C), erm(E) and erm(F) [22]. All ten determinants were quantified in the first experiment, whereas only tet(M), tet(W), erm(F), bla_{TEM}, and bla_{SHV}, and bla_{CTX-M} (based on current results and previous experience) were analysed in the second experiment.

Two microlitres of DNA template and appropriate primers were combined with iQ Supermix PCR reagent (BioRad, Hercules, CA) and molecular-grade water to create 25-ml volumes. Analyses were then performed using a BioRad iCycler equipped with iQ fluorescence detector and software (BioRad). Temperature cycles were 95uC (10 min), and then 40 cycles of 94uC (20 sec), annealing temperatures (tet- and erm- determinants: 60uC; bla_{TEM}: 50uC, and 55uC for the remaining bla genes) for 60 seconds, and 72uC (for bla-genes only) for an additional 30 seconds. Samples were analysed in duplicate; any samples with a major discrepancy (high analytical variability, i.e., greater than one-cycle difference) were re-analysed. Typical duplicate values ranged within ±0.3 (log scale). SYBR-Green I, a non-specific fluorescent dye, was used and followed with a post-analytical temperature melt curve to verify reaction quality (50–95uC, DT = 0.1uC/second).

All reactions were run with serially diluted plasmid-DNA standards of known quantity, created from gene-positive bacteria. QPCR reaction efficiencies were determined by spiking sample with known amounts of DNA template; these results were compared with efficiencies of ''neat'' standards (plasmids dissolved in nanopure water). All samples were diluted, either 1:100 or 1:1000, to minimise inhibitory effects, often caused by substances from soils co-eluting with the DNA. Correlation coefficients (r^2) for all standard curves were $\geq$0.99; and log gene-abundance values (except those below detection limits) were within the linear range of the calibration curves. Any sample values below detection were not included in any statistical analyses.

Data analysis

ARG abundances were normalised to 16S-rRNA gene abundances (a surrogate measure of 'total bacteria') to minimise variance caused by differential extraction and analytical efficiencies, and differences in background bacterial abundances. These normalised values are then log-transformed to normalise the data (Kolmogorov-Smirnov test).

All statistics were conducted using SPSS™ version 18. Factors for analyses included: log-transformed abundances, which were determined *de novo* from retrieved archive samples. Other information, from archived records, included: ''total metal'' content of chromium, copper, molybdenum, nickel, and lead (Table S2); extractable measures of zinc and iron (no total values available); and soil quality characteristics—pH, organic carbon, total phosphate, sand, silt, clay and ash content (Table S1).

Results and Discussion

The primary soil series was obtained from a wide range of soil conditions in Scotland. With these soils, we assessed the correlation of numerous metals and other soil parameters (e.g., organic carbon, total phosphorus, dry ash, pH, and silt, sand and clay contents) (Table 1), and the relative abundance of ARG in soils (Table S3). A bivariate correlation analysis (Table 2; other correlations can be found in Supplemental Table S4) showed that eight of the eleven quantified ARG positively correlated with soil copper levels; five ARG being highly significant ($p \geq 0.05$), including tet(M), tet(W), bla_{OXA}, erm(B) and erm(F) (all genes normalised to 16S-rRNA gene abundances). In addition, chromium positively correlated with relative tet(M), bla_{CTX-M} and bla_{OXA} gene abundances. Other metals also showed positive correlations, but were gene specific; e.g., nickel linked with tet(W), and tet(M) associated with nickel, lead and extractable iron, whereas only erm(B) had negative correlations with any metals (lead, zinc and iron). Overall, tet(M) level was most consistently linked to soil metal conditions, and copper most strongly influenced ARG abundances.

Table 1. Soil properties of samples collected throughout Scotland.

	Mean and 95% confidence intervals (in brackets)	Minimum – maximum
Cobalt (mg/kg)	10.2 (6.7)	0–140
Chromium (mg/kg)	53.1 (20.4)	0–250
Copper (mg/kg)	21.2 (9.2)	0–140
Nickel (mg/kg)	25.8 (7.4)	0–100
Lead (mg/kg)	52.4 (44.9)	10–1000
Zinc, extractable (meq/L)	2.5 (2.1)	0–38
Iron, extractable (meq/L)	23.0 (9.0)	0–115
Carbon, organic (%)	5.2 (2.1)	0–45.3
Phosphorus, total (mg/kg)	354 (145)	0–1610
pH	5.8 (0.2)	4.3–7.2
Sand (%)	45.3 (6.7)	0–88.0
Silt (%)	26.9 (4.5)	0–64.0
Clay (%)	14.3 (2.7)	0–45.6
Ash (%)	88.3 (3.7)	22–97

doi:10.1371/journal.pone.0027300.t001

Despite these significant individual correlations, we suspected that groups of factors were more likely to influence AR gene abundance in complex soil environments. Therefore, multiple-linear regression (MLR) also was performed on the data set (Fig. 2; Supplemental Table S5). Based on the MLR, stronger associations between soil conditions and ARG levels (i.e., $R \geq 0.70$) become apparent, although these correlations are consistent with bivariate correlation predictions. As examples, observed tet(M) (Figure 2A; R = 0.70, p = 0.11), bla_{CTX-M} (Figure 2B; R = 0.73, $p \geq 0.01$) and erm(F) (Figure 2C; R = 0.66, p = 0.05) abundances correlated to a model based on five metals (chromium, copper, nickel, lead and iron) and pH, and explained $\geq$50% of the variability in gene abundances. Other MLR relationships between metals and relative gene abundances were also found, but observations were not as strongly linked to predictions (See Supplemental Fig. S1).

Regardless of statistical approach, soil copper appears to be a key major factor in soil ARG (as well as chromium and iron; Supplemental Table S5) with 2–3 orders of magnitude of differences in intrinsic ARG levels attributable to five metals and pH. It should be noted our coefficients of determination (R^2) for tet(M), bla_{CTX-M}, and erm(F) suggest only about 50% of the variance is explained by the model. However, this correlation is impressive given the complexity of soil environments and the fact it is difficult to parameterise all possible factors that might contribute to AR. Given the age and limited information on most of the soils, this correlation is surprisingly strong and suggests that similar key factors may contribute to intrinsic ARG levels.

To assess how ARG patterns in the soils from across Scotland compared with more metal-impacted soils (e.g., with Cu), soils from experimental plots that had been provided Cu-amended sewage sludge at different levels and sites were quantified for ARG. Significant treatment differences were found at the Hartwood site

Table 2. Bi-variate correlations among geochemical properties and normalised gene abundances (log-transformed).

	tet(M) /16S	tet(Q) /16S	tet(W) /16S	bla_{TEM} /16S	bla_{SHV} /16S	bla_{CTX} /16S	bla_{OXA} /16S	erm(B) /16S	erm(C) /16S	erm(E) /16S	erm(F) /16S
Carbon, organic	2.123	.189	.227	2.080	.222	2.139	.094	2.764	.444*	2.058	.223
Phosphorus, total	.357	2.047	2.081	2.264	2.267	2.278	2.041	2.169	2.075	2.293	2.392 *
Sand	2.404	.145	.287	2.026	.447 **	.033	.064	2.366	.270	.183	.248
Silt	2.021	.238	2.138	2.140	2.151	.521 **	.172	.038	2.229	.415 **	.307
Clay	.377	.260	2.177	.042	2.398 *	.061	.000	.562	2.297	.153	2.005
Ash	2.076	.095	2.043	.017	.007	.308 *	2.021	.189	2.248	.322 *	.327
pH	.221	.251	2.154	2.059	.064	.602 **	.312 *	.923 *	2.053	.438 **	.208
Cobalt, total	.399	.179	.237	.167	.138	2.116	.137	2.115	2.173	2.085	.191
Chromium, total	.492 *	2.017	2.067	.045	2.039	.324 *	.390 **	2.101	.163	.053	.046
Copper, total	.439 *	.281	.296 *	.276	.280	.038	.408 **	.845 *	2.052	2.028	.432 *
Nickel, total	.336	.262	.363 *	.191	.148	2.121	.281	2.158	2.269	2.011	.066
Lead, total	.407	2.107	.105	.245	2.077	.047	.037	2.703	2.328	.110	2.014
Zinc, extractable	.157	2.333	.206	.192	.236	2.138	.215	2.583	.307	2.139	.211
Iron, extractable	.597 **	.241	.156	.060	.113	2.286	.124	2.621	.126	2.298	2.270

*Significant at $P > 0.05$ level ($a = 5\%$).
**Significant at $P > 0.01$ level ($a = 1\%$).
doi:10.1371/journal.pone.0027300.t002

(North Lanarkshire; Fig. 3; Supplemental Table S6), which had relatively higher bla_{CTX-M} relative gene abundances (ANOVA; $F_{4,6} = 3.17$, $p = 0.10$) when Cu levels were supplemented. Further, bla_{TEM} and bla_{SHV} gene abundances were elevated when Cu levels were greater than 150 mg/kg at Hartwood (ANOVA; bla_{TEM}: $F_{4,7} = 3.41$, $p = 0.07$; bla_{SHV}: $F_{4,7} = 4.54$, $p = 0.04$). Tet(M) and tet(W) also showed general upward trends at this site, but were not significant (ANOVA; all $p > 0.10$). The second site (Auchincruive, South Ayrshire; Fig. 4; Supplemental Table S6) did not show any significant evidence of elevated resistance potential with increased Cu-content for many genes (ANOVA; $p > 0.17$).

We cannot rule out that bacteria with resistance traits in the original sludge or residual antibiotics in the sludge might have been sources of the ARG. However, these samples were collected in 2008, and the elevated Cu content in the treatments was still apparent from soil analysis ten years after Cu amendments. Overall, Cu-amended plots had slightly higher ARG than plots without Cu amendment, although metal effects were less well defined than in the broader soil archive. Also, MLR equations from the first soil series poorly predicted ARG levels in the impacted soils, which imply artificial metal additions may alter intrinsic ARG and metal patterns, and may not accurately reflect native ARG-metal relationships in soils.

