Antibiotic resistance in human impacted environments

Antti Karkman

Academic Dissertation in Microbiology

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


The publications are referred to in the text by their roman numerals.
*Equal contribution

AUTHOR CONTRIBUTION

I Antti Karkman participated in the design of the work, executed most of the experimental work, with the exception of the HPLC analyses. He participated in interpreting the results and writing the manuscript.

II Antti Karkman participated in the design of the study and experimental work. He analyzed the results together with the co-authors and participated in writing the manuscript.

III Antti Karkman designed the work and did the experimental work except run of samples with the qPCR array. He analyzed the results and wrote the manuscript.

IV Antti Karkman participated in the design of the study, in the experimental work, in interpreting the results and writing the manuscript.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARB</td>
<td>Antibiotic resistant bacteria</td>
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<tr>
<td>ARG</td>
<td>Antibiotic resistance gene</td>
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<tr>
<td>CE</td>
<td>Common Era</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum ß-lactamase</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>MDR</td>
<td>Multi drug resistant</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SSN</td>
<td>Sequence similarity network</td>
</tr>
<tr>
<td>UWTP</td>
<td>Urban wastewater treatment plant</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Increasing microbial resistance against antibiotics is threatening their efficiency in the future and we might be heading back to pre-antibiotic era. Infectious diseases still treatable with antibiotics might soon become life threatening.

There is a strong correlation between antibiotic use and antibiotic resistance occurrence. Hotspots for antibiotic resistance are mainly man made such as wastewater treatment plants, animal farms and aquaculture. Aquaculture is the fastest growing food industry in the world and uses antibiotics to treat and prevent fish diseases. The use of antibiotics is linked to increase in antibiotic resistance at the farms. At urban wastewater treatment plants microbes from various sources can mix and exchange genetic material. In addition wastewaters contain antibiotics that can further select for resistant microbes.

In this work hundreds of antibiotic resistance genes were quantified with cutting-edge molecular methods. The global patterns of antibiotic resistance gene movement were determined using publicly available metagenomic data. In addition, the famous Baas-Becking hypothesis ‘everything is everywhere, but the environment selects’ was tested on gene level.

Aquaculture increases the amount of antibiotic resistance genes in the farms. The resistance genes persist in the aquaculture sites without a clear selection pressure, however the impact is only local. Urban wastewater treatment plants efficiently removed antibiotic resistance genes from wastewaters. The release of wastewater had only a limited impact on the sediment resistome near the release site. When looking at the metagenomic data, antibiotic resistance genes were found to have different dispersal pattern compared to other genes in the metagenomes. Antibiotic resistance genes can cross taxonomical and geographical barriers with ease, possibly explaining their wide dispersal in the environment and the clinic. These results show that antibiotic resistance is ubiquitous in the environment and the anthropogenic activities affect the incidence of antibiotic resistance.
TIIVISTELMÄ

Antibioottien kasvava ja jopa holtiton käyttö terveydenhuollossa ja eläintuotannossa on johtanut antibiooteille vastustuskykyisten bakterien yleistymiseen ja leviämiseen maailmanlaajuisesti. Pahimmassa tapauksessa olemme palamassa aikaan, jolloin bakterien aiheuttamille sairauksille ei ole tehokasta hoitoa ja jopa keuhkokuume voi olla kohtalokas. Jo nyt vaarassa ovat immuunipuolustuskyvyttäen heikentyneet ihmiset, kuten vanhuksent ja vakavasti sairattut.


