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VEIKO VOOLAID

Aquatic environment: primary reservoir, link, or sink of antibiotic resistance?

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Aquatic environment: primary reservoir, link, or sink of antibiotic resistance?

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Supervisor: Tanel Tenson, PhD
Institute of Technology, University of Tartu, Estonia

Veljo Kisand, PhD
Institute of Technology, University of Tartu, Estonia

Opponent: Célia Maria Manaia Rodrigues, PhD
Universidade Católica Portuguesa, Portugal

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To my lovely wife, and my sons Albert and "Robert/Lennart"

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to by their Roman numerals.

I. **Voolaid V**, Jõers A, Kisand V & Tenson T (2012) Co-occurrence of resistance to different antibiotics among aquatic bacteria. *BMC microbiology* 12: 225.

II. **Voolaid V**, Tenson T & Kisand V (2013) Aeromonas and Pseudomonas species carriers of ampC FOX genes in aquatic environments. *Applied and environmental microbiology* 79: 1055–1057.

III. Laht M, Karkman A, **Voolaid V**, Ritz C, Tenson T, Virta M & Kisand V (2014) Abundances of Tetracycline, Sulphonamide and Beta-Lactam Antibiotic Resistance Genes in Conventional Wastewater Treatment Plants (WWTPs) with Different Waste Load. PLoS One 9: e103705.

Author's contributions:

I. Performed all the experiments, participated in data analysis, and writing the manuscript

II. Performed all the experiments and data analysis, participated in writing the manuscript.

III. Participated in sample collection, extraction, and analysis. Helped to write the manuscript

LIST OF ABBREVIATIONS

AB	–	antibiotic
AR	–	antibiotic resistance
ARG	–	antibiotic resistance gene
ESBL	–	extended spectrum β-lactamases
EUCAST	–	The European Committee on Antimicrobial Susceptibility Testing
HGT	–	horizontal gene transfer
MGE	–	mobile genetic element
MIC	–	minimum inhibitory concentration
MLST	–	multilocus sequence typing
MRSA	–	methicillin resistant *Staphylococcus aureus*
qPCR	–	quantitative PCR
WWTP	–	wastewater treatment plant

INTRODUCTION

The discovery of antibiotics in the beginning of 20th century created hope in both the medical community and broader public that bacterial infectious diseases will be soon eradicated. It was quickly realized that bacteria develop resistance against the new "miracle drugs". Initially, this was not much of a problem because many new drugs were discovered and rapidly entered the market. Today, the development of new antimicrobial drugs has decreased due to both the high cost of development and low profit margin of antibiotic drugs. Simultaneously, antibiotic resistance is emerging and spreading more rapidly around the globe.

While humans discovered antibiotics and began using them as drugs in the 20th century, microorganisms in the environment have produced these compounds for many millions of years. The main role that antibiotics play in the environment is the same as in medicine, to fend off unwanted microorganisms. Resistance genes are as ancient as antibiotics and have not just appeared with the human use of antibiotics, however, the development of resistance genes and their spread has been accelerated by the stronger selection created by man.

Currently, antibiotic resistance is a worldwide problem that causes both treatment failures and increases the costs in our medical systems. The problems caused by antibiotic resistance mostly impact our medical practice; however, medicinal use of antibiotics is not the only cause of resistance. Resistance is influenced by antibiotic usage in veterinary medicine, agriculture, and fisheries. All of this impacts the resistome, which is a term describing all of the known and unknown resistance determinants, including determinants in natural environment. The impact on the natural environment caused by the emergence and spread of resistance is not well understood. Medically relevant antibiotic resistance genes are found in the environmental resistome, however, it is not clear how our medical use of antibiotics impacts on the resistome, or conversely, how the resistome within the natural environment impacts the development of medically relevant resistance.

Understanding how resistance genes transfer would inform us about how to fight antibiotic resistance in our hospitals. This work aims to characterize the antibiotic resistant bacterial population in aquatic environments to determine which phylogenetic bacteria contain resistant strains, which types of antibiotic resistance are prevalent, if any, and if there is a link between antibiotic resistance and the prevalence of pathogens. In addition, this study attempts to assess the impact of human activities on the aquatic environment through wastewater management.

REVIEW OF LITERATURE

Antibiotic origins and resistance

Definition and origins of antibiotic compounds

Infectious diseases have caused many epidemics throughout the history of mankind. Several plagues have wiped out large proportions of the historic population. The Black Death plague (1345–1351), caused by a pathogen *Yersinia pestis*, killed more than a third of the European population (24 million) and 40 million people worldwide. Not all regions were hit with similar death tolls. Southern European countries were savaged more than Northern and Eastern countries (Nelson & Williams 2007; McNeill 1976). Infections have also been one of the reasons why life expectancy was considerably lower in the past than it is today. Surgeries were attempted less frequently, especially more complicated procedures, because almost all wounds became infected and roughly half of all patients died (Alexander 1985). The death toll caused by infections remains significant today, despite advancements in medicine and drug development. It has been estimated that more than 20% of deaths annually are caused by infectious diseases (Morens et al. 2004; WHO 2008).

Antimicrobial techniques and substances that are used to treat infections have been used for centuries. For example, bone samples from a Sudanese Nubia population from between 350 and 550 CE revealed traces of tetracycline (Nelson et al. 2010). Antibiotics were not the only treatment available against infection. Before the discovery and purification of modern antibiotics, antibiosis and antiseptics were used to treat and prevent infections. Antibiosis is the treatment of infection with another organism that has an antagonistic effect on the infection while curing the patient. Pyocyanase, developed in the 1890s (Hays et al. 1945) may have been the first antibiotic (AB) treatment that was "discovered" through a study of antibiosis. However, pyocyanase was not identified as a purified molecule but rather a cell lysate from a *Pseudomonas aeruginosa* cell culture; it was discovered through the observation that *Bacillus anthracis* could not develop in the presence of *P. aeruginosa*. Antiseptics were used externally to treat wounds and to maintain asepsis. The first recommended antiseptic treatment was sodium hypochlorite in the middle of the 19th century (Bryskier 2005).

The first antibiotic substance, Salvarsan, was taken in to use by Paul Ehrlich to fight against syphilis at the beginning of the 20th century. By observing dyes that specifically colored bacterial, human, or animal cells Ehrlich inferred that chemicals could specifically kill these cells. He tested many different compounds before finding Salvarsan. His methods formed the basis for future systematic and large scale screens to find antimicrobial compounds that target specific infectious bacteria (Aminov 2010). Penicillin on the other hand was discovered by accident by Alexander Fleming; his *Staphylococcus aureus* containing petri dishes became contaminated with *Penicillium notatum* while on

holiday. In 1929 he published his findings on an active antimicrobial substance – penicillin. The publication did not attract attention and was forgotten for a number of years; the first penicillin treatment occurred no sooner than 1941 (Table 1) (Bryskier 2005). Various problems with the extraction and purification of penicillin were largely to blame for the long delay from discovery to use. Penicillin is probably the most well-known antimicrobial substance, however, the first very successful antibacterial agent was the synthetic substance sulfonamide. Sulfonamide was developed by Gerhard Domagk using systematic screening methods and is effective against a broad spectrum of bacteria: it affects Gram-negative and Gram-positive bacteria. Sulfonamides have been in use since 1936 and continue to be used today (Aminov 2010; Bryskier 2005). The discovery of streptomycin in 1944 from *Streptomyces griseus*, a species in the phylum *Actinobacteria*, started a systematic screening for antibiotics from the genus *Streptomyces*. The search for new antimicrobials from the group actinomycete continues to this day. The period from the 1950s to the 1970s was a golden era for antibiotic discovery (Aminov 2010) with a peak of finding new substances in ca. 1970 (Watve et al. 2001). Since this time only a few new classes of antibiotics have been discovered, including the purely synthetic linezolid and a fermentation product of *Sterpotmyces roseoporus*, daptomycin (Raja et al. 2003; Beiras-Fernandez et al. 2010; Brickner 1996). The importance of actinomycete in the discovery of antibiotics is illustrated by the large proportion of antibiotics produced by this group of bacteria. Eighty per cent of antibiotics discovered between 1955 and 1962 originated from actinomycete bacteria, the majority from *Streptomyces* (Watve et al. 2001). They have contributed to the majority of various classes of antibiotics, including β-lactams, glycopeptides, aminoglycosides, macrolides, and tetracyclines (Kohanski et al. 2010).

Selman Waksman defined antibiotics as small molecules produced by microbes that inhibit the growth of, or kill, other microbes, with the added property of not being toxic to the host (Waksman 1947). Later it was elaborated that antibacterial activity should occur at low concentrations and semisynthetic molecules should also be included in the definition. Synthetic antibacterial agents were historically defined as synthetic antibacterial agents and not antibiotics, although today there is no discrimination between the two (Bryskier 2005). Antibiotics can either be bacteriostatic or bactericidal, with the former acting to stall bacterial growth. When a bacteriostatic drug is removed, the cells are free to grow again, however, when a bactericidal drug is removed the cells will not continue growing.