Regardless of specific correlations, both experiments show that soil conditions can affect the intrinsic potential for AR in associated microbial communities, which is noteworthy because it is the first demonstration that relatively low total metal levels correlate with ARG abundance in soils. Even more interestingly, our results suggest that even low metal levels may co-select for antibiotic resistance. In reality, metal impacts on AR signatures at environmental sites are not new; however, all previous work has been done at heavily polluted sites. For example, Stepanauskas et al. [2] found higher levels of AR in the water leaving a settling basin, which contained ash from coal-fired power stations known to have elevated metals. Further to this, Wright et al. [21] looked at how AR in a metal polluted river compared to that of a pristine reference stream. They found metal and antibiotic resistance to be highest in the sediment bacteria. Similar results were found by Graham et al. [18]; elevated levels of resistance were found surrounding discharges of metal-laden leachate from landfill in

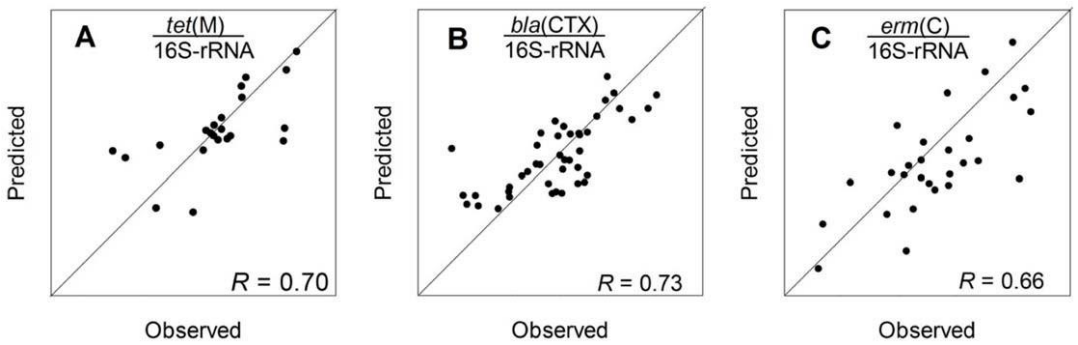

Figure 2. Comparison of multi-linear regression predictions and observed relative abundances of A) tet(M), B) bla_{CTX-M} and C) erm(F) genes. Factors entered into the regression included: total chromium, copper, nickel, lead, extractable iron, and pH.
doi:10.1371/journal.pone.0027300.g002

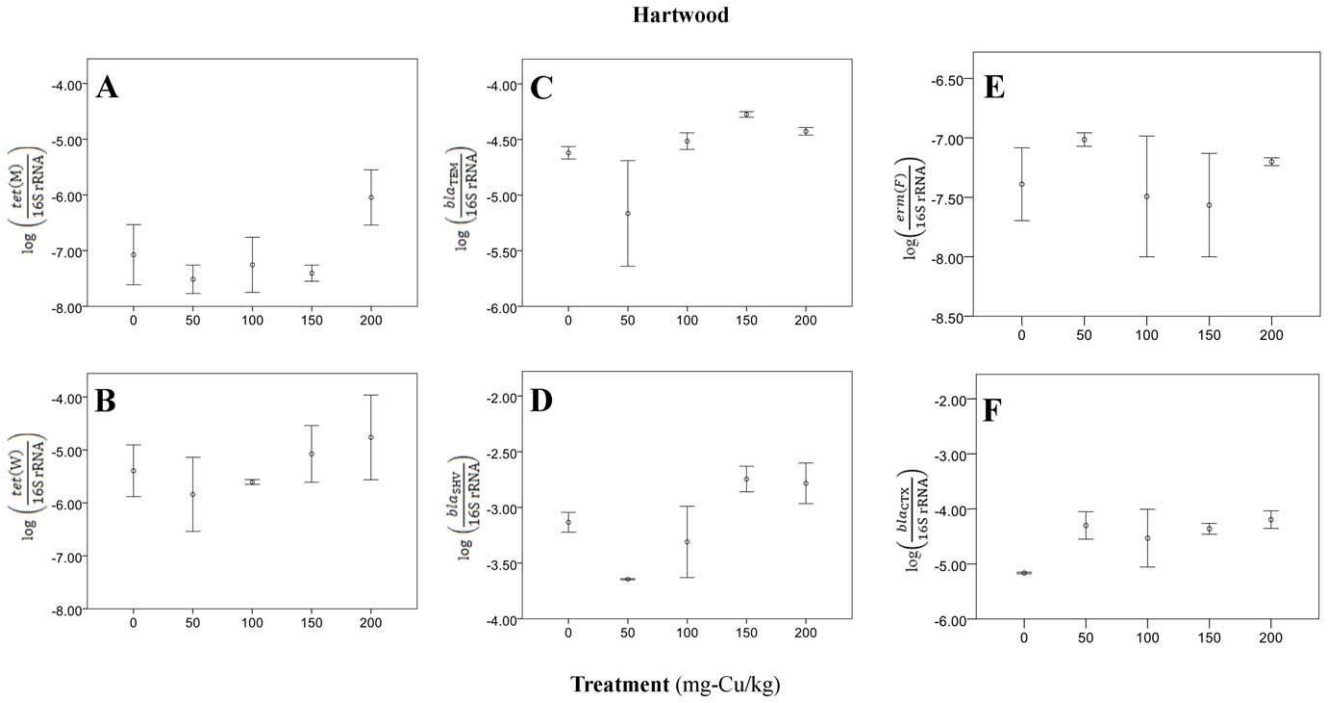

Figure 3. Relative resistant gene levels in experimental plots, located at Hartford, North Lanarkshire, receiving copper-amended sewage sludge: 0, 50, 100 or 200 mg-copper/kg.
doi:10.1371/journal.pone.0027300.g003

Cuba. Noteworthy, Berg et al. [5] linked increased soil copper exposure to increased levels of antibiotic resistance. In all aforementioned cases, sediment acted as a sink for metals and promoted higher concentrations of AR-related genes. In another experiment taking advantage of defined copper sulphate additions to soils [6], researchers found significantly higher ampicillin resistance in susceptibility tests among copper-resistant bacteria. AR patterns can be related to total metals and also to bioavailable

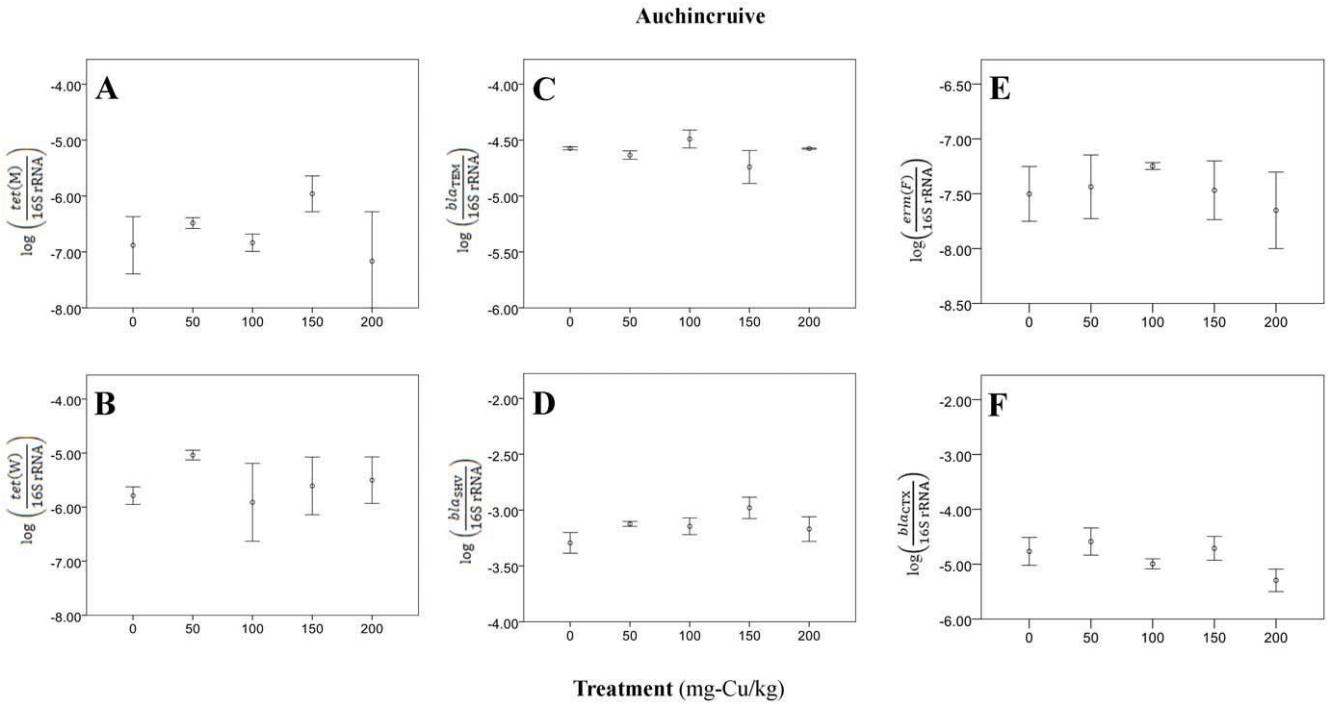

Figure 4. Relative resistant gene levels in experimental plots, located at Auchincruive, South Ayrshire, receiving copper-amended sewage sludge: 0, 50, 100 or 200 mg-copper/kg.
doi:10.1371/journal.pone.0027300.g004

copper levels [22]; however, it should be noted total metal concentrations are often poor predictors of bioavailability and toxicity; many factors in soil are attributed to this [23].

However, here we show that specific ARG concentrations themselves significantly correlate with metal conditions, but relationships between metal conditions in natural soils differ than impacted soils, which is critical for assessing broader AR risk from soil sources. Clearly, elevated AR can be found in polluted areas, but Figure 2 shows that lower metal concentrations influence intrinsic AR potential and that influence differs from impacted soils. Therefore, investigations such as ours are important to better understand the risks of AR, mechanisms that promote AR propagation, and background source inputs to AR in anthropogenically important species.

Environmental bacteria can harbour resistance genes, and the constant exposure to metals can increase their frequency in the gene pool [24]. With gene transfer occurring among soil bacteria, the chance of acquiring resistant pathogens, therefore, is increased. Epidemiological studies are now suggested to further examine whether intrinsic resistance actually does contribute to increased AR, especially at landscape and larger scales. As such, this new information from soils becomes crucial for developing management strategies to reduce the global risk of AR and protecting agricultural and aqua-cultural economies by better knowing where AR might germinate.

Supporting Information

Figure S1 Comparison of multiple linear regression predictions and observed relative abundances of genes.
(PDF)

Table S1 Original location and physical-chemical properties of archived samples.
(DOCX)

Table S2 Metal concentrations of archive soil used in the study.
(DOCX)

Table S3 Distribution of physical and chemical conditions.
(DOCX)

Table S4 Bi-variate correlations among physical-chemical properties.
(DOCX)

Table S5 Results of the multi-linear regression analyses.
(DOCX)

Table S6 One-way ANOVA statistics describing treatment differences in Hartwood and Auchincruive Cu-amended agricultural plots.
(DOCX)

Author Contributions

Conceived and designed the experiments: BKS DWG CWK. Performed the experiments: CWK SMM GH. Analyzed the data: CWK SMM. Contributed reagents/materials/analysis tools: CDC GH BKS. Wrote the paper: SMM CWK DWG BKS. Contributed by reviewing the manuscript and making corrections: CWK SMM BKS CDC GH DWK.