Table 1 Development of antibiotic resistance following its introduction. From (Palumbi 2001)

Antibiotic	Year deployed	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late 1960s

Antibiotic resistance

Antibiotic resistance is a phenomenon where bacteria are able to grow in the presence of the drug. Currently, antibiotic resistance is a serious problem in hospitals and within susceptible communities such as those suffering from immunodeficiency. The hope that antibiotics are the "magic bullet", that will eradicate infectious diseases, is long gone. The phrase "magic bullet" was originally coined by Paul Ehrlich to describe the desired property of a drug to selectively target only disease-causing organisms (Aminov 2010). Resistance to new antibiotics has rapidly emerged after introduction of the drug with only the time of emergence varying. For example, penicillin resistance was discovered months after its introduction (Walsh 2003), methicillin resistance took a relatively short time of three years to emerge (Davies et al. 2009), while vancomycin resistance was observed more than 30 years after its introduction (Table 1). The long delay for vancomycin resistance is explained in part by the restricted use of the drug (Walsh 2003; Chambers & Deleo 2009).

To determine if a bacterial population is able to grow at or near concentrations relevant in medicine, the minimum inhibitory concentration (MIC) of the bacteria is usually measured by observing the growth of bacteria in the presence of an antibiotic at several concentrations. The MIC is defined as the smallest concentration where the bacteria are not able to grow; concentrations above the MIC will either inhibit the growth or kill the bacteria, depending on whether the antibiotic is bacteriostatic or bactericidal. The breakpoint concentration above which bacteria are considered resistant is determined by clinical breakpoint tests defined by the Clinical and Laboratory Standards Institute (CLSI) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST). In addition, epidemiological breakpoints are also defined (EUCAST). The breakpoint for a bacterial species is defined based

on MIC tests on the population. Clinical breakpoints are species, disease, and strain specific and may change over time. They may even be defined differently by different agencies. Clinical breakpoints define the likelihood of a positive outcome of antibiotic treatment. Epidemiological breakpoints are species and population specific and will not change over time. The epidemiological breakpoint is defined as the highest MIC value of the wild-type population that has not acquired resistance genes (EUCAST; Kahlmeter et al. 2003).

MIC is measured by observing bacterial growth at specific AB concentrations. The first concentration where there is no growth is the MIC. However, this method cannot be used to find tolerant bacteria. Tolerance is a phenomenon where bacteria act as if they were being treated by a bacteriostatic antibiotic when they are being exposed to a lower concentration of a bactericidal antibiotic. They do not grow in the presence of the antibiotic, but resume growth when the antibiotic is removed. This impairs effective treatment because a sub-population of the initial population persists. This type of sub-population can be found by measuring the minimum bactericidal concentration using similar technique as in the determination of MIC with the added step of plating out the surviving population on growth media that does not contain antibiotics (Levin & Rozen 2006).

Once resistance has emerged in an environment it can persist even in the absence of selection pressures. For example, resistance to sulfonamide antibiotics did not decrease during a period of longer than eight years, even though the use of these antibiotics was greatly reduced or even banned within UK healthcare systems (Enne et al. 2001; Bean et al. 2005). A different effect was observed in Finland where the use of erythromycin and other macrolides was restricted due to high levels of resistance to these antibiotics. A significant decrease in resistance levels was observed only a few years after the restriction (Seppälä et al. 1997; Andersson & Hughes 2011). A similar trend was observed after the ban of avoparcin, an analog of vancomycin which also gave rise to cross-resistance, after its use as an animal food additive in Taiwan. A statistically significant decrease in the number of vancomycin resistant enterococci was observed in the farms tested although, resistant isolates were still found (Lauderdale et al. 2007).

There are three main types of resistance mechanisms: antibiotic detoxification, alteration of the drug binding site, and reducing the accumulation of the drug. Antibiotic detoxification involves modifying or degrading the drug. For example, β-lactamases cleave the lactam ring of β-lactam antibiotics which hinders their binding to transpeptidases which is required for their action (Vicente et al. 2006; Kümmerer 2009b). Alteration of the drug binding site is a widespread mechanism of resistance. Occasionally, the addition of a single methyl group to the right place will render a bacterium resistant, as is the case with erythromycin from the family of macrolides; methylating a single adenine in the 23S rRNA in the 50S ribosomal subunit makes the organism resistant to erythromycin (Gupta et al. 2013). There can also be active transport of the drug

out of the cell using efflux pumps not specific to antibiotics whose primary role is to export metabolites and toxic substances (Walsh 2003). For example *Pseudomonas aeruginosa* intrinsically possesses efflux pumps that are able to transport β-lactams, macrolides, and tetracyclines (Poole 2001). The change in the composition of the outer membrane can reduce the permeability of the cell envelope to antibiotics. The outer membrane of Gram-negative bacteria is significant barrier for hydrophobic antibiotics such as aminoglycosides and macrolides, which cross the membrane by diffusion. Lipopolysaccharides in the membrane will inhibit diffusion by giving it hydrophilic properties and antibiotics will not acquire a sufficient concentration near the target sites in the cell (Delcour 2009).

Intrinsic antibiotic resistance

Resistance can be classified as intrinsic or acquired. This distinction is important when determining the mechanism of resistance. Intrinsic resistance is a type of resistance that a bacterium has acquired because of its physiology; a trait that is encoded by chromosomal genes, is independent of antibiotic selection, and not influenced by horizontal gene transfer (Cox & Wright 2013). For example, organisms that produce antibiotics are intrinsically resistant to these antibiotics. Resistance is required to protect themselves from the self-produced antibiotic. Some researchers have argued that some form of intrinsic resistance is a characteristic of almost all bacterial species (Leclercq et al. 2013).

A common example of intrinsic antibiotic resistance is a multi-drug resistant phenotype exhibited by Gram-negative bacteria against many classes of antibiotics effective against Gram-positive bacteria. This phenomenon is caused by the outer membrane of Gram-negative bacteria, which is impermeable to many molecules, and efflux pumps, that are capable of effectively reducing the concentration of many antibiotics in the cell (Cox & Wright 2013). For example, erythromycin has no effect against *Escherichia coli* and tigecycline against *Pseudomonas aeruginosa* because of their efflux pumps (Piddock 2006). Acquired resistance would occur if the same efflux pump coding gene that gives resistance to *P. aeruginosa* against tigecycline would be horizontally transferred into a susceptible species (Normark & Normark 2002). Lack of an AB target would also allow for intrinsic resistance to a specific AB. Isoniazid, an important AB treating *Mycobacterium tuberculosis* infections, inhibits the synthesis of mycolic acid, which is required to produce the mycobacterial cell wall. Other bacteria do not have mycolic acid in their cell wall and are thus intrinsically resistant against isoniazid (Verschoor et al. 2012; Lee et al. 2012).

Decreased permeability of the outer-membrane and active efflux by efflux pumps are thought to be the main causes of intrinsic resistance, however, recent studies suggest that deleting some unrelated genes could also make the bacteria more susceptible (Cox & Wright 2013). A screen of transposon library of

Acinetobacter baylyi revealed genes that were not previously thought to be involved in providing intrinsic resistance, however, their disruption made the mutants hypersusceptible to the AB. The genes involved were *gshA*, involved in glutathione biosynthesis, and *recD*, involved in recombination and repair. This phenomenon seems to be species specific because *E. coli* knock-outs of homologous genes did not show significant changes in antibiotic MICs (Gomez & Neyfakh 2006). This shows that the phenomenon of intrinsic resistance is complex and involves more genes than previously thought. Intrinsic resistance cannot be classified based on a single gene. The same gene can provide intrinsic resistance in one species but acquired resistance in another.

Acquired antibiotic resistance

Acquired antibiotic resistance is a type of resistance that is acquired by mutation of bacterial chromosomal genes or by horizontal gene transfer of resistance genes (Palmer & Kishony 2013). All three different resistance mechanisms play an important part in acquired resistance; the actual mechanism depends on the antibiotic. For β-lactams the most common mechanism is drug modification by β-lactamases (Smet et al. 2010). Tetracycline resistance is mainly provided by drug efflux or by a ribosomal protection protein that helps to release tetracycline from the ribosome (Thaker et al. 2010; Roberts & Schwarz 2009). The most frequent resistance mechanisms that work against chloramphenicol, another protein synthesis inhibiting antibiotic, are drug modification and efflux (Murray & Shaw 1997; Roberts & Schwarz 2009). To differentiate acquired resistance from intrinsic resistance we need to determine if the resistance is species specific or population specific. The latter indicates that resistance has been acquired via natural selection in relatively recent times (McDermott et al. 2003). Usually, the term resistance in medicine and animal husbandry implies acquired resistance (Levy & Marshall 2004).

The rapid and wide dissemination of newly arisen resistance genes is, in part, due to mobile genetic elements (MGE) and horizontal gene transfer mechanisms (which are discussed later) (Stokes & Gillings 2011). There are many types of MGEs that resistance genes can move with between organisms. These include transposons, integrons, integrative conjugative elements, plasmids, and genomic islands (see Figure 1 for hierarchical complexity of mobile elements), however, only plasmids are able to autonomously replicate. Other elements require integration into the host's chromosome or into a plasmid through either site specific or homologous recombination or via transposition. The resistance genes are not fixed to the MGEs and are able to move from one organism to other, thus creating MGEs that confer resistance to several antibiotics (Stokes & Gillings 2011). For example, genes for AmpC enzymes are found in plasmids ranging in size from 7 to 180 kb. Besides *ampC*, these plasmids often carry resistance genes for tetracycline, sulfonamide, aminoglycosides, chloramphenicol, and trimethoprim (Philippon et al. 2002).