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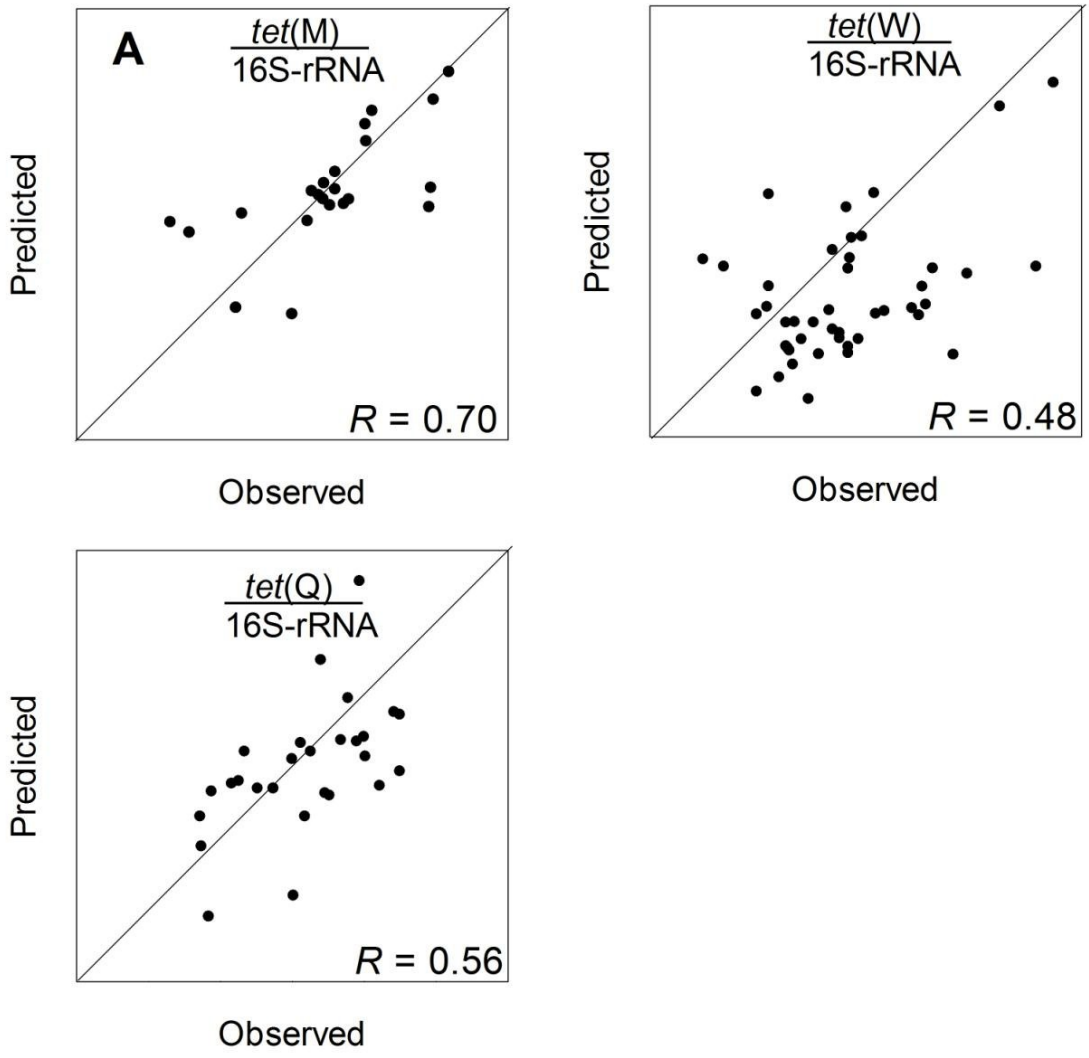
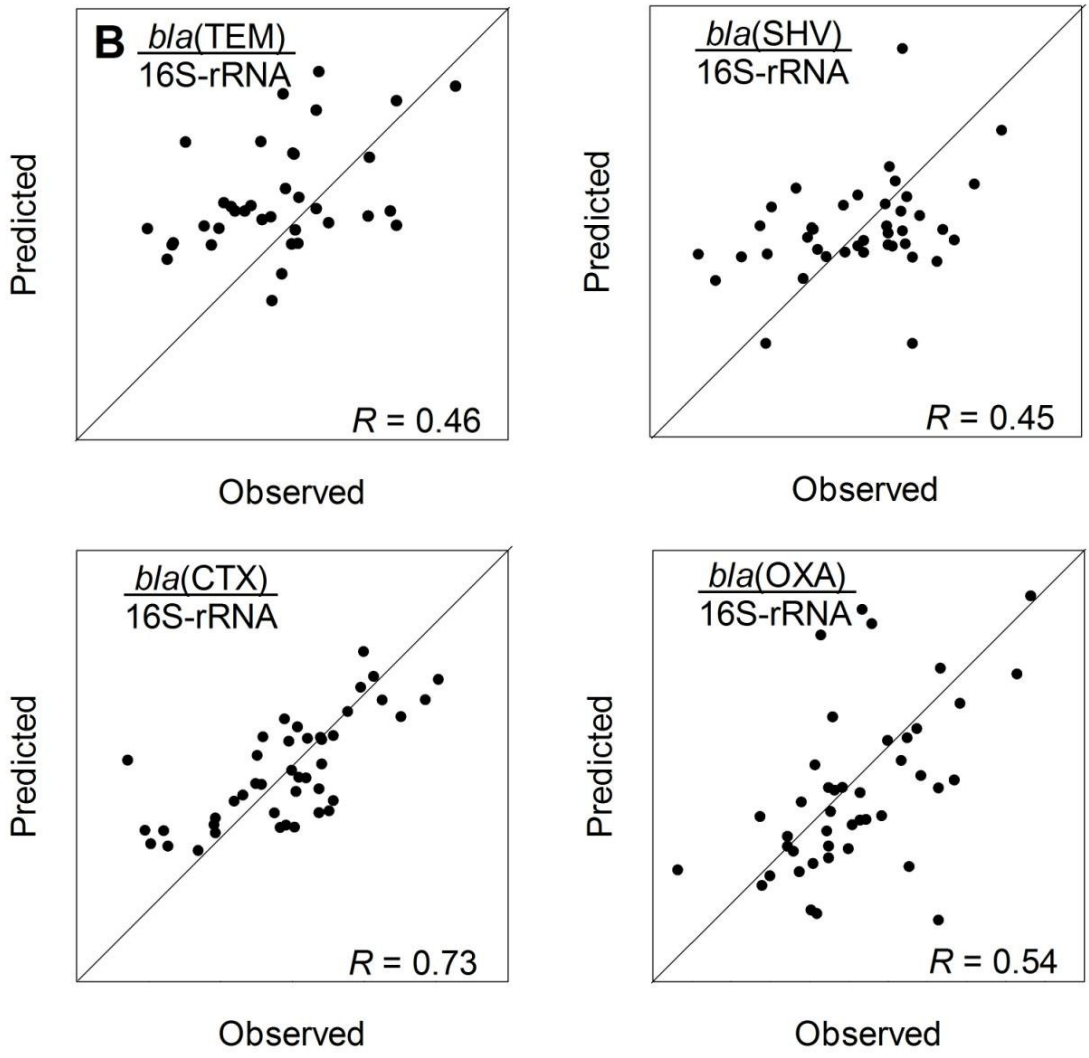
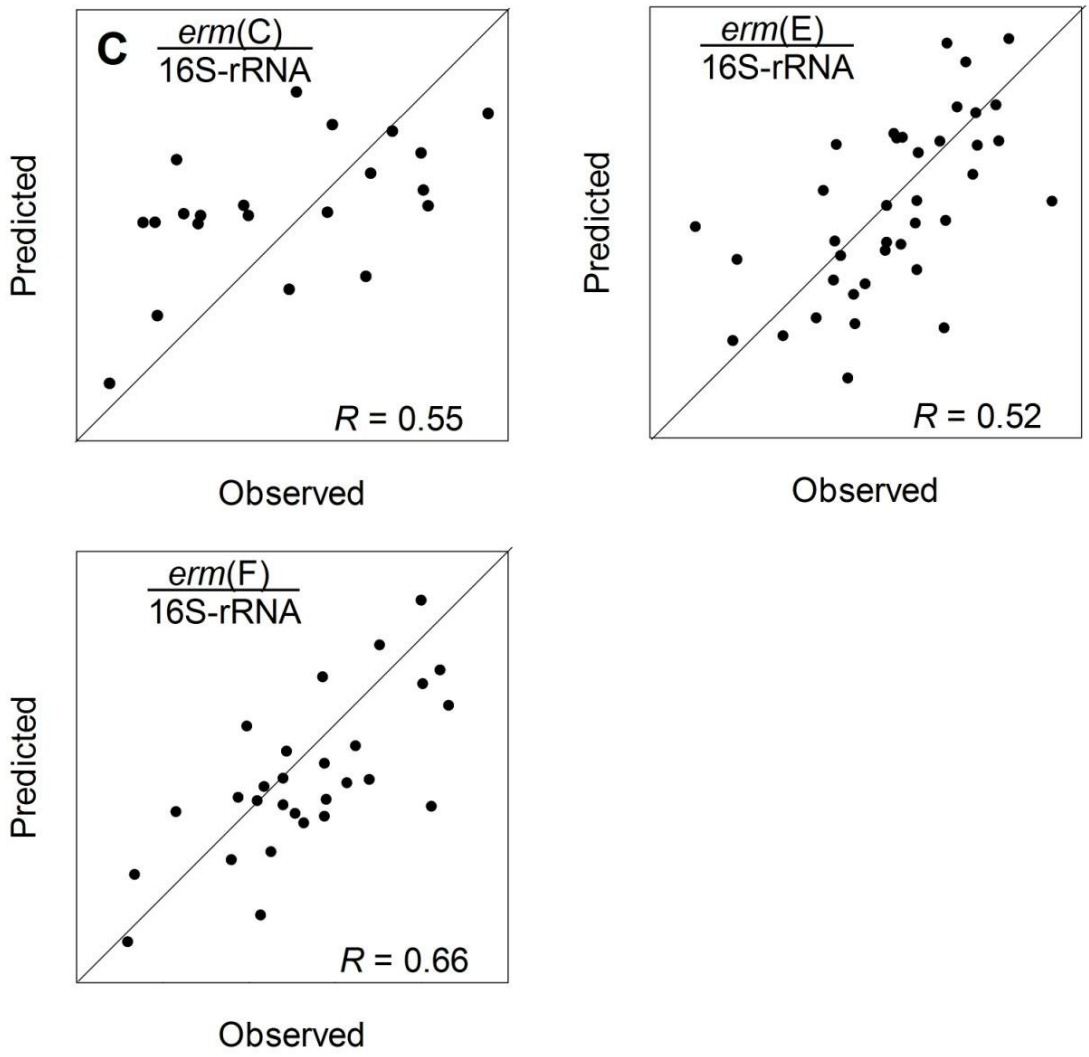
Appendix 2


A
tet(M)
16S-rRNA
Predicted
Observed
R = 0.70
tet(W)
16S-rRNA
Predicted
Observed
R = 0.48
tet(Q)
16S-rRNA
Predicted
Observed
R = 0.56


B
bla(TEM)
16S-rRNA
R = 0.46
Predicted
Observed
bla(SHV)
16S-rRNA
R = 0.45
Predicted
Observed
bla(CTX)
16S-rRNA
R = 0.73
Predicted
Observed
bla(OXA)
16S-rRNA
R = 0.54
Predicted
Observed


C
erm(C)
16S-rRNA
Predicted
Observed
R = 0.55
erm(E)
16S-rRNA
Predicted
Observed
R = 0.52
erm(F)
16S-rRNA
Predicted
Observed
R = 0.66

Supplemental Table S1. Original location and physical-chemical character of archived samples.

Sample	Year	Location[a] Easting	Northing	Characteristics	pH	Organic Carbon	Total phosphate	Sand	Silt	Clay	Ash
54146	1946	Unknown	unknown	Unknown	5.5	20.3	1280	45.7	8.4	15.8	65
54147	1946			Unknown	5.3	11.1	755	68.6	12.5	10.7	83
61616	1947	383200	783200	Humus-iron podzol	5.2	7.2	1160	55.3	19.8	17.0	85
79395	1949	356200	854900	Non-calcareous gley	5.8	2.2	695	0.0	0.0	17.8	93
82036	1950	245100	641000	Non-calcareous gley	5.5	4.3	1330	0.0	0.0	39.9	84
82203	1950	227300	646200	Humic gley	6.0	0.0	1410	0.0	0.0	0.0	65
111073	1956	365500	626400	Brown forest soil	5.4	7.5	885	45.9	24.7	13.9	79
114415	1956	365700	769900	Brown forest soil with gleying	6.7	1.6	0	35.6	36.5	25.4	96
114420	1956	365800	770000	Non-calcareous gley	6.6	1.6	0	51.2	28.0	18.5	95
119387	1957	385400	842100	Humus-iron podzol	6.1	6.0	1610	33.1	49.0	11.5	87
126831	1958	387200	787300	Brown forest soil with gleying	6.2	4.7	253	28.2	33.0	32.7	90
136234	1959	344300	673700	Brown forest soil with gleying	5.9	1.5	0	57.0	22.2	17.6	95
152064	1961	329300	732300	Brown forest soil	6.5	3.9	0	37.2	39.6	18.0	90
152083	1961	349200	735300	Brown forest soil	6.2	4.5	0	39.9	39.7	14.4	88
152084	1961	349200	735300	Brown forest soil	6.2	3.7	0	36.8	43.1	14.9	90
155615	1961	281700	874000	Humus-iron podzol	5.8	2.0	0	61.0	24.0	13.0	96
155616	1961	281700	874000	Humus-iron podzol	5.1	1.6	0	53.0	31.0	16.0	97
155684	1961	271100	869900	Humus-iron podzol	6.2	5.3	0	52.0	30.0	14.0	92
155756	1961	359100	679400	Non-calcareous gley	6.8	3.9	821	3.8	43.9	45.6	87
164260	1962	271100	870600	Immature raised beach soil	5.4	5.5	0	63.0	27.0	5.0	89
164261	1962	271100	870600	Immature raised beach soil	5.4	5.3	0	63.0	24.0	8.0	90
164320	1962	286600	879400	Humus-iron podzol	5.6	2.5	0	60.0	26.0	11.0	95
164338	1962	287300	879400	Not applicable	5.8	2.4	0	65.0	20.0	12.0	95
165456	1962	348400	679900	Brown forest soil	6.0	1.9	0	69.8	14.3	13.2	96
189123	1965	276800	693200	Non-calcareous gley	5.6	3.6	670	13.1	53.4	29.0	92
191195	1965	266602	702800	Brown forest soil with gleying	5.8	3.9	883	42.3	34.9	17.4	91
194665	1964	285600	855900	Humus-iron podzol	5.6	1.6	0	88.0	7.0	5.0	97
205659	1966	386600	837600	Humus-iron podzol	5.3	2.6	1300	55.1	20.2	20.0	91
205664	1966	387100	837200	Iron podzol	5.8	9.8	840	39.8	33.1	11.0	79

Sample	Year	Location[a] Easting	Northing	Characteristics	pH	Organic Carbon	Total phosphate	Sand	Silt	Clay	Ash
207571	1967	341500	849900	Non-calcareous gley	4.8	2.8	136	58.5	23.8	14.0	93
208386	1966	333700	712900	Brown forest soil with gleying	6.1	3.9	0	63.2	19.1	13.4	91
209070	1967	207400	547600	Brown forest soil	6.0	5.2	0	69.0	12.0	13.0	89
241845	1971	276700	843800	Humus-iron podzol	5.1	3.7	0	56.0	32.0	8.0	93
242310	1971	236500	728700	Not applicable	4.3	45.3	0	0.0	0.0	0.0	22
243105	1971	294600	850900	Humus-iron podzol	5.8	1.9	0	60.0	30.0	10.0	97
245014	1971	325300	841800	Humus-iron podzol	5.7	2.4	311	65.0	22.6	9.2	94
245052	1971	326200	839800	Humus-iron podzol	7.2	6.0	442	34.4	52.2	6.4	87
250136	1971	300400	634300	Alluvial soil (silty)	6.1	1.8	0	22.0	64.0	12.0	95
253915	1972	172300	731400	Brown forest soil	5.2	9.2	0	30.6	43.4	10.8	82
254187	1972	317100	842800	Non-calcareous gley	6.3	3.7	104	36.0	52.0	9.0	92
269249	1973	309500	858800	Humus-iron podzol	5.8	2.2	144	64.0	28.0	8.0	95
269264	1973	318500	830900	Humus-iron podzol	6.9	3.9	216	67.0	25.0	8.0	92
279796	1974	318900	839600	Non-calcareous gley	6.3	3.4	173	80.0	15.0	5.0	93
279820	1974	329100	846500	Alluvial soil (sandy)	6.1	1.8	163	67.0	17.0	16.0	97

[a] Geographic Cartesian coordinates based on the UK Ordnance Survey.

Supplemental Table S2. Metal concentrations of archive soils used in the study.

Sample	Total Cobalt	Total Chromium	Total Copper	Total Nickel	Total Lead	Extractable Zinc	Extractable Iron
54146	140	65	140	100	35	0.0	55
54147	40	100	100	80	20	0.0	40
61616	20	200	50	70	200	0.0	115
79395	0	0	0	0	0	0.0	35
82036	0	0	0	0	0	0.0	60
82203	10	100	15	25	150	0.0	60
114415	15	20	40	40	15	0.0	20
114420	0	0	30	0	0	0.0	0
119387	0	0	20	0	0	0.0	0
126831	7	70	6	30	10	0.0	0
136234	20	200	40	60	90	5.0	58
152064	10	30	30	30	100	0.0	21
152083	25	250	60	40	18	0.0	20
152084	20	200	80	40	20	0.0	34
155615	20	200	100	40	20	0.0	36
155616	bdl	0	2	15	6	0.4	30
155684	bdl	0	2	20	10	0.4	24
155756	bdl	0	3	15	10	0.0	0
164260	0	100	8	25	50	0.0	0
164261	bdl	0	2	20	8	6.6	51
164320	3	0	3	30	4	0.0	0
164338	bdl	0	3	20	15	0.0	24
165456	bdl	0	2	15	10	0.7	68
189123	10	30	20	40	60	0.0	27
191195	15	150	10	40	40	0.0	42
194665	10	0	10	40	30	0.0	49
205659	bdl	10	2	-10	50	0.5	31
205664	15	100	20	30	15	0.0	15
207571	6	50	2	20	15	0.0	59
208386	4	40	8	15	30	38	7
209070	6	60	4	20	25	10	bdl
241845	20	0	30	80	100	17	13
243105	bdl	10	4	0	50	0.0	13
245014	0	0	0	0	0	25	36
245052	bdl	10	3	10	20	0.0	16
250136	0	0	0	0	0	0.0	bdl
253915	6	60	20	15	15	0.0	27
254187	15	0	25	60	1,000	1.0	17
269249	15	100	25	40	8	0.0	bdl
269264	0	0	0	0	0	0.0	bdl
279796	3	20	3	4	10	0.0	bdl
279820	3	40	3	10	15	0.0	bdl

Supplemental Table S3. Distribution of physical and chemical conditions among sampled soils.

	Minimum	25% (Q1)	50% (median)	75% (Q3)	Maximum
$\log\left[\frac{tet(\mathrm{M})}{16SrRNA}\right]$	-4.91	-3.31	-3.03	-2.64	-1.68
$\log\left[\frac{tet(\mathrm{Q})}{16SrRNA}\right]$	-5.14	-4.79	-4.37	-4.04	
$\log\left[\frac{tet(\mathrm{W})}{16SrRNA}\right]$	-3.65	-3.19	-2.90	-2.64	-1.66
$\log\left[\frac{bla_{\mathrm{TEM}}}{16SrRNA}\right]$	-5.96	-5.25	-4.83	-4.55	-3.42
$\log\left[\frac{bla_{\mathrm{SHV}}}{16SrRNA}\right]$	-5.16	-4.38	-3.94	-3.73	-3.05
$\log\left[\frac{bla_{\mathrm{CTX-M}}}{16SrRNA}\right]$	-3.64	-2.84	-2.48	-2.29	-1.48
$\log\left[\frac{bla_{\mathrm{OXA}}}{16SrRNA}\right]$	-4.07	-3.38	-3.23	-2.90	-2.27
$\log\left[\frac{erm(\mathrm{C})}{16SrRNA}\right]$	-5.06	-4.75	-4.31	-3.97	-3.48
$\log\left[\frac{erm(\mathrm{E})}{16SrRNA}\right]$	-5.02	-3.70	-3.28	-2.93	-2.20
$\log\left[\frac{erm(\mathrm{F})}{16SrRNA}\right]$	-5.20	-4.44	-4.21	-3.86	-3.34
Carbon, organic	0.0	2.5	3.7	5.3	45.3
Phosphorus, total	0.0	0.0	52.0	710	1610
Sand	0.0	35.3	52.5	63.1	88.0
Silt	0.0	18.6	25.5	35.3	64.0
Clay	0.0	9.2	13.1	17.1	45.6
Ash	22.0	87.0	91.5	95.0	97.0
pH	4.3	5.5	5.8	6.2	7.2
Cobalt, total	0.0	0.0	5.0	15.0	140
Chromium, total	0.0	0.0	25.0	85.0	250
Copper, total	0.0	2.8	7.0	26.3	140
Nickel, total	0.0	9.5	20.0	40.0	100
Lead, total	0.0	9.5	15.0	36.3	1000
Zinc, extractable	0.0	0.0	0.0	0.7	38.0
Iron, extractable	0.0	0.0	20.5	37.0	115

Supplemental Table S4. Bi-variate correlations among physical-chemical properties of archived soil samples.