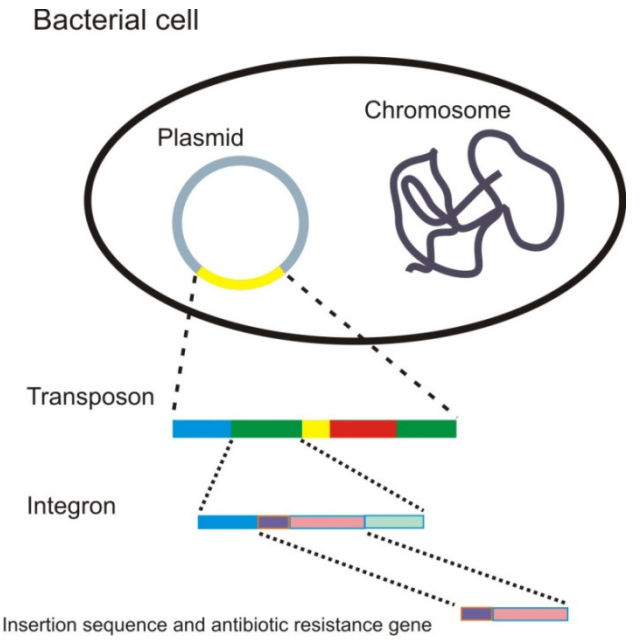

Figure 1. Hierarchical complexity of mobile resistance elements participating in the mobilization, spread, and maintenance of antibiotic resistance genes, with modifications from (Cantón et al. 2012).

Many resistance genes are not always associated with MGEs; they can also be found in the bacterial chromosomal DNA without any association with MGEs. In β-lactamases within the class D OXA family, similarity based on the amino acid sequence of the protein varies from 19 to 96%. The majority of class D plasmid borne genes are from *Eneterobacteraceae, Pseudomonas* spp. and *Acinetobacter* spp. while chromosomal genes are obtained from *Aeromonas*, *Legionella, Shewanella*, and *Campylobacter* species (Poirel et al. 2010a). It is not easy to find the origin of a medical resistance gene, however, in a few cases a culprit has been proposed. The origin of *qnrA*, a gene giving resistance to quinolones by protecting DNA gyrase and topoisomerase IV, is linked with an environmental species *Shewanella algae* (Poirel et al. 2005). For some class A CTX-M type plasmid encoded β-lactamases, the progenitor originates from *Kluyvera ascorbata*, an opportunistic pathogen with low clinical significance. The chromosomally encoded gene of *K. ascorbata* had a high sequence similarity with CTX-M sequences, including parts of the flanking sequences of the genes (Humeniuk et al. 2002). CTX-M type genes belong to the extended-spectrum β-lacatamses (ESBLs), which are able to hydrolyze expanded spectrum cephalosporins and monobactams. Because of their wide and fast dissemination, beginning in the year 2000, the phrase "CTX-M pandemic" has been used. The rapid spread is facilitated by MGEs and successful bacterial clones (Cantón et al. 2012).

Antibiotic resistance in the natural environment

Antibiotic resistance is a concern because it increases mortality and morbidity in both healthcare (Vicente et al. 2006) and veterinary settings, and induces a significant economic cost (Levy & Marshall 2004; Smith & Coast 2013). Resistance in medicine and animal husbandry has been the primary concern and few consider that the spread of AR in the environment is either a problem or threat to either human health or the environment.

Most instances of antibiotic resistance in the environment have been discovered in soil bacteria. Resistance genes have functioned in the environment well before humans began using antibiotics in medicine or perhaps before mammals evolved from therapsids for that matter. Using phylogenetic analysis of β-lactamase protein sequences, we now understand that these resistance genes are millions of years old. These genes also existed on mobile genetic elements before the human use of antibiotics (Hall & Barlow 2004; Barlow & Hall 2002). We ought to study resistant organisms in the natural environment because all of the various environments that house antibiotic genes are connected (Figure 2). Resistance that has been selected for in one environment can 'hitchhike', one way or another, to other environments. The movement of antibiotic resistance between environments is discussed in more detail below.

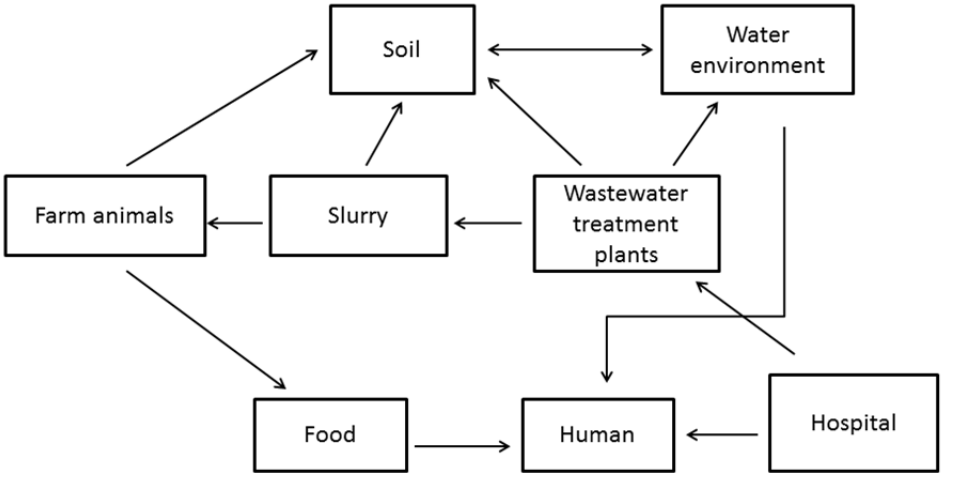

Figure 2. A structured compartmental view of how antibiotics and antibiotic resistance genes are linked between the human and natural environment. Modified from (Davies & Davies 2010; Hawkey & Jones 2009)

Antibiotic resistance in soil

As mentioned above, most of the antibiotics in use today originate directly from soil dwelling bacteria or are derivatives of these compounds. It is estimated that more than 80% of the antibiotics in clinical use originate from soil bacteria (Torres-Cortés et al., 2011).

Knapp et al. (2010) found that the quantity of ARGs in soils has increased during the time humans have used antibiotics. The study made use of archived soil samples from 1940 to 2008 to look for the number of antibiotic resistance genes quantitatively using qPCR. The authors saw an increase in the number of resistance genes, however, the increase was not universal, and differed between both resistance genes and soil types. The increase of β-lactamases and tetracyclines was more significant over the same time period compared with erythromycin resistance genes (Knapp et al. 2010). Although their study shows that antibiotic resistance in soil environment has increased during the use of antibiotics by humans, it also shows that antibiotic resistance genes could have been selected by the pre-human-occurrence of antibiotics in soils.

The presence of antibiotic resistance genes in the environment before humans began using the drugs is clearly shown by D'Costa *et al*. (2011) who used non-culture methods to PCR amplify putative resistance genes from permafrost cores that were about 30,000 years old. They were able to amplify tetracycline, β-lactam, and vancomycin resistance genes, thereby demonstrating a link between 30,000 year old and contemporary resistance genes (D'Costa et al. 2011). Barlow and Hall estimate that the age of β-lactamase genes can be in millions, instead of thousands of years (Hall & Barlow 2004). Antibiotic resistance is not only found in ancient and old samples, but also from locations untouched by anthropogenic activity. This shows that resistance genes and resistant organisms can be found in pristine soil environments (Allen et al. 2009; Bhullar et al. 2012).

In light of this evidence it seems logical that resistance against antibiotics or against modified isoforms of natural antibiotics may be easy to find in the environment. However, the environment also contains resistance genes for synthetic antibiotics. During their search for resistance genes in the genus *Streptomyces,* D'costa *et al*. (2006) found that all isolates tested were resistant to more than one antibiotic, including synthetic antibiotics, some long in use and others new to the market (D'Costa et al. 2006). There is also no difference in the rate that resistance emerges against natural antibiotics and synthetic antibiotics (Table 1 and (Bryskier 2005)).

Antibiotic resistance is ancient and has been mostly found in soil bacteria. While superficially similar resistance is found in healthcare settings, direct proof that resistance genes in pathogens and environmental bacteria share a common origin has only recently been produced. Forsberg *et al.* (2012) found a direct link between soil bacteria and clinical pathogens using a metagenomic approach to find resistance genes from cultivated environmental bacteria, mainly *Proteobacteria*. They found 18 genes that had 100% and 32 that had

>90% amino acid identity to entries from pathogens in GenBank. This level of similarity implies horizontal gene transfer (HGT) because the organisms studied were only distantly related on an evolutionary scale. In addition, the majority of the resistance genes discovered were formerly unknown (Forsberg et al. 2012).

The study by Forsberg et al. (2012) is one of only a few that has linked environmental and clinical resistance genes, however, it is not the first to make use of metagenomics to enrich our knowledge regarding the diversity of resistance genes in the soil environment (Allen et al. 2009; Lang et al. 2010; Donato et al. 2010). Metagenomics has proven to be an effective tool for both, to discover new resistance genes and exploring the diversity of the resistome in the environment. Currently, culture based methods are not as effective in this search (Monier et al. 2011; Lefevre et al. 2008) because only 1 – 5% of bacteria are culturable (Amann et al. 1995).

Antibiotic resistance in the aquatic environment

Aquatic environments are important reservoirs for antibiotic resistance because resistant organisms and MGEs from a variety of sources, together with increased concentrations of antibiotics from our waste stream, all come together in this environment. AR bacteria enter the aquatic environment via both treated and untreated sewage and agricultural run-off (Baquero et al. 2008). Production of antibiotics in current use have not been found in aquatic bacteria, however, traces of clinical antibiotics end up in the aquatic system from our waste stream. In some conditions between 70 to 80% of the antibiotic can pass through the organism (animal or human) unchanged when the antibiotic is orally administered (Sarmah et al. 2006; Kümmerer & Henninger 2003) and end up in the sewage system which is also not able to completely remove these compounds (Kümmerer 2009a; Kümmerer & Henninger 2003). The concentration of antibiotic compounds in surface water can range from a few ng per L to more than a µg and typically hundreds of ng per L can be found in ground water. Concentrations differ between antibiotics and specific environments (Kümmerer 2009a).

Antibiotic resistance and antibiotic resistance genes have been studied extensively in aquatic environments. Culture based methods are most commonly employed and many studies concentrate on medically relevant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and other *Pseudomonas* spp., *Aeromonas* spp., and *Enterococcus* spp. (Morris et al. 2012; Roberts et al. 2013; de Vicente et al. 1990). More recently, PCR based methods have been used to determine the resistance genes involved (Dhanji et al. 2011; Girlich et al. 2011). This can only be performed on genes that are known and most studies target medically relevant genes such as *vanA* that provides resistance to vancomycin, a drug considered as a "last resort" drug (Cytryn 2013), *mecA* that encodes for a penicillin binding protein that has reduced affinity for β-lactams (Hartman &

Tomasz 1981), and other less infamous genes. With the use of culture based methods it was established that antibiotic resistance in the aquatic environment is prevalent (Boon & Cattanach 1999; Blasco et al. 2008). Resistant organisms were found among opportunistic pathogens (Girlich et al. 2011; Blasco et al. 2008) and organisms that did not relate to pathogens (Henriques et al. 2006), which shows that resistance in the water is as widespread as it is in the soil and does not confine itself to a few bacterial genera. The use of moleuclar methods has broadened the picture, including information about organisms that do not grow on conventional media or laboratory conditions. Suzuki et al. (2013) found, by measuring the quantity of *sul1*, *sul2*, and *sul3* genes in both fresh water and marine water that both the presence and quantity of antibiotic genes differs between the cultivable population and the entire population. Using culture based methods, *sul3* was not found in marine samples, however, was present when the gene was amplified from community DNA.

Once antibiotic resistance has emerged it is seen to persist and can persist in a formerly polluted site in the absence of selection. Tamminen et al. (2011) used quantitative PCR (qPCR) to observe that the copy numbers of tetracycline resistance genes remained elevated in a fish farm even though antibiotics had not been administered for several years. Antibiotic resistance genes are also found in pristine aquatic and related environments (Dib et al. 2009; Segawa et al. 2013) as was the case with pristine soil environments. For example resistant organisms were found in alpine lakes. Moreover, most of the isolated and cultured organisms were resistant to several antibiotics (Dib et al. 2009).

Antibiotic resistance Hot-Spots

Having established the ubiquity of antibacterial resistance in both human and natural environments we shift the discussion to the nature of how resistance is transferred in environments where the potential of resistance transfer is the highest. A number of potential hot-spots for both the presence and spread of antibiotic resistance are healthcare settings, veterinary facilities, and waste-water treatment plants (WWTP) and their surrounding areas. These environments are highlighted because of the simultaneous presence of antibiotic selection, the presence of both pathogenic and non-pathogenic bacteria, and the possibility of transferring resistance between organisms, people and the natural environment.

Healthcare settings as a potential hot-spot

Healthcare settings are the most obvious hot-spots for antibiotic resistance to develop. The consumption of antibiotics is high, thus inducing strong selection, patients turnover rapidly, and many pathogens grow in close proximity within an often confined and relatively small space.

It is important to consider antibiotic resistance in healthcare settings because many patients are admitted with one illness and subsequently acquire nosocomial infections. Because of the presence of strong selection in this environment, these infections can be resistant to several antibiotics administered at high concentrations. Resistance can develop through both mutations and HGT, as previously mentioned, and pathogenic bacteria with higher mutation rates have been observed, although no correlation between higher mutation rates and antibiotic resistance was found (Denamur et al. 2002; Henderson-Begg et al. 2010). Antibiotic treatment itself will select for a resistant population, even when the treatment uses a combination of antibiotics. This was shown *in vitro* by Pena-Miller et al. (2013) where a resistant subpopulation was present after treatment. Mwangi et al. (2007) observed that resistance can appear during treatment in a stepwise manner. HGT via MGEs has been shown to be a common mechanism whereby bacteria acquire resistance (Nakamura et al. 2004). Many of the resistance genes are carried by integrons that are not able to actively move between organisms, however, they can insert themselves into mobile plasmids and transposons. Integrons are bacterial genetic elements that can contain and express many resistance genes (Stalder et al. 2012; Cantón et al. 2003). Of primary concern are the resistance and virulence integron cassettes that hold genes for several antibiotics. Such cassettes can travel together to susceptible organisms making them multiresistant with a single transfer event (Partridge et al. 2009). Selection preserves all genes on an MGE even if only one resistance gene is under selection. This preserves multiple resistance genes simultaneously thereby nullifying the strategy of rotating antibiotics within clinics and hospitals. Although many clinically relevant pathogenic species can also be found in the environment, few studies have found direct contacts whereby resistance is transferred between pathogenic and environmental bacteria, as mentioned above. A few studies report a link between resistance genes from clinical pathogens and other environments by comparing the sequences of the resistance genes (Poirel et al. 2005; Bonnet 2004; Forsberg et al. 2012).

Both resistant bacteria and the genetic material that confers resistance can be disseminated to other environments through both patient transfer and waste water as was observed with *Enterococcus sp.* which moved into the natural environment (Novais et al. 2005; Varela et al. 2013). There are also reports that show the presence of organisms that have acquired resistance to the same antibiotics as clinical bacteria in the absence of a direct clonal connection between the environments (Morris et al. 2012; Leclercq et al. 2013).

Agriculture as a potential hot-spot

The use of antibiotics in agriculture accounts for approximately 80% of all antibacterial use in the USA and 30% in the EU (Kemper 2008). In the EU, the use of antibiotics as growth enhancers was banned in 2005 (Zheng et al. 2011),

however, it is still common practice in the US and other countries (Sarmah et al. 2006). Because of the widespread use of antibiotics in agriculture and both direct and indirect contact between agricultural products, humans, and other environments, agriculture is considered to be an important contributor to the global antibiotic resistome. Agriculture contributes to the resistome through both the dissemination of resistant bacteria directly to humans and by leakage of agricultural waste containing resistance determinants, organisms, and antibiotics into the surrounding environment.

The transfer of bacteria from animals to farmers was nicely shown by Oppliger et al. (2012) where they collected nasal samples to look for the presence of *S. aureus* from pigs, their farmers, and a few veterinarians. The dominant genotypes found in the pigs were also present in the humans tested, although the genotypic variability was higher in humans. The occurrence of antibiotic resistance, including multiple AB resistant bacteria, was similar between pigs and farmers and differed from healthy subjects without contact to agriculture (Oppliger et al. 2012; Armand-lefevre et al. 2005). In South-Korean chicken farms, antibiotic resistant *Salmonella* spp. was found in samples taken from egg shells and boots which are both likely to have contact with humans . Multi-resistance was common among the isolates and only 14% were susceptible to the antibiotics tested (Rayamajhi et al. 2010).

Between 25 to 75% of the administered AB dosage passes through the organism, is excreted with the feces in an unaltered or partially altered form, and can persist in the soil after the manure is used as a fertilizer (Chee-Sanford et al. 2001). In addition to applying antibiotics with the manure onto the fields, the manure itself contains resistant bacteria and resistance determinants (Jechalke et al. 2013). The effect on the local resistome after applying manure does not last for very long; the number of resistance genes and resistant organisms in the soil decreases over time (Jechalke et al. 2013; Heuer & Smalla 2007; Sengeløv et al. 2003). However, this does provide an opportunity for AR to spread with the use of manure. Chee-Sanford et al. (2001) looked for tetracycline resistance genes in both lagoons and underlying groundwater near two swine production facilities. They found tetracycline resistance genes from sampling points downstream of the facilities while only one gene was found in a control well upstream. This shows that leakage from production facilities has an impact on the groundwater downstream (Chee-Sanford et al. 2001).