	Total cobalt	Total chromium	Total copper	Total nickel	Total Lead	Extract. zinc	Extract. iron
Sand	-.053	-.205	-.018	-.006	-.167	-.008	-.128
Silt	-.099	.241	.022	.079	.311**	-.212	-.304**
Clay	.107	.262*	.102	.151	-.025	-.150	.101
Ash	-.305**	-.072	-.194	-.149	.057	-.354**	-.206
pH	-.063	.167	.033	-.076	.054	-.409***	-.165
Phosphorus, total	.371**	.208	.183	.290*	-.035	-.208	.387***
Carbon, organic	.324**	-.007	.211	.157	-.099	.398***	.119
Cobalt, total	-	.333**	.821***	.741***	.080	-.067	.207
Chromium, total	.333**	-	.565***	.463***	-.011	-.120	.225
Copper, total	.821***	.565***	-	.737***	.071	-.124	.240
Nickel, total	.741***	.463***	.737***	-	.321**	-.018	.279*
Lead, total	.080	-.011	.071	.321**	-	-.008	.099
Zinc,extractable	-.067	-.120	-.124	-.018	-.008	-	-.046
Iron, extractable	.207	.225	.240	.279*	.099	-.046	-

***** Significant at $P < 0.100$ level ($\alpha = 10\%$).

****** Significant at $P < 0.050$ level ($\alpha = 5\%$).

******* Significant at $P < 0.010$ level ($\alpha = 1\%$).

Supplemental Table S5. Results of multi-linear regression.

$$\log\left[\frac{tet(\mathrm{M})}{16\mathrm{SrRNA}}\right]$$

R = .695	R^2 = .480	P = 0.112

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-4.54	2.13		-2.13	.051
Chromium, total	.0018	.0056	.163	.318	.755
Copper, total	.0053	.0106	.169	.494	.629
Nickel, total	-.0012	.0173	-.026	-.069	.946
Lead, total	.0054	.0105	.136	.515	.615
Iron, extractable	.0128	.0067	.427	1.91	.077
pH	.175	.348	.105	.503	.622

$$\log\left[\frac{tet(\mathrm{Q})}{16\mathrm{SrRNA}}\right]$$

R = .555	R^2 = .308	P = .234

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-6.61	1.16		-5.71	.000
Chromium, total	-.0024	.0016	-.367	-1.45	.164
Copper, total	.0026	.0046	.199	.558	.583
Nickel, total	.0069	.0066	.345	1.04	.309
Lead, total	-.0022	.0028	-.168	-.798	.434
Iron, extractable	.0023	.0030	.160	.763	.454
pH	.356	.190	.395	1.87	.076

$$\log\left[\frac{tet(\mathrm{W})}{16\mathrm{SrRNA}}\right]$$

R = .482	R^2 = .232	P = .124

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-2.81	.746		-3.77	.001
Chromium, total	-.0022	.0011	-.367	-2.00	.053
Copper, total	.0031	.0032	.236	.988	.330
Nickel, total	.0056	.0041	.336	1.36	.181
Lead, total	-.0001	.0004	-.031	-.190	.850
Iron, extractable	.0011	.0021	.082	.528	.601
pH	-.029	.124	-.037	-.235	.816

$$\log\left[\frac{bla_{\mathrm{TEM}}}{16\mathrm{SrRNA}}\right]$$

R = .456	R^2 = .208	P = .344

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-4.94	1.16		-4.26	.000
Chromium, total	-.0033	.0021	-.398	-1.58	.125
Copper, total	.0132	.0064	.593	2.06	.049
Nickel, total	-.0045	.0067	-.183	-.674	.506
Lead, total	.0057	.0032	.436	1.80	.084
Iron, extractable	-.0026	.0036	-.149	-.729	.472
pH	.001	.195	.001	.004	.997

$$\log\left[\frac{bla_{\mathrm{SHV}}}{16\mathrm{SrRNA}}\right]$$

R = .451	R^2 = .203	P = .298

	Coefficient (B)	**Standard Error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-4.85	.98		-4.98	.000
Chromium, total	-.0031	.0015	-.456	2.02	.052
Copper, total	.0097	.0046	.524	2.11	.043
Nickel, total	.0012	.0054	.053	.214	.832
Lead, total	-.0005	.0006	-.169	-.911	.370
Iron, extractable	.0019	.0026	.127	.735	.468
pH	.125	.160	.136	.784	.439

$$\log\left[\frac{bla_{\mathrm{CTX-M}}}{16\mathrm{SrRNA}}\right]$$

R = .725	R^2 = .526	P = .000

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-5.20	.72		-7.24	.000
Chromium, total	.0030	.0011	.409	2.84	.007
Copper, total	.0006	.0030	.034	.182	.856
Nickel, total	-.0045	.0039	-.220	-1.14	.263
Lead, total	.0004	.0004	.133	1.04	.305
Iron, extractable	-.0044	.0020	-.269	-2.20	.035
pH	.456	.119	.469	3.82	.001

$$\log\left[\frac{bla_{\mathrm{OXA}}}{16\mathrm{SrRNA}}\right]$$

R = .539	R^2 = .291	P = .043

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-4.54	.62		-7.37	.000
Chromium, total	.0008	.0009	.164	.932	.358
Copper, total	.0032	.0026	.280	1.22	.230
Nickel, total	.0005	.0034	.037	.157	.876
Lead, total	.0000	.0004	-.007	-.043	.966
Iron, extractable	.0006	.0017	.052	.348	.730
pH	.210	.102	.308	2.05	.048

$$\log\left[\frac{erm(\mathrm{C})}{16\mathrm{SrRNA}}\right]$$

R = .550	R^2 = .302	P = .456

	Coefficient (B)	**Standard Error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (p)**
Constant	-4.30	1.33		-3.22	.006
Chromium, total	.0041	.0026	.630	1.60	.131
Copper, total	-.0025	.0097	-.083	-.256	.801
Nickel, total	-.0185	.0104	-.786	-1.79	.096
Lead, total	.0004	.0008	.176	.501	.624
Iron, extractable	.0068	.0056	.332	1.22	.244
pH	-.003	.218	-.004	-.014	.989

$$\log\left[\frac{erm(\mathrm{E})}{16\mathrm{SrRNA}}\right]$$

R = .522	R^2 = .272	P = .096

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (*p*)**
Constant	-6.29	1.22		-5.18	.000
Chromium, total	.0006	.0016	.065	.339	.737
Copper, total	-.0022	.0047	-.107	-.459	.650
Nickel, total	.0029	.0060	.119	.495	.624
Lead, total	.0004	.0006	.096	.566	.575
Iron, extractable	-.0052	.0031	-.273	-1.68	.103
pH	.506	.204	.399	2.49	.018

$$\log\left[\frac{erm(\mathrm{F})}{16\mathrm{SrRNA}}\right]$$

R = .663	R^2 = .440	P = .049

	Coefficient (B)	**Standard error**	**Standardised Coefficients (β)**	***t*-score**	**Signific. (*p*)**
Constant	-4.83	.99		-4.90	.000
Chromium, total	-.0029	.0015	-.478	-1.90	.073
Copper, total	.0150	.0047	.811	3.24	.004
Nickel, total	-.0011	.0047	-.049	-.224	.825
Lead, total	-.0002	.0005	-.083	-.415	.682
Iron, extractable	-.0051	.0027	-.323	-1.87	.076
pH	.118	.165	.127	.711	.485

Supplementary Table S6. One-way *ANOVA* statistics describing treatment differences in Hartwood and Auchincruive Cu-amended agricultural plots.

Hartwood Site		Sum of squares	d.f.	Mean squares	F	Sig.
$\log\left(\frac{tet(\mathbf{M})}{16S\ \mathbf{rRNA}}\right)$	Between groups	2.789	4	0.697	1.442	0.327
	Within groups	2.901	6	0.484		
	Total	5.691	10			
$\log\left(\frac{tet(\mathbf{W})}{16S\ \mathbf{rRNA}}\right)$	Between groups	1.760	4	0.440	0.451	0.770
	Within groups	6.830	7	0.976		
	Total	8.590	11			
$\log\left(\frac{bla_{\mathbf{TEM}}}{16S\ \mathbf{rRNA}}\right)$	Between groups	0.954	4	0.238	3.411	0.075
	Within groups	0.489	7	0.070		
	Total	1.443	11			
$\log\left(\frac{bla_{\mathbf{SHV}}}{16S\ \mathbf{rRNA}}\right)$	Between groups	1.238	4	0.310	4.535	0.040
	Within groups	0.478	7	0.068		

	Total	1.716	11			
$\log\left(\frac{bla_{CTX}}{16S\ rRNA}\right)$	Between groups	1.576	4	0.394	3.168	0.100
	Within groups	0.746	6	0.124		
	Total	2.322	10			
$\log\left(\frac{erm(F)}{16S\ rRNA}\right)$	Between groups	0.406	4	0.102	0.416	0.792
	Within groups	1.466	6	0.244		
	Total	1.872	10			

Auchincruive Site		Sum of squares	d.f.	Mean squares	F	Sig.
$\log\left(\frac{tet(M)}{16S\ rRNA}\right)$	Between groups	2.252	4	0.563	1.168	0.393
	Within groups	3.855	8	0.482		
	Total	6.106	12			
$\log\left(\frac{tet(W)}{16S\ rRNA}\right)$	Between groups	1.232	4	0.308	0.742	0.590
	Within groups	3.323	8	0.415		

	Total	4.555	12			
$\log\left(\frac{bla_{\mathrm{TEM}}}{16S\ \mathrm{rRNA}}\right)$	Between groups	0.087	4	0.022	1.147	0.401
	Within groups	0.153	8	0.019		
	Total	0.240	12			
$\log\left(\frac{bla_{\mathrm{SHV}}}{16S\ \mathrm{rRNA}}\right)$	Between groups	0.150	4	0.038	2.078	0.176
	Within groups	0.144	8	0.018		
	Total	0.294	12			
$\log\left(\frac{bla_{\mathrm{CTX}}}{16S\ \mathrm{rRNA}}\right)$	Between groups	0.716	4	0.179	1.259	0.361
	Within groups	1.138	8	0.142		
	Total	1.854	12			
$\log\left(\frac{erm(\mathrm{F})}{16S\ \mathrm{rRNA}}\right)$	Between groups	0.170	4	0.043	0.219	0.920
	Within groups	1.554	8	0.194		
	Total	1.724	12			

Correlations

Notes

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Correlations

		log_m_16S	log_W_16S	log_tem_16S	log_shv_16S
log_m_16S	Pearson Correlation	1	.111	.048	.068
	Sig. (2-tailed)		.622	.847	.763
	N	22	22	19	22
log_W_16S	Pearson Correlation	.111	1	.620**	.215
	Sig. (2-tailed)	.622		.000	.156
	N	22	45	36	45
log_tem_16S	Pearson Correlation	.048	.620**	1	.388*
	Sig. (2-tailed)	.847	.000		.019
	N	19	36	36	36
log_shv_16S	Pearson Correlation	.068	.215	.388*	1
	Sig. (2-tailed)	.763	.156	.019	
	N	22	45	36	45
cu	Pearson Correlation	.443*	.239	.236	-.121
	Sig. (2-tailed)	.039	.115	.166	.430
	N	22	45	36	45
phW	Pearson Correlation	.247	-.272	-.169	-.182
	Sig. (2-tailed)	.268	.071	.324	.231
	N	22	45	36	45
clay	Pearson Correlation	.333	-.288	-.067	-.355*
	Sig. (2-tailed)	.130	.055	.700	.017
	N	22	45	36	45

Correlations

		cu	phW	clay
log_m_16S	Pearson Correlation	.443*	.247	.333
	Sig. (2-tailed)	.039	.268	.130
	N	22	22	22
log_W_16S	Pearson Correlation	.239	-.272	-.288
	Sig. (2-tailed)	.115	.071	.055
	N	45	45	45
log_tem_16S	Pearson Correlation	.236	-.169	-.067
	Sig. (2-tailed)	.166	.324	.700
	N	36	36	36
log_shv_16S	Pearson Correlation	-.121	-.182	-.355*
	Sig. (2-tailed)	.430	.231	.017
	N	45	45	45
cu	Pearson Correlation	1	.044	.090
	Sig. (2-tailed)		.774	.558
	N	45	45	45
phW	Pearson Correlation	.044	1	.192
	Sig. (2-tailed)	.774		.207
	N	45	45	45
clay	Pearson Correlation	.090	.192	1
	Sig. (2-tailed)	.558	.207	
	N	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

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Correlations

Control Variables			log_m_16S	log_W_16S	log_tem_16S
clay	log_m_16S	Correlation	1.000	.162	-.026
		Significance (2-tailed)	.	.521	.919
		df	0	16	16
	log_W_16S	Correlation	.162	1.000	.565
		Significance (2-tailed)	.521	.	.015
		df	16	0	16
	log_tem_16S	Correlation	-.026	.565	1.000
		Significance (2-tailed)	.919	.015	.
		df	16	16	0
	log_shv_16S	Correlation	.250	.456	.478
		Significance (2-tailed)	.316	.057	.045
		df	16	16	16
	cu	Correlation	.372	.597	.536
		Significance (2-tailed)	.129	.009	.022
		df	16	16	16

Correlations

Control Variables			log_shv_16S	cu
clay	log_m_16S	Correlation	.250	.372
		Significance (2-tailed)	.316	.129
		df	16	16
	log_W_16S	Correlation	.456	.597
		Significance (2-tailed)	.057	.009
		df	16	16
	log_tem_16S	Correlation	.478	.536
		Significance (2-tailed)	.045	.022
		df	16	16
	log_shv_16S	Correlation	1.000	.412
		Significance (2-tailed)	.	.089
		df	0	16
	cu	Correlation	.412	1.000
		Significance (2-tailed)	.089	.
		df	16	0

Partial Corr

Notes

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Correlations

Control Variables			log_m_16S	log_W_16S	log_tem_16S
phW	log_m_16S	Correlation	1.000	.111	-.013
		Significance (2-tailed)	.	.662	.961
		df	0	16	16
	log_W_16S	Correlation	.111	1.000	.538
		Significance (2-tailed)	.662	.	.021
		df	16	0	16
	log_tem_16S	Correlation	-.013	.538	1.000
		Significance (2-tailed)	.961	.021	.
		df	16	16	0
	log_shv_16S	Correlation	-.118	.399	.306
		Significance (2-tailed)	.642	.101	.217
		df	16	16	16
	cu	Correlation	.410	.560	.529
		Significance (2-tailed)	.091	.016	.024
		df	16	16	16

Correlations

Control Variables			log_shv_16S	cu
phW	log_m_16S	Correlation	-.118	.410
		Significance (2-tailed)	.642	.091
		df	16	16
	log_W_16S	Correlation	.399	.560
		Significance (2-tailed)	.101	.016
		df	16	16
	log_tem_16S	Correlation	.306	.529
		Significance (2-tailed)	.217	.024
		df	16	16
	log_shv_16S	Correlation	1.000	.194
		Significance (2-tailed)	.	.440
		df	0	16
	cu	Correlation	.194	1.000
		Significance (2-tailed)	.440	.
		df	16	0

Partial Corr

Notes

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Correlations

Control Variables			log_m_16S	log_W_16S	log_tem_16S
clay & phW	log_m_16S	Correlation	1.000	.111	-.114
		Significance (2-tailed)	.	.672	.662
		df	0	15	15
	log_W_16S	Correlation	.111	1.000	.542
		Significance (2-tailed)	.672	.	.024
		df	15	0	15
	log_tem_16S	Correlation	-.114	.542	1.000
		Significance (2-tailed)	.662	.024	.
		df	15	15	0
	log_shv_16S	Correlation	.074	.444	.417
		Significance (2-tailed)	.778	.074	.095
		df	15	15	15
	cu	Correlation	.324	.578	.505
		Significance (2-tailed)	.205	.015	.039
		df	15	15	15

Correlations

Control Variables			log_shv_16S	cu
clay & phW	log_m_16S	Correlation	.074	.324
		Significance (2-tailed)	.778	.205
		df	15	15
	log_W_16S	Correlation	.444	.578
		Significance (2-tailed)	.074	.015
		df	15	15
	log_tem_16S	Correlation	.417	.505
		Significance (2-tailed)	.095	.039
		df	15	15
	log_shv_16S	Correlation	1.000	.348
		Significance (2-tailed)	.	.171
		df	0	15
	cu	Correlation	.348	1.000
		Significance (2-tailed)	.171	.
		df	15	0

Appendix 3 - Chapter 4 qPCR data

All values averaged and corrected for dilution

Sample	16S	Tet1	Tet2	Tet3	TEM	CTX
1	3.40E+08	4.21E+07	5.42E+06	5.15E+07	3.52E+06	3.87E+09
2	3.05E+08	2.57E+07	3.22E+06	2.52E+07	3.62E+06	3.30E+09
3	3.02E+08	2.68E+07	1.34E+06	3.86E+07	3.21E+06	4.12E+09
4	3.59E+08	2.67E+07	4.03E+06	1.11E+07	3.28E+06	9.08E+08
5	3.28E+08	2.95E+07	4.28E+06	3.40E+07	3.35E+06	1.17E+09
6	2.00E+08	1.70E+07	8.62E+05	2.63E+07	1.76E+06	1.24E+09
7	2.67E+08	4.61E+07	6.69E+06	5.64E+07	3.67E+06	2.32E+09
8	2.00E+08	2.55E+07	2.81E+06	3.21E+07	1.41E+06	2.47E+08
41	2.48E+08	3.61E+07	4.25E+06	5.28E+07	3.33E+06	5.27E+08
42	3.30E+08	3.21E+07	4.51E+06	5.22E+07	5.53E+06	2.20E+09
43	3.28E+08	3.32E+07	3.05E+06	1.02E+08	2.21E+06	4.69E+08
44	2.98E+08	4.96E+07	8.60E+06	5.35E+07	2.70E+06	8.52E+08
45	3.34E+08	4.33E+07	7.09E+06	5.95E+07	3.79E+06	2.21E+09
46	3.03E+08	4.19E+07	4.57E+06	6.82E+07	2.39E+06	2.86E+07
47	3.25E+08	1.99E+08	7.59E+06	5.30E+07	3.10E+06	8.53E+08
48	1.10E+08	2.49E+07	3.91E+06	2.41E+07	1.51E+06	6.77E+08
49	9.65E+07	9.96E+06	2.11E+06	5.75E+06	1.56E+05	1.07E+06
50	1.59E+08	1.59E+07	3.71E+06	1.28E+07	1.87E+06	1.14E+07
51	1.55E+08	1.51E+07	2.34E+06	2.00E+07	1.40E+06	1.31E+07
52	3.36E+08	4.90E+07	8.89E+06	6.61E+07	1.68E+06	4.24E+07
53	7.27E+08	5.90E+07	1.36E+07	1.23E+08	1.33E+07	1.25E+10
54	3.65E+08	5.52E+07	3.43E+06	1.10E+08	5.66E+06	1.24E+09
55	6.40E+08	1.32E+08	5.32E+06	2.95E+08	1.03E+07	2.40E+09
56	6.61E+08	6.84E+07	3.61E+06	1.61E+08	1.20E+07	9.23E+09
57	2.51E+08	2.03E+07	2.92E+06	3.98E+07	2.78E+06	2.51E+08
58	2.30E+08	1.67E+07	1.20E+06	3.08E+07	1.31E+06	3.48E+07
59	3.90E+08	2.43E+07	3.07E+06	4.47E+07	4.90E+06	4.92E+07
60	2.54E+08	2.08E+07	1.60E+06	4.86E+07	3.17E+06	1.60E+06
61	3.30E+08	5.42E+07	3.45E+06	1.52E+08	3.68E+06	1.74E+06
62	4.85E+08	4.21E+07	3.53E+06	1.01E+08	5.70E+06	3.29E+07
63	6.50E+08	4.25E+07	3.39E+06	1.40E+08	3.27E+06	2.04E+07
64	3.49E+08	2.63E+07	2.53E+06	8.72E+07	2.76E+06	3.66E+06

Appendix 4 - Chapter 5 plate count data

corrected for dilution			corrected for dilution			corrected for dilution			corrected for dilution		
week 6	PCA	PCA	week 6	Cu	Cu	week 6	Tet	Tet	week 6	Amp	Amp
R1	2.37E+06	2.83E+06	R1	1.54E+06	1.78E+06	R1	9.90E+03	9.50E+03	R1	2.13E+06	2.01E+06
R2	4.17E+05	3.66E+05	R2	2.07E+06	2.11E+06	R2	4.50E+04	3.90E+04	R2	3.10E+05	2.64E+05
R3	3.46E+06	2.88E+06	R3	2.34E+06	2.41E+06	R3	1.14E+04	9.10E+03	R3	2.34E+06	2.63E+06
R4	3.65E+06	2.66E+06	R4	1.64E+06	2.24E+06	R4	8.80E+03	8.50E+03	R4	2.87E+06	2.67E+06
R5	2.41E+06	3.12E+06	R5	1.65E+06	1.68E+06	R5	1.30E+03	1.27E+03	R5	2.80E+06	2.66E+06
R6	2.57E+06	2.96E+06	R6	1.68E+06	1.38E+06	R6	8.70E+03	7.80E+03	R6	2.56E+06	2.69E+06
R7	2.79E+06	3.02E+06	R7	1.76E+06	2.18E+06	R7	1.46E+05	1.65E+05	R7	2.41E+06	2.34E+06
R8	1.22E+05	2.71E+05	R8	1.70E+05	1.73E+05	R8	3.80E+04	4.00E+04	R8	2.86E+06	2.27E+06
week 8	PCA	PCA	week 8	Cu	Cu	week 8	Tet	Tet	week 8	Amp	Amp
R1	3.70E+05	4.50E+05	R1	1.49E+05	2.20E+05	R1	4.70E+04	4.50E+04	R1	2.21E+05	2.40E+05
R2	2.07E+06	1.42E+06	R2	1.21E+06	1.14E+06	R2	4.80E+04	4.30E+04	R2	1.38E+06	8.70E+05
R3	1.61E+06	1.72E+06	R3	7.90E+05	1.12E+06	R3	2.70E+04	6.20E+04	R3	9.00E+05	1.54E+06
R4	1.79E+06	2.20E+06	R4	1.09E+06	1.22E+06	R4	5.10E+04	5.80E+04	R4	1.21E+06	2.02E+06
R5	1.44E+06	1.86E+06	R5	7.00E+05	6.30E+05	R5	1.50E+03	2.00E+03	R5	9.50E+05	6.70E+05
R6	1.94E+06	1.77E+06	R6	6.20E+05	6.30E+05	R6	3.80E+03	3.70E+03	R6	9.80E+05	9.20E+05
R7	2.43E+05	2.08E+05	R7	8.60E+05	6.60E+05	R7	9.80E+03	8.40E+03	R7	1.57E+05	1.69E+05
R8	1.52E+06	2.00E+06	R8	6.60E+05	6.60E+05	R8	4.70E+03	3.20E+03	R8	1.02E+06	9.80E+05