WWTP as a hot-spot

WWTP are considered hot-spots because they receive waste and sewage water from large urban areas, including hospitals, and house an environment with dense bacterial loads (Schlüter et al. 2007). Sewage contains different species of both pathogenic and non-pathogenic bacteria that can be classified as either resistant or non-resistant. Some of these are released into the environment after the treatment process (Varela & Manaia 2013)

The presence of resistance genes in both the activated sludge and outflow water of WWTPs has been observed many times, however, Szczepanowski et al. (2009) stands out as a large scale study. They extracted plasmids from resistant bacteria, which were then selected with culturing methods, followed by a scan for resistance genes using PCR amplification. The genes they amplified provide resistance to medically important groups of antibiotics. They were able to detect 140 different resistance genes that, upon sequencing, allowed them to identify sequences that were identical or highly similar to known sequences from various databases (medical strains). Using metagenomics, Zhang et al. (2011) showed that most resistance genes found in activated sludge are tetracycline, macrolide, and multidrug resistance genes. The WWTP reduces the total number of bacteria and also the numbers of resistant bacteria, however, a few studies observe that the relative numbers of some species significantly increase in the effluent. In addition, waste water treatment can select for a specific resistance, for example resistance to fluoroquinolones (Łuczkiewicz et al. 2010). Resistance to other antibiotics have also been observed, but not with increased frequency (Ferreira da Silva et al. 2006; Łuczkiewicz et al. 2010). Many studies make use of culture based methods and typically use specific species such as *Escherichia coli, Enterococci* spp*.,* and *Acinetobacter* spp. as representative of the larger population (Rizzo et al. 2013). It is important to note a major limitation of applying culture dependent methods to describe the population: of the majority of species within the bacterial population are not cultivatable.

The final effluent is not always simply released into the environment. In more arid environments effluent is often used to irrigate crops. This potentially increases the spread of AR by inoculating the fields with resistant organisms and genetic agents, however, the group led by E. Cytryn disproved this hypothesis. They studied several fields that were irrigated with either treated wastewater or fresh water and found that the number of resistant organisms found in the soil was similar between fields. The field treated with fresh water had a significantly higher number of resistant organisms, however, the total number of bacteria, including resistant bacteria, was orders of magnitude higher in the treated wastewater. Of the resistance genes tested, no significant differences were observed in the copy numbers between soils irrigated with treated wastewater or freshwater (Negreanu et al. 2012).

Dissemination of antibiotic resistance

As mentioned above, antibiotic resistance is not a local phenomenon. The rise of resistance in one place will influence the rise of resistance in other places. Dissemination of resistance can, broadly speaking, occur by both the physical spread of resistant organisms and horizontal gene transfer.

Horizontal gene transfer and the dissemination of antibiotic resistance

Horizontal gene transfer (HGT) is a process where the recipient organism acquires extra genetic material in some process other than cell division. HGT is one of the main reasons why antibiotic resistance is both rapidly and widely spread. There are three mechanisms whereby HGT occurs: transformation, which is the active uptake of extracellular DNA from the environment (Lorenz & Wackernagel 1994); conjugation, which involves DNA transfer requiring energy and cell contact (Waters & others 2001); and transduction, which is bacteriophage mediated transfer of genetic material (Jiang & Paul 1998).

HGT is important in dissemination because it helps multiresistance to arise and resistance to spread faster (Barlow 2009; Roberts 2008). Many of the resistance genes that have been found on MGEs are able to transfer between organisms and species (Marti & Balcázar 2012; Pérez-Pérez & Hanson 2002; Poirel et al. 2005; Schlüter et al. 2007). The relative frequency rates of the three HGT mechanisms are currently not known in the natural environment, however, if one considers the number of bacteria in the various environments, smaller transfer rates could still add up to a considerable number of total HGT events. Jiang & Paul (1998) deduced from their measurements that up to 100 transduction events per liter per day can occur in a marine environment under ideal conditions. Using chromosomal DNA and plasmid DNA in mesocosm experiments, the frequency rate of transformation was found to be in the range of $10^{-10} – 10^{-4}$ transformation events per viable cell. Both the frequency and efficiency depend on environmental factors such as pH, humidity, and the type of soil (Lorenz & Wackernagel 1994).

Dissemination of antibiotic resistant organisms

Although bacteria themselves cannot travel long distances using their own power, they are small enough to be carried by physical forces as wind and water and also by other organisms. All of these physical mechanisms can relocate material great distances, and with it bacteria, viruses, and DNA that can affect the new environment.

Both animals and birds can act as vehicles for bacteria. MRSA can transfer from farm animals to farmers (Oppliger et al. 2012; Fluit 2012). Wild animals that live closer to humans were shown to carry more antibiotic resistant bacteria than animals living further away from human influence (Gilliver et al. 1999; Österblad et al. 2001). Dib et al. (2009) discuss that resistant bacteria could be dispersed by birds because resistant organisms were found in both natural water and bird feces.

The most prominent vehicles for bacterial movement are, of course, humans. We consume a large portion of the antibiotics administered and thus create a selection environment within our bodies. We also travel large distances and are

in contact with others. The rapid spread of viral diseases around the globe is a prime example of how effective we are as disease carriers (Enserink 2013). This also applies to bacterial infections, albeit not as rapidly. The consumption of agricultural products that are produced in remote locations across the globe is also facilitating the dissemination of antibiotic resistant bacteria and genetic material (Buchholz & Bernard 2011).

RESULTS AND DISCUSSION

Aims of the study

Current levels of antibiotic resistance already constitute a major problem in medicine and several studies predict this problem will become worse over time with the development of resistance genes and resistant organisms in various environments (McKennan 2013; Smith & Coast 2013). Despite the magnitude of the problem with antibiotic resistance in medicine, it is not known if the natural environment either contributes to the medical resistome or acts as sink where resistance genes eventually end up.

The aim of this dissertation is to characterize antibiotic resistance within an aquatic bacterial population to establish a potential link between resistance genes found in human pathogens and aquatic bacteria. Specific aims are as follows:

- to study the variability and phylogenetic affiliations within a population of freshwater bacteria that displays antibiotic resistance;
- to determine the occurrence of multidrug resistance in the aquatic environment;
- to assess if there are associations between environmental and clinical resistance genes;
- to study the potential impact of WWTPs in disseminating antibiotic resistance genes to the downstream environment.

Co-occurrence of resistance to different antibiotics among aquatic bacteria (I)

Studies of antibiotic resistance are biased for medical/veterinary or medically related bacteria and perhaps rightfully so. On the other hand, the wider discussion about the dissemination of resistance requires a broader characterization of antibiotic resistance within environmental populations.

We chose to characterize the antibiotic resistance of a random population of fresh-water river bacteria in Estonia using cultivation-based methods. While cultivation methods are biased (Kaeberlein et al. 2002), they provide both an opportunity to study the phenotype of the selected isolates and the possibility of linking antibiotic resistance and phylogenetic affiliation. The medium used for the isolation of environmental bacteria was formed from four volumetric parts of filtered natural water and one part distilled water. It was further supplemented with 5g/l of peptone and 1g/l of yeast extract. This medium allows one to isolate a wide phylogenetic and physiological range of bacteria. Five antibiotics were chosen as selective agents: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (20 μg/ml), norfloxacin (2 μg/ml), and tetracycline (20 μg/ml). These antibiotics were chosen to cover a range of drug

targets: DNA replication, protein synthesis, and cell wall synthesis. The concentrations of these antibiotics were selected to be close to the EUCAST MIC cutoff values for resistance (EUCAST).

Sampling was carried out at two locations in duplicate on five different dates over four months from July to October 2008, thus providing 10 samples (Figure 3). Each sample was plated out to five different selective media, each containing one of the five antibiotics mentioned above. The isolates (n = 760) were selected for analysis by choosing morphologically different colonies, but no less than 10 colonies per plate; differences were evaluated visually. The phylogeny of the isolates was assessed by sequencing the 16S rRNA gene and comparing with data from Ribosome Database Project (Wang et al. 2007). The isolates belonged to nine phylogenetic classes and 59 genera (I, Figure 1). Almost half (49%) of the 760 isolates belonged to Gammaproteobacteria and more than half of these (58%) were *Pseudomonas* spp. The selection of Gammaproteobacteria is a known plating bias of aquatic bacterial communities (Eilers et al. 2000) and the phylogenetic composition of the population we studied is in accordance with previous studies (Pinhassi et al. 1997; Simu et al. 2005; Selje et al. 2005).

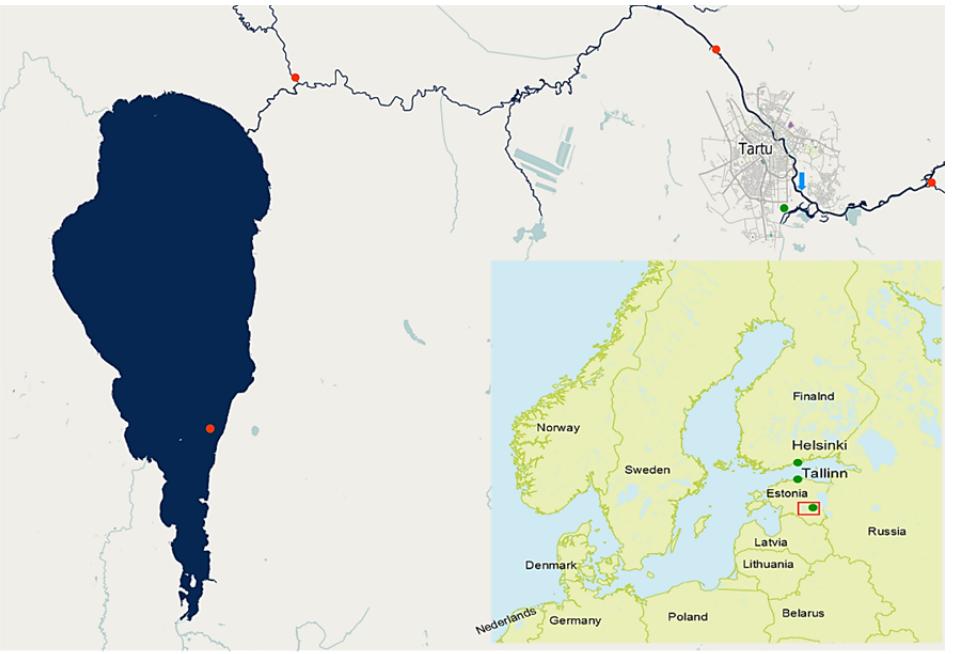

Figure 3. Map of different sampling stations. Red circles indicate the stations for freshwater samples; green circle indicates the cities where the WWTP samples were taken and in Tartu the location of the WWTP. Blue arrow indicates the effluent runoff to the river Suur-Emajõgi in Tartu. Figure is a modification and combination from www.OpenStreetMap.org and www.FreeVectorMaps.com.

For every isolate we tested the concentration dependence of antibiotic resistance using six antibiotics (ampicillin – 10, 25, 100 μg/ml; meropenem – 0.3, 3, 30 μg/ml; norfloxacin – 0.5, 2, 10 μg/ml; chloramphenicol – 1, 5, 30 μg/ml; kanamycin – 1, 5, 20 μg/ml; tetracycline – 1, 5, 20 μg/ml). In addition to the antibiotics used in the initial selection, meropenem, of the family carbapenem, was included because carbapenem resistance is a characteristic problem in Estonian medical settings (Lõivukene et al. 2006). Because of the variability in the growth rates of environmental bacteria, we did not employ standard MIC analysis. Instead, we tested the antibiotic resistance by growing the bacteria in the presence and absence of the antibiotic over a longer timescale while measuring the optical density (OD). For each isolate and antibiotic pair, a resistance value (R) was calculated using the ratio of the lowest OD value in the presence of the antibiotic to the lowest OD value in the control experiment. For each antibiotic at each concentration we calculated the sum of all pairwise differences (S) between resistance values:

$$S = \sum_{i=1}^{759} \sum_{\substack{j=2 \\ i>j}}^{760} \mathrm{abs}\left(R_i - R_j\right)$$

For each antibiotic we selected the concentration with the largest sum of all pairwise differences (S), thus choosing the concentration where the diversity in resistance between isolates was the greatest. The concentration with the largest S provides the greatest chance of differentiating resistant bacteria from susceptible bacteria.

Resistance was considered at the genus level with genera that contained more than 20 isolates (n=8). In some genera, almost all isolates were resistant to a particular drug with an average resistance value above 0.8. This could indicate intrinsic resistance for the entire genus. We also observed that some genera contain isolates that are mostly all sensitive to a given antibiotic (I, Figure 2A). One exception is resistance to meropenem. This antibiotic displays an average resistance value above 0.5 for all genera analyzed, thus potentially indicating that the concentration with the maximum S was not high enough to differentiate resistant bacteria from susceptible bacteria. No genus was found to be fully susceptible or fully resistant; none of the antibiotics was able to inhibit the growth of all isolates in a genus. In every genus analyzed we found at least one antibiotic that was able to inhibit the growth of most isolates while some of the other antibiotics were not able to inhibit any of the isolates (I, Figure 2B).

To evaluate the diversity of resistance values within each genus we constructed a histogram of the R values (I, Figure 3 and Figure S1). In some cases a wide distribution of resistance values was formed of two sub-populations that did and did not display resistance. Two sub-populations within a genus suggest that the resistance observed is acquired. Some genera (e.g.

Stenotrophomonas, *Chryseobacterium*, *Flavobacterium*) displayed high levels of resistance to several antibiotics. We tested if this is either a result of "superbugs" or a result of a random distribution of intrinsic and natural resistance levels. We tested this by summing up the resistance values of all six antibiotics for every isolate within a genus and plotting a histogram of these values (I, Figure 4). The distribution of summed resistance values form a normal distribution, which suggests that multidrug resistance happens by chance and there is no selection for it in the freshwater environment studied.

Although resistance levels are randomly grouped, it does not exclude the possibility that some forms of resistance cannot group together. To test this, we calculated correlation coefficients between all antibiotic pairs in the dataset (I, Figure 5). In total, we identified eight significant ($p < 0.05$) positive and four negative correlations. The antibiotic pair tetracycline and chloramphenicol displayed the highest correlation coefficient ($r=0.669$, $p=0.001$). However, the resistance correlation using all isolates can be influenced by a small number of phylogenetic groups that dominate the correlation. To evaluate this we looked for resistance correlations among the eight largest genera with >20 isolates (I, Figure 5). A strong positive correlation between resistance to tetracycline and chloramphenicol was found in six out of eight genera. In addition, there were resistance correlations within a given genus that differ from the overall test population. The results of this analysis suggest that resistance for tetracycline and chloramphenicol may be facilitated by a common multidrug efflux pump or, alternatively, the two separate resistance determinants are found on the same MGE that is capable of efficient HGT.

Aeromonas and Pseudomonas Species as Carriers of ampC FOX Genes in Aquatic Environments (II)

The use of β-lactams, a class of commonly used antibiotics, has always been followed by emergence of antibiotic resistance that is mostly driven by β-lactamases (Bradford 2001; Jacoby & Munoz-price 2005; van Hoek et al. 2011). *ampC* genes have been found among pathogenic enterobacteria and is an important resistance gene in medical pathogens (Philippon et al. 2002). β-lactams and β-lactamases are evolutionarily ancient and thus originate from the environment. This might manifest itself in their wider dissemination in the environment. To test this hypothesis, we investigated if the resistance genes from the class *ampC* could be found in environmental freshwater bacteria.

This study was performed using the previously mentioned population of river bacteria with additional bacteria from Lake Võrtsjärv and river Pede. In total, 214 plus 760 isolates were analyzed. All bacteria were isolated from selective media containing at least one antibiotic as described in Paper I. The *ampC* genes were detected using multiplex PCR (Pérez-Pérez & Hanson 2002) that is able to amplify all major families of plasmid-carried *ampC* genes (e.g.,

those for CMY, DHA, MOX, FOX, and ACC). We detected *ampC* genes in 48 isolates and all of these belonged to the FOX family. Forty five of these isolates were isolated from ampicillin containing media and the other three were from tetracycline containing media.

The 16S rRNA gene in these 48 isolates was amplified using universal primers (Lane 1991, Paper I) and sequenced to determine the phylogenetic affiliation of the FOX positive isolates; the affiliation was assessed using data from the Ribosome Database Project (Wang et al. 2007). This showed us that 30 (65%) out of 48 isolates were *Aeromonas* spp. and 12 (25%) were *Pseudomonas* spp. (II, Figure 1A). Over the entire study population, 7% are *Aeromonas* spp. and 23% are *Pseudomonas* spp. It could be supposed that the high percentage of *Pseudomonas* spp. and *Aeromonas* spp. in the study population is the reason we found FOX-positive isolates belonging to these genera. However, this is not likely to be true because we did not find FOX-positive isolates from other genera with equally high isolate representation such as *Stenotrophomonas* and *Chryseobacterium*.

The PCR products from the FOX genes in the 48 isolates were compared with FOX variants described in public databases (Genbank and EMBL-EBI). By clustering the gene variants using Bayesian inference of phylogeny (MrBayes; II, Figure 1B), the FOX genes from our population clustered into four distinct groups with an intragroup similarity of 100% and were phylogenetically distinct from a separate node formed from the mostly enterobacterial FOX genes found in the public databases. We mapped the four FOX gene variants onto the 16S rRNA gene phylogenetic tree of the isolates (II, Figure 1A) to see how they are distributed among the isolates. This mapping demonstrated that one variant (VAR1) dominated over all others; in addition, all variants were represented among *Aeromonas* spp., and only two were found in *Pseudomnas* spp.

We only analyzed the relation of the population structure and distribution of FOX gene variants in *Aeromonas* isolates because not all of the FOX variants were found in *Pseudomnas* spp. The population structure was studied using multilocus sequence typing (MLST), using six houskeeping genes: *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA* (Martino et al. 2011). Every *Aeromonas* isolate ($n = 71$) was included in the MLST gene amplification. Because MLST was unable to function as expected for all of the six housekeeping genes (PCR amplification from all isolates was not achieved), we chose isolates where three of the MLST genes (gltA, metG, recA) were successfully amplified. With these genes 19 FOX-containing isolates and 23 FOX negative isolates were included in the analysis. The three MLST gene sequences were concatenated, aligned, followed by the construction of a maximum parsimony tree (II, Figure 2). From this we saw that most FOX-positive isolates grouped together and were separate from FOX-negative isolates. We did not observe an association between the FOX gene variants and the location of origin of the isolate (chi-square test, $P > 0.05$), however, we did find that more FOX gene variants were found in

autumn samples as opposed to summer samples (P = 0.02). The seasonal dependence may be influenced by autumn rains that tend to bring together material from adjacent agricultural fields and other streams. After making a phylogenetic tree using the 16sRNA gene sequences, we observe two populations. This suggests an inflow of a resistant population from elsewhere. Because the clustering of FOX-positive and FOX-negative isolates was found to be distinct, we conclude that there has been no recent horizontal transfer of FOX genes because phylogenetically closer isolates were seen to carry a specific FOX gene variant.