week 10	PCA	PCA	week 10	Cu	Cu	week 10	Tet	Tet	week 10	Amp	Amp
R1	2.68E+06	2.61E+06	R1	3.56E+06	3.28E+06	R1	4.20E+05	4.00E+05	R1	3.15E+06	2.89E+06
R2	2.32E+06	2.02E+06	R2	2.80E+06	2.45E+06	R2	2.50E+05	2.80E+05	R2	2.07E+06	2.67E+06
R3	2.20E+06	1.30E+06	R3	1.74E+06	2.02E+06	R3	3.20E+05	3.50E+05	R3	1.98E+06	2.86E+06
R4	1.99E+06	1.74E+06	R4	1.65E+06	1.04E+06	R4	1.40E+05	2.20E+05	R4	1.40E+06	1.54E+06
R5	1.72E+06	1.62E+06	R5	2.26E+06	2.10E+06	R5	2.10E+04	1.60E+04	R5	2.52E+06	1.87E+06
R6	1.54E+06	1.20E+06	R6	2.08E+06	2.05E+06	R6	1.20E+04	8.00E+03	R6	2.88E+06	2.31E+06
R7	2.81E+06	2.40E+06	R7	1.20E+06	1.21E+06	R7	1.20E+04	1.10E+04	R7	2.59E+06	2.23E+06
R8	2.30E+06	2.37E+06	R8	1.60E+06	1.40E+06	R8	1.60E+04	1.50E+04	R8	2.12E+06	2.23E+06
week 12	PCA	PCA	week 12	Cu	Cu	week 12	Tet	Tet	week 12	Amp	Amp
R1	2.31E+06	2.11E+06	R1	3.41E+06	2.51E+06	R1	2.90E+05	2.10E+05	R1	2.53E+06	2.50E+06
R2	4.01E+06	4.04E+06	R2	4.86E+06	4.49E+06	R2	3.20E+05	1.60E+05	R2	2.73E+06	2.65E+06
R3	2.57E+06	2.00E+06	R3	2.36E+06	2.47E+06	R3	2.40E+05	1.60E+05	R3	3.29E+06	2.93E+06
R4	2.18E+06	3.45E+06	R4	2.12E+06	2.81E+06	R4	1.40E+05	2.40E+05	R4	3.45E+06	3.03E+06
R5	2.70E+06	2.88E+06	R5	1.27E+06	1.79E+06	R5	2.40E+03	5.10E+03	R5	3.22E+06	3.26E+06
R6	2.09E+06	1.98E+06	R6	3.04E+06	2.85E+06	R6	1.40E+04	1.30E+04	R6	2.78E+06	2.63E+06
R7	3.73E+06	3.64E+06	R7	3.50E+06	3.32E+06	R7	1.20E+03	1.10E+03	R7	3.75E+06	3.48E+06
R8	4.04E+06	3.61E+06	R8	4.27E+06	4.40E+06	R8	2.70E+04	1.60E+04	R8	3.98E+06	3.62E+06

week 14	PCA	PCA	week 14	Cu	Cu	week 14	Tet	Tet	week 14	Amp	Amp
R1	2.38E+06	1.86E+06	R1	1.72E+06	1.57E+06	R1	4.40E+05	4.40E+05	R1	2.63E+06	2.72E+06
R2	2.72E+06	2.78E+06	R2	2.33E+06	2.18E+06	R2	5.50E+05	4.00E+05	R2	1.46E+06	2.64E+06
R3	4.27E+06	4.71E+06	R3	2.04E+06	1.06E+06	R3	3.60E+05	4.40E+05	R3	1.72E+06	2.05E+06
R4	2.21E+06	2.46E+06	R4	2.26E+06	2.16E+06	R4	3.00E+05	2.70E+05	R4	2.97E+06	2.76E+06
R5	2.14E+06	2.16E+06	R5	1.96E+06	1.84E+06	R5	2.40E+04	2.60E+04	R5	2.43E+06	2.66E+06
R6	2.50E+06	2.48E+06	R6	1.91E+06	1.74E+06	R6	2.20E+04	3.80E+04	R6	2.58E+06	2.26E+06
R7	2.65E+06	2.51E+06	R7	1.50E+06	1.55E+06	R7	3.90E+04	4.30E+04	R7	2.45E+06	2.15E+06
R8	2.45E+06	2.34E+06	R8	1.41E+06	1.36E+06	R8	2.80E+04	2.90E+04	R8	1.62E+06	1.98E+06
week 16	PCA	PCA	week 16	Cu	Cu	week 16	Tet	Tet	week 16	Amp	Amp
R1	2.42E+06	2.11E+06	R1	1.20E+05	6.40E+05	R1	3.00E+05	3.30E+05	R1	1.98E+06	2.24E+06
R2	2.08E+06	2.31E+06	R2	2.09E+06	1.91E+06	R2	3.90E+05	2.80E+05	R2	2.03E+06	2.01E+06
R3	2.10E+06	1.76E+06	R3	1.22E+05	1.77E+06	R3	3.40E+05	2.90E+05	R3	2.02E+06	2.85E+06
R4	2.11E+06	1.71E+06	R4	2.67E+06	2.39E+06	R4	3.90E+05	3.90E+05	R4	1.60E+06	1.90E+06
R5	1.90E+06	1.86E+06	R5	2.13E+06	2.05E+06	R5	3.20E+04	2.70E+04	R5	1.93E+06	2.26E+06
R6	2.15E+06	1.78E+06	R6	1.97E+06	1.87E+06	R6	3.90E+04	3.10E+04	R6	1.89E+06	2.10E+06
R7	2.14E+06	2.04E+06	R7	1.66E+06	1.49E+06	R7	4.00E+04	4.10E+04	R7	2.04E+06	2.54E+06
R8	2.19E+06	2.04E+06	R8	1.56E+06	1.95E+06	R8	3.00E+04	4.20E+04	R8	1.92E+06	1.50E+05

week 18	PCA	PCA	week 18	Cu	Cu	Week 18	Tet	Tet	week 18	Amp	Amp
R1	2.13E+06	1.72E+06	R1	2.39E+06	2.20E+06	R1	4.00E+05	4.00E+05	R1	1.76E+06	1.66E+06
R2	1.95E+06	2.01E+06	R2	2.01E+06	2.50E+06	R2	3.20E+05	3.00E+05	R2	1.69E+06	1.84E+06
R3	1.52E+06	1.76E+06	R3	1.96E+06	1.09E+06	R3	1.90E+05	2.20E+05	R3	1.72E+06	1.63E+06
R4	1.88E+06	1.73E+06	R4	2.08E+06	1.82E+06	R4	4.00E+05	3.60E+05	R4	1.76E+06	1.89E+06
R5	2.28E+06	1.87E+06	R5	2.14E+06	2.17E+06	R5	1.70E+04	3.30E+04	R5	1.92E+06	1.73E+06
R6	2.30E+06	1.83E+06	R6	2.31E+06	1.89E+06	R6	2.10E+04	3.10E+04	R6	1.75E+06	1.24E+06
R7	1.92E+06	2.11E+06	R7	2.08E+06	1.91E+06	R7	3.60E+04	2.60E+04	R7	2.15E+06	2.21E+06
R8	1.97E+06	1.42E+06	R8	2.19E+06	1.97E+06	R8	2.00E+04	2.60E+04	R8	2.31E+06	2.16E+06

week 20	PCA	PCA	week 20	Cu	Cu	week 20	Tet	Tet	week 20	Amp	Amp
R1	1.23E+06	1.31E+06	R1	1.27E+06	1.12E+06	R1	4.90E+05	6.00E+05	R1	1.60E+06	1.55E+06
R2	2.55E+06	1.53E+06	R2	1.57E+06	1.55E+06	R2	6.00E+05	8.70E+05	R2	2.59E+06	1.06E+06
R3	3.53E+06	3.50E+06	R3	2.18E+06	1.76E+06	R3	3.00E+05	3.30E+05	R3	3.35E+06	3.78E+06
R4	3.33E+06	3.54E+06	R4	1.64E+06	1.32E+06	R4	2.90E+05	1.80E+05	R4	3.19E+06	3.11E+06
R5	4.51E+06	4.38E+06	R5	1.77E+06	2.47E+06	R5	4.10E+04	4.20E+04	R5	4.04E+06	3.95E+06
R6	3.24E+06	3.38E+06	R6	1.67E+06	1.95E+06	R6	1.70E+04	3.00E+04	R6	2.40E+06	2.99E+06
R7	3.61E+06	3.55E+06	R7	3.00E+06	3.01E+06	R7	3.90E+04	3.00E+04	R7	3.09E+06	3.37E+06
R8	3.56E+06	3.71E+06	R8	2.37E+06	2.33E+06	R8	3.80E+04	3.60E+04	R8	3.34E+06	3.38E+06

week 22	PCA	PCA	week 22	Cu	Cu	week 22	Tet	Tet	week 22	Amp	Amp
R1	1.84E+06	1.55E+06	R1	1.82E+06	1.54E+06	R1	4.60E+05	4.60E+05	R1	2.10E+06	1.66E+06
R2	2.07E+06	1.95E+06	R2	1.68E+06	1.53E+06	R2	4.10E+05	5.70E+05	R2	1.62E+06	1.32E+06
R3	2.78E+06	2.88E+06	R3	1.99E+06	2.04E+06	R3	5.20E+05	4.00E+05	R3	2.51E+06	3.27E+06
R4	3.14E+06	3.12E+06	R4	2.54E+06	2.53E+06	R4	5.20E+05	5.20E+05	R4	3.38E+06	2.87E+06
R5	4.11E+06	4.28E+06	R5	2.22E+06	2.06E+06	R5	5.00E+04	4.30E+04	R5	3.48E+06	3.47E+06
R6	3.48E+06	3.51E+06	R6	1.84E+06	1.98E+06	R6	3.20E+04	4.10E+04	R6	3.18E+06	3.09E+06
R7	3.94E+06	3.69E+06	R7	2.69E+06	2.32E+06	R7	2.10E+04	3.30E+04	R7	2.53E+06	3.07E+06
R8	3.69E+06	3.35E+06	R8	3.71E+06	3.61E+06	R8	3.50E+04	3.60E+04	R8	3.41E+06	3.36E+06