Relative abundance of antibiotic resistance genes in conventional wastewater treatment plants (III)

WWTPs are considered to be important hot-spots for the selection and spread of antibiotic resistance (Jury et al. 2011; Moura et al. 2010). It has been shown that high concentrations of both antibiotic compounds and resistance genes coexist in WWTPs (Kim & Aga 2007). The spread of antibiotic resistance could happen by resistant organisms and/or genetic material carrying resistance genes. Because the WWTP is a link between human community and natural water environment, we aimed to investigate if and how the quantities of the resistance genes change during the sewage treatment process and if the WWTP contributes to the resistome in the natural environment.

We sampled three different WWTPs, all from the Baltic Sea catchment area. Two WWTPs were located in Estonia (Tallinn and Tartu) and one in Finland (Helsinki). The WWTP in Tartu is the smallest and serves a population of ~100,000 while the WWTP in Helsinki was the largest and serves a population of ~800,000. The WWTP in Tallinn serves a population of ~350,000. The process technology employed in all three is similar. Five groups of samples were taken from December 2011 to December 2012, with each group representing one season (winter was sampled twice). Each sampling group consisted of three sampling dates that were separated by one to three days. Multiple sampling dates were used to obtain a better representation of the seasonal average while minimizing weather and community fluctuations.

We quantified seven resistance genes using quantitative PCR (qPCR), two resistance genes for sulphonamide (*sul1* and *sul2)*, two for tetracycline (*tetM* and *tetC)*, and three giving resistance to β-lactams (bla_{oxa-58}, bla_{shv-34}, *and* $bla_{ctx-m-32}$). These antibiotics and their respective resistance genes represent clinically important resistance genes, have been found in MGEs, and all of the associated antibiotics are widely used. The selection of resistance genes was based on an initial experiment where we screened for the presence of additional tetracycline and β-lactamase genes at the Tartu WWTP and a similar screening from the Helsinki plant. Some of the resistance genes provide resistance against antibiotics that have been used for a long period of time (e.g. tetracycline and

sulphonamide) while some provide resistance against newer antibiotics. The β-lactamases studied provide resistance against extended spectrum β-lactams such as carbapenems, and 3rd and 4th generation cephalosporins (Poirel et al. 2010b; Szczepanowski et al. 2009; Pfeifer et al. 2010).

We quantified the number of bacteria in both the inflow and outflow of each WWTP using the 16S rDNA copy number in the extracted DNA. As expected, the 16S rDNA quantities were orders of magnitudes lower in the effluent than in the inflow (III, Figure 1A). The highest drop was in the Tallinn WWTP with 1×10^{10} 16S rDNA copies per ml (SD 3×10^{10}) in the inflow and 1×10^{8} (SD 2×10^{8}) in the effluent. The differences in the quantity of 16S rDNA between the inflow and effluent were statistically significant in all studied WWTPs (adjusted $p<0.01$) (III, Figure 1A).

The raw copy numbers of resistance genes per ml were decreased during the purification process in the WWTPs and the levels of detected resistance genes were lower in the effluent than in inflow (III, Figure 1B), although, the decrease is not statistically significant for all resistance gene in every WWTP. For the WWTP in Helsinki, the decrease in detected resistance genes was statistically significant ($p<0.01$) for every resistance gene. In Tallinn WWTP, the decrease was significant ($p<0.01$) for all resistance genes except bla_{shv-34} ($p > 0.05$). In Tartu WWTP the raw copy numbers for the resistance genes studied decreased from the inflow to effluent but only three were statistically significant: $bla_{ctx-m-32}$ *and tetM* ($p < 0.01$), and, to a lesser degree, bla_{oxa-58} ($p = 0.03$). The decrease in raw copy number is similar to previously published data (Gao et al. 2012; Zhang et al. 2009; Auerbach et al. 2007). We found in our study that the resistance genes for "older" antibiotics (except *tetC*) were more commonly detected. sul1, sul2, and tetM were present above limit of quantification in all sites and samples (III, Table 3 and Supplemental Figure 2).

To both avoid inconsistencies between qPCR assays and allow one to compare results between various extraction methods, we normalised the raw resistance gene copy numbers with 16S rDNA copy numbers. It is known that the raw qPCR numbers differ between extraction times and these methods are not directly comparable, however, copy number ratios tend to be more reliable (Kubo et al. 2012; Lloyd et al. 2013). The amplification efficiencies between quantification assay 1 and 2 for the various genes in our study is close (III, Table 1). Comparing relative abundances, only four cases were statistically significant ($p<0.01$) (III, Figure 2). bla_{oxa-58} and *tet*C from the Helsinki plant and *tet*M from the WWTP in Tallinn decreased after purification in the effluent samples. In addition, we observe an increase in bla_{shv-34} resistance genes in the Tallinn WWTP, which is statistically significant. For all other resistance genes studied, in all samples, the purification process had no significant effect on the relative copy numbers of the resistance genes. From this we conclude that there is neither considerable enrichment nor removal of antibiotic resistance genes during the processes in all three WWTPs (Figure 2) at the community level. Our observation that the abundance of resistance genes changes in accordance with

bacterial abundance has been observed in previous quantitative studies that normalised their results with the quantity of 16S rDNA (Munir et al. 2011; Gao et al. 2012).

Previous studies have reported that there is a relationship between the change in ARG abundance and nutrient removal efficiency and temperature (Nõlvak et al. 2013; Novo & Manaia 2010). In our study we also examined if water quality measures, such as total nitrogen, total phosphorus, biological oxygen demand, and suspended solids, are able to predict the efficiency of ARG removal. However, we did not find any relationship between the quality measures monitored and the abundances of ARGs (III, Supplemental Figure 4). This means that in our case, the changes in ARG abundance did not depend on either the treatment technology employed or operating conditions in the WWTPs, but rather in the general decrease in the total population of bacteria.

CONCLUSIONS

- Bacterial genera from aquatic samples display a heterogeneous antibiotic resistance distribution. Each genus displayed a continuum of resistance coefficients ranging from 0 to 1 and multidrug resistance was randomly distributed in both the population and between genera.
- Despite the random distribution of multi drug resistance genes, some forms of resistance display a statistically significant ($p<0.05$) correlation. Chloramphenicol and tetracycline display a highly significant positive correlation over the entire study population, with even higher correlation among specific genera (e.g. *Aeromonas*).
- Medically relevant beta-lactam class *ampC* FOX family genes can be found in freshwater bacterial populations. However, the environmental FOX genes cluster separately from medical sequences using data obtained in public databases.
- *Aeromonas* spp. and *Pseudomonas* spp. are the main carriers of FOX resistance genes. Based on the results of multilocus sequence typing, FOX gene carriers group separately from non-carriers in the genus *Aeromonas*.
- Resistance genes for antibiotics that have been in use for longer were more commonly detected in the wastewater treatment plant than resistance genes against newer antibiotics.
- The raw abundance of resistance genes decreased for all resistance genes during the wastewater treatment process, however, the decrease of relative abundance was significant only for bla_{oxa-58} and *tetC* in Helsinki plant, and decrease for *tetM* and increase for bla_{shv-34} in Tallinn plant.

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SUMMARY IN ESTONIAN

Kas veekeskkond on antibiootikumiresistentsuse allikas, vahelüli või lõpp-punkt?

Antibiootikumide kasutuselevõtuga eelmise sajandi alguses arvati, et bakteriaalsetele haigustele on suudetud lõplik löök anda. Enne antibiootikumide kasutuselevõttu võis tänapäeval kergelt ravitav haigus olla raskesti ravitav või lausa surmav. Seetõttu nimetati antibiootikumid koheselt ka imeravimiteks ning ootused maailmaparandamisele olid suured nii spetsialistide hulgas kui ka üldise elanikkonna seas.

Enamus tänapäeval kasutuses olevaid antibiootikume on esialgselt avastatud keskkonna mikroorganismidelt. Looduses arvatakse antibiootikumide roll olevat kaitse konkurentsi eest. Penitsilliini ja streptomütsiini avastamine tõi endaga kaasa massilise ravimikandidaatide sõelumise mikroorganismidest ja kõige rohkem tänaseid ravimeid on avastatud ühest bakteriperekonnast – *Streptomyces*est.

Juba kohe alguses kui antimikroobsed ained kasutusele võeti oli neid, kes hoiatasid resistentsuse eest, mis tähendab, et ravim ei suuda kõrvaldada haigustekitajat bakteripopulatsiooni – haigus ei allu ravile. Uusi ravimeid avastati üha juurde ja turulejõudmine oli samuti kiire, mis lubas resistentsuse vastu võidelda uue ravimi kasutuselevõtuga. Tänaseks on paljugi muutunud. Uusi antibiootikumide klasse ei ole avastatud peale 1980ndaid aastaid. Põhjused peituvad arenduse ja turuletoomise kalliduses ning resistentsuse kiires esiletõusus ja levikus üle maailma, mis ei lase teenida tagasi arenduskulusid ja pärsib ravimifirmade huvi.