Appendix 4 - Chapter 5 qPCR data

All values averaged and corrected for dilution

Sample	16S	Tet1	Tet2	Tet3	TEM	CTX
1	2.21E+07	9.24E+06	1.45E+06	1.78E+06	4.62E+04	1.99E+06
2	1.15E+07	1.38E+07	1.99E+06	1.52E+06	2.89E+04	2.96E+05
3	1.18E+07	4.91E+07	7.18E+06	3.36E+06	1.35E+05	1.04E+06
4	4.12E+07	2.74E+07	2.49E+06	3.59E+06	8.60E+04	1.20E+06
5	3.31E+07	3.79E+07	3.55E+06	3.64E+06	1.40E+05	1.72E+06
6	2.35E+07	5.68E+07	5.75E+06	3.60E+06	3.05E+05	3.09E+06
7	9.31E+07	6.40E+07	5.88E+06	6.30E+06	1.21E+05	4.26E+06
8	1.97E+07	1.31E+07	1.60E+06	2.12E+06	1.94E+04	3.69E+05
9	1.69E+07	4.81E+06	4.47E+05	1.11E+06	1.80E+04	2.17E+05
10	1.62E+07	1.71E+07	3.13E+06	2.63E+06	5.18E+04	1.34E+06
11	5.97E+07	4.06E+07	3.59E+06	3.08E+06	3.62E+04	1.40E+06
12	3.96E+07	7.96E+07	7.53E+06	2.24E+07	7.28E+04	3.06E+06
13	1.05E+07	1.77E+07	1.25E+06	2.09E+06	1.85E+04	4.01E+05
14	3.58E+07	1.55E+07	1.75E+06	3.32E+06	1.16E+04	2.77E+05
15	3.40E+07	4.24E+07	4.07E+06	1.17E+07	5.99E+04	1.29E+06
16	6.98E+06	4.79E+06	4.36E+05	1.80E+06	5.93E+03	4.09E+04
17	2.90E+07	3.37E+07	1.89E+06	1.32E+06	1.47E+04	9.19E+05
18	8.36E+07	4.27E+07	2.48E+06	3.81E+06	3.47E+04	5.09E+05
19	4.03E+07	5.01E+07	2.98E+06	2.94E+06	1.96E+04	8.13E+05
20	7.50E+08	5.22E+07	4.22E+06	3.83E+05	3.88E+04	1.28E+06
21	1.82E+08	1.93E+07	1.98E+06	4.55E+06	2.37E+04	1.30E+06
22	1.21E+08	1.37E+07	1.90E+06	2.61E+06	2.92E+04	1.16E+06
23	2.56E+08	2.65E+07	3.13E+06	3.69E+06	3.39E+04	1.65E+06
24	1.03E+08	1.50E+07	1.54E+06	2.47E+06	3.04E+04	1.69E+06
25	1.33E+07	8.97E+06	8.09E+05	1.23E+06	1.89E+04	3.38E+05
26	1.25E+07	1.62E+07	1.04E+06	1.07E+06	5.75E+03	7.72E+04
27	2.98E+08	2.83E+06	1.41E+06	4.42E+04	1.03E+04	1.18E+05
28	6.26E+07	1.92E+06	1.07E+06	3.26E+04	7.51E+03	7.78E+04
29	1.96E+08	7.70E+05	7.66E+05	1.25E+05	7.10E+03	7.56E+05
30	1.19E+08	1.60E+06	2.07E+06	2.68E+05	1.01E+04	3.75E+05
31	1.07E+09	3.75E+06	5.57E+06	6.96E+05	5.18E+04	5.36E+06
32	5.36E+08	1.05E+06	1.17E+06	1.53E+05	9.76E+03	4.19E+05
33	2.13E+08	2.00E+06	6.72E+05	5.93E+04	4.24E+04	2.26E+05
34	1.57E+08	2.04E+06	1.65E+06	4.76E+04	2.19E+04	1.72E+05
35	2.65E+07	6.87E+06	2.43E+06	4.35E+04	1.49E+04	2.01E+05
36	3.03E+08	7.54E+05	6.98E+05	3.10E+04	1.48E+04	1.38E+05
37	3.55E+08	4.63E+05	1.02E+06	4.82E+05	6.70E+03	3.05E+05
38	1.78E+09	1.42E+06	3.21E+06	1.72E+06	1.08E+04	3.42E+05
39	5.12E+08	1.48E+06	4.01E+06	5.15E+05	6.30E+03	2.20E+05
40	4.98E+08	9.09E+05	1.94E+06	8.84E+05	6.93E+03	5.19E+05
41	2.27E+09	9.58E+06	2.61E+07	3.07E+06	1.63E+05	8.03E+05

42	1.91E+08	2.48E+06	4.57E+06	4.89E+05	1.35E+04	1.15E+05
43	2.83E+08	2.38E+06	1.12E+06	9.27E+05	4.83E+04	2.01E+05
44	4.15E+08	1.82E+06	9.19E+05	8.98E+05	3.16E+04	1.60E+05
45	1.65E+09	8.95E+05	2.34E+06	1.13E+06	7.15E+03	3.36E+05
46	7.31E+08	7.53E+05	2.34E+06	1.10E+06	1.15E+04	5.84E+04
47	5.38E+08	1.28E+06	3.65E+06	1.61E+06	1.23E+04	2.10E+05
48	3.56E+08	8.01E+05	1.85E+06	9.71E+05	4.39E+03	8.32E+04
49	6.43E+07	1.85E+05	5.78E+05	4.29E+05	5.03E+03	2.82E+03
50	6.16E+07	1.07E+05	2.05E+05	2.54E+05	4.20E+03	6.69E+03
51	3.71E+06	1.21E+05	8.02E+04	2.53E+05	5.40E+03	5.14E+03
52	4.92E+06	7.34E+04	1.14E+05	2.84E+05	6.88E+03	5.19E+04
53	1.06E+08	6.23E+05	1.88E+05	7.82E+05	4.86E+03	3.68E+06
54	2.60E+07	2.51E+05	2.47E+05	7.68E+05	4.97E+03	7.81E+05
55	1.37E+08	2.99E+05	3.49E+05	6.47E+05	4.64E+03	3.91E+06
56	2.44E+07	3.26E+05	1.77E+05	1.50E+08	4.12E+03	6.47E+05
57	9.63E+06	2.03E+06	2.10E+06	1.11E+06	8.61E+03	1.15E+07
58	1.80E+07	9.64E+05	9.50E+04	3.06E+05	4.13E+03	7.07E+05
59	4.32E+07	1.53E+05	1.48E+05	5.45E+05	1.28E+04	1.93E+06
60	9.12E+06	3.35E+05	1.84E+05	1.37E+06	3.23E+03	5.88E+05
61	6.13E+07	2.02E+05	1.20E+05	5.36E+05	6.32E+03	9.42E+05
62	4.34E+07	1.16E+05	8.77E+04	8.32E+05	8.51E+03	1.39E+06
63	5.03E+07	2.12E+05	1.81E+05	6.75E+05	3.80E+03	1.64E+06
64	8.28E+07	5.18E+05	1.19E+06	1.01E+06	6.56E+03	2.88E+06
65	2.84E+08	1.97E+06	1.41E+06	1.14E+06	7.69E+03	5.41E+06
66	5.29E+07	5.94E+05	2.10E+05	8.62E+05	4.78E+03	1.30E+06
67	6.46E+07	1.83E+05	2.37E+05	3.54E+05	5.30E+03	1.47E+06
68	6.18E+07	4.12E+05	2.98E+05	3.55E+05	5.23E+03	2.72E+06
69	3.81E+07	6.47E+04	2.12E+05	9.19E+05	3.20E+03	2.63E+06
70	7.53E+07	9.68E+04	2.35E+05	7.33E+05	4.80E+03	2.84E+06
71	2.78E+08	8.63E+05	9.81E+05	1.25E+06	7.07E+03	2.17E+07
72	1.21E+08	8.49E+05	1.09E+06	2.66E+06	9.57E+03	1.39E+08

Appendix 5 - Chapter 6 qPCR data

Values averaged and corrected for dilution

Sample	16S	Tet1	Tet2	Tet3	CTX	TEM
1	7.20E+07	3.45E+05	1.38E+05	3.76E+04	1.12E+05	3.14E+04
2	3.43E+09	1.45E+08	3.73E+06	1.40E+06	4.96E+07	1.41E+07
3	5.53E+08	1.98E+08	3.52E+06	4.91E+05	9.94E+06	4.39E+06
4	1.51E+09	2.06E+08	1.09E+07	1.05E+06	7.45E+06	2.20E+06
5	1.26E+08	8.55E+07	4.20E+05	3.52E+05	3.40E+06	7.48E+05
6	5.34E+08	1.28E+08	4.48E+06	8.64E+05	1.76E+06	4.95E+05
7	1.99E+09	1.60E+08	4.49E+06	7.72E+05	1.88E+07	6.36E+06
8	2.80E+08	8.00E+07	2.14E+06	3.37E+05	2.00E+06	5.95E+05
9	2.15E+09	2.01E+08	1.86E+07	7.52E+05	3.22E+07	7.70E+06
10	6.03E+08	1.80E+07	7.89E+05	5.64E+05	5.43E+06	1.33E+06
11	6.47E+08	4.56E+07	4.24E+06	5.00E+05	3.14E+06	4.24E+05
12	9.62E+08	1.53E+08	8.41E+06	6.69E+05	3.18E+06	7.75E+05
13	5.56E+08	9.41E+07	4.13E+06	5.87E+05	2.79E+06	1.83E+08
14	1.08E+09	1.80E+08	1.24E+07	7.66E+05	1.05E+07	1.48E+09
15	1.68E+09	2.40E+08	2.46E+07	1.96E+06	6.43E+07	4.75E+09
16	5.84E+08	4.56E+07	5.01E+06	5.73E+06	9.16E+06	5.88E+08
17	3.22E+08	7.21E+07	4.78E+06	3.00E+05	2.89E+06	2.71E+08
18	1.31E+09	2.90E+08	2.50E+07	9.01E+05	1.50E+07	9.86E+08
19	5.27E+08	2.93E+08	2.17E+07	1.73E+06	8.44E+06	7.08E+08
20	2.33E+09	1.30E+08	1.20E+07	6.49E+05	3.66E+06	2.80E+08
21	1.71E+09	4.59E+07	5.60E+06	1.33E+06	4.51E+06	3.08E+08
22	9.90E+07	1.95E+07	3.92E+06	2.15E+06	1.73E+06	1.14E+08
23	6.64E+08	3.92E+07	5.65E+06	8.36E+05	8.18E+06	5.52E+08
24	6.08E+08	1.48E+08	1.79E+07	1.82E+06	1.79E+06	1.26E+08
25	1.09E+08	1.29E+08	1.21E+07	7.20E+05	3.22E+06	4.12E+08
26	1.18E+08	5.74E+07	4.44E+06	3.90E+05	2.75E+06	1.82E+08
27	8.65E+08	1.65E+07	6.10E+06	8.83E+07	2.35E+06	1.71E+07
28	7.63E+08	1.11E+07	4.77E+06	2.00E+09	2.05E+06	1.05E+07
29	1.83E+09	2.15E+07	9.15E+06	9.01E+07	3.53E+06	1.36E+07
30	4.13E+08	1.60E+07	5.72E+06	3.31E+07	3.90E+05	1.82E+06
31	6.62E+08	4.60E+07	1.49E+07	1.64E+08	1.40E+06	7.24E+06
32	3.17E+08	2.80E+07	8.19E+06	7.88E+07	9.16E+05	4.15E+06
33	3.98E+08	8.10E+06	4.90E+06	1.27E+08	1.07E+06	6.52E+06
34	4.10E+08	4.71E+06	1.65E+06	5.58E+08	6.52E+05	3.77E+06
35	7.94E+08	1.24E+07	7.81E+06	3.95E+08	2.11E+06	1.14E+07
36	1.59E+08	8.49E+06	2.94E+06	3.83E+08	7.03E+05	2.72E+06
37	2.98E+08	1.27E+07	5.43E+06	4.54E+07	2.02E+05	2.26E+06
38	8.27E+08	2.19E+07	8.23E+06	5.78E+08	1.66E+06	1.21E+07