Nii nagu antibiootikumid on pärit loodusliku keskkonna mikroorganismidelt, on ka resistentsus antibiootikumidele evolutsioneerunud looduses koos antibiootikumide evolutsiooniga. Samas, tuleb mainida, et antimikroobsete ravimite kasutamine inimeste poolt on mõjutanud ka resistentset populatsiooni keskkonnas. Uuring, mis viidi läbi Hollandi pinnaseproovidega näitas, et resistentsete bakterite proportsioon on ajas järkjärgult suurenenud. Resistentsete bakterite levik ei ole piiratud inimlähedaste keskkondadega. Baktereid, kes suudavad üle elada ravikuuri on leitud kaugetest maailmanurkadest: näiteks Andide mäestikujärvest ja liustiku proovidest üle maailma. Üks osa antud tööst oli iseloomustada resistentset bakteripopulatsiooni Eestis, Suur-Emajões. Selleks võeti proove neljal järjestikusel kuul 2008 aastal. Proovivõtu kohti oli kaks: Tartust ülesvoolu ja allavoolu. Proovid külvasime me selektiivsöötmele (sisaldasid ühte antibiootikumi) ja kasvatasime ülesse bakterikolooniad ning valitud kolooniatel määrasime fülogeneetilise kuuluvuse.

Leidsime, et suure hulga meie valitud kogumist moodustasid *Gammaproteobacteria*, mille hulgast olid kõige arvukam perekond *Pseudomonas*. Lisaks olid arvukamad perekonnad *Stenotrophomonas*, *Chryseobacterium* ja

Aeromonas. Kõikide nimetatud perekondade hulgas leidub ka olulisi patogeenseid liike.

Üks suurimaid muresid antibiootikumiresistentsete patogeenide juures on see, et nad võivad olla resistentsed mitmetele antibiootikumidele. Kui resistentsus oleks ainult ühe antibiootikumi suhtes, oleks ravi siiski suhteliselt lihtne – võta järgmine ravim. Multiresistentsus, resistentsus mitme ravimi suhtes, seda teha ei võimalda. Lisaks kulutab kõiksugu testimine ja sobiva ravimi leidmine väärtuslikku aega. Multiresistentsus ei esine ainult seal, kus antibiootikume laialt kasutatakse, vaid on laialt levinud ka looduses. Seda on näidanud nii varasemad tööd kui ka meie enda töös. Nägime, et enamus perekondi olid resistentsed rohkem kui ühe antibiootikumi suhtes. Samas leidsime, et testitud 6 antibiootikumi suhtes ei olnud ükski vaadeldud perekondadest täielikult resistentne. Vähemalt üks ravim oli võimeline enamust bakteritest perekonnas hävitama. Multiresistentsus oli normaaljaotusega, mis näitab, et ühte superresistentset populatsiooni uuritavate bakterite hulgas ei olnud. Uurisime ka resistentsuste koosesinemist organismide kaupa ja leidsime, et tetratsükliini ja klooramfenikooli koosesinemisel oli statistiliselt oluline ($p<0,05$) kõrge korrelatsioonikoefitsient. Seda nii üle kogu uuritava populatsiooni kui ka üksikute perekondade seas. Selline seotus võib viidata resistentsusgeenide paiknemisele ühel mobiilsuselemendil. Geneetilised mobiilsed elemendid on molekulid, millega on võimalik üle kanda geneetilist materjali ühest mitte-järglasrakust teise. Selline kandumine on ka üheks antibiootikumi resistentsuse kiireks levikuks ühest organismist teise ja ka erinevate geograafiliste keskkondade vahel.

Resistentsusgeenide liikumine, kas geneetilise materjalina või organismidega, ja selle suund on oluline küsimus takistamaks resistentsuse levikut ja teket. Geenid, mis tagavad resistentsuse on keskkonnas olemas. On olemas nii vanade ja keskkonnas pärit antibiootikumide suhtes kui ka uute täissünteetiliste vastu. Ühe osana üritab ka antud töö vaadata inimese mõju keskkonnale antibiootikumide resistentsuse seisukohalt. Seda, et resistentsed organismid on vesikeskkonnas olemas me näitasime. Näitasime ka resistentsusgeenide olemasolu, mis on sarnane patogeensetest tüvedest leitud geeniga. Selline otsene seos viitab selgelt keskkondade seotusele ja võimalikule ohule, mis loodus võib meditsiinis põhjustada.

Kolmandana uurisime kas reovee puhastusjaamas toimuvad protsessid, mille järel lastakse puhastatud vesi taas loodusesse, võib mõjutada sealset kooslust. Teaduskirjanduses avaldatu põhjal võib teha järelduse, et reovee puhastusjaamad on kohad, kus resistentsus võib levida erinevate organismide vahel ning sealt edasi keskkonda. Puhastusjaama jõudvas reovees on antibiootikumide jääke ja patogeenseid liike ning puhastusjaamas on soodsad tingimused resistentsusgeenide vahetuseks erinevate organismide vahel. Meie uurisime kvantitatiivselt resistentsusgeenide hulga muutust puhastatud vees võrreldes reoveega. Võrdlesime seitset erinevat antibiootikumidele resistentsust põhjustavat geeni: kaks tetratsükliini, kaks sulfonamiidi ja kolm geeni beta-laktaamide

vastu. Proovid olid võetud kolmest erinevast puhastusjaamast – Tartust, Tallinnast ja Helsingist, Soomest – ja proove võeti aasta jooksul, igal aastaajal. Kõik resistentsusgeenid olid kõikidest puhastusjaamadest leitavad, kuid mitte kõikides proovides. Kui me vaatasime uuritud geenide arvukuse toorandmeid, siis kõikide arvukus oli puhastatud vees langenud võrreldes reoveega, kuid muutus ei olnud kõikidel kordadel statistiliselt oluline. Toorandmete võrdlemine erinevate tööde, DNA eraldusmeetodite ja mõõtmistingimuste vahel ei ole parim praktika. Kitsaskoha vältimiseks mõõtsime samuti bakteri ribosomaalse geeni hulka proovides, mis näitab meile kaudselt proovis olevate bakterite hulka. Peale geeni hulkade normaliseerimist bakteriaalse arvukusega oli näha, et tegelikult mingit suhtelist resistentsusgeenihulga suurenemist ega vähenemist ei toimunud. Geenide hulk vähenes bakterite hulga vähenemisega puhastatavas vees. Tulemused näitavad, et uuritud geenide hulgad reoveepuhastusjaamas ei suurene, siis mingi osa jõuab siiski ka loodusesse.

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PUBLICATIONS

CURRICULUM VITAE

Name: Veiko Voolaid
Date of birth: 14 June, 1983
Citizenship: Estonian
Contact: Jaama 54–1, 51009 Tartu
veiko.voolaid@ut.ee
+372 56216924

Education and professional employment:

Education:

2009– current	University of Tartu, PhD studies, Technology of Biomedicine
2006–2008	University of Tartu, Master's degree, Gene Tehcnology
2002–2006	University of Tartu, Bachelor's degree, Gene Technology

Work experience:

2008–2009	University of Tartu, Institute of Technology; Laboratory technician
2013–2014	University of Tartu, Institute of Technology; Research assistant
2014– current	Dresden University of Technology, Institute of Hydrology, Postdoc

Belonging to organizations:

2009–2013	Member of the COST Action TD0803 DARE

Scientific work:

My main research interests have been the presence of antibiotic resistance among bacteria in natural water environment and the potential relation with medical settings.

List of publications:

Voolaid V, Jõers A, Kisand V & Tenson T (2012) Co-occurrence of resistance to different antibiotics among aquatic bacteria. *BMC microbiology* 12: 225.

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ELULOOKIRJELDUS

Nimi: Veiko Voolaid
Sünniaeg: 14.06.1983
Kodakondsus: Eesti
Kontaktinfo: Jaama 54–1, 51009 Tartu
veiko.voolaid@ut.ee
+372 56216924

Haridus:

2009 –…	Tartu Ülikool, Doktoriõpe, Biomeditsiini tehnoloogia erialal
2006–2008	Tartu Ülikool, Magistriõpe, Geenitehnoloogia erialal
2002–2006	Tartu Ülikool, Bakalaureuseõpe, Geenitehnoloogia erialal
1990–2002	Otepää Gümnaasium

Teenistuskäik:

2008–2009	Tartu Ülikool, Tartu Ülikooli Tehnoloogiainstituut; Laborant
2013–2014	Tartu Ülikool, Tartu Ülikooli Tehnoloogiainstituut; Laborant
2014–…	Dresdeni Tehnika Ülikool, Hüdroloogia instituut; Järeldoktorantuur

Teadustegevus:
Minu uurimistöö on keskendunud antibiootikumiresistentsetele bakteritele looduslikus vesikeskkonnas ja kuidas võib looduses esinev resistentsus olla seotud resistentsusega meditsiinis.

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