1. INTRODUCTION

1.1 Brief history of antibiotics and antibiotic resistance

Antibiotics are one of the most important discoveries that have affected human and animal health in the history of mankind. Since antibiotics are mainly natural products synthesized by microbes, it's evident that they have existed for ages (Davies & Davies, 2010; Aminov, 2010). Probably antibiotics have been used already in 350 – 550 CE based on traces of tetracycline on humans skeletal remains (Nelson et al, 2010). Also traditional Chinese medicine has used herbs with antimicrobial activity (Cui & Su, 2009). The antibiotic era began in the early 20th century with the discovery of Salvarsan, a synthetic drug against syphilis. Synthetic dyes staining microbes selectively gave Paul Ehrlich the idea of “magic bullets” that could target pathogenic microbes selectively. Erlich systematically screened chemical compounds to find a cure to the common and almost untreatable disease caused by a spirochete Treponema pallidium. This kind of systematic screening approach became popular in drug search and was also used in the discovery of sulfa drugs in the early 1920's.

The most important discovery in the history of antibiotics was the discovery of penicillin by Alexander Fleming in 1928 from Penicillium mold. Penicillin was not immediately taken into use in the clinic. The problems with purification and stability took 12 years and Fleming already abandoned the idea in 1940. Fortunately on the same year another group in Oxford came up with a solution in purifying enough of penicillin for clinical testing. The purification protocol of the Oxford group eventually led to the mass production of penicillin, which was further fueled by the huge need during World War II (Davies & Davies, 2010; Aminov, 2010). From there started the golden era of antibiotics and several new classes and compounds were discovered (Figure 1).

Antibiotics have revolutionized medicine and saved many lives, but it was evident from the beginning of the antibiotic era that bacteria can become resistant to antibiotics. Already Alexander Fleming observed resistant strains soon after the discovery of penicillin. Fleming said in 1946: "There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring fastness (resistance)” (Levy & Marshall, 2004). It took only 5 years after the clinical use of penicillin had started when the first clinically relevant resistant bacteria was observed. Bacteria have developed resistance to all antibiotics in use, few years after antibiotics are introduced to the clinic, clinically significant resistance appears (Figure 1). The rapid evolution of microbial antibiotic resistance is ending or has already ended the golden era of antibiotics (Clatworthy et al, 2007).

Bacteria have produced antibiotics probably for over 500 million years, already from the Cambrian period (Allen et al, 2010; Baltz, 2008), but still it is unclear what are the roles of antibiotics in the natural environments. The concentrations in nature are far lower than what is used in the clinic and antibiotics probably have different functions than warfare in nature. (Allen et al, 2010; Aminov, 2009). Low antibiotic concentrations induce biochemical pathways and the natural roles of antibiotics are related to signaling, regulation and quorum sensing (Allen et al, 2010; Aminov, 2009; Clardy et al, 2009). From anthropocentric viewpoint antibiotics have been considered to have hostile roles and act as weapons in nature (Allen et al, 2010; Davies & Davies, 2010). However there are only few examples of hostile antibiotic functions in nature (Allen et al, 2010; Currie et al, 1999; Neeno-Eckwall et al, 2001).
1.2 Origins and genetics of antibiotic resistance

Antibiotic resistance genes are thought to originate from antibiotic-producing bacteria or from bacterial housekeeping genes through mutation. Natural producers of antibiotics have a protection mechanism against the antibiotics they produce often located on the same genetic element as the antibiotic synthesis genes (Benveniste & Davies, 1973; Martin & Liras, 1989). Although these bacteria can be a reservoir for new resistance determinants, no clear evidence exist for clinical antibiotic resistance to originate from natural producers (Aminov & Mackie, 2007). Strains isolated before the widespread use of antibiotics have shown that also non-producer strains had resistance determinants (Allen et al, 2010; Smith, 1967).

Bacterial antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is naturally occurring in the host, such as multidrug efflux pumps or physical barriers preventing the entry of the antibiotic. Acquired resistance involves spontaneous mutations and transfer of the resistance genes from other bacteria through transformation, conjugation or transduction (horizontal gene transfer, HGT). The selection pressure caused by the ever-increasing use of antibiotics fastens the evolution of resistance genes, selects for resistant phenotypes and even induces the horizontal transfer of the genes through several mechanisms (Hastings et al, 2004; Roberts & Mullany, 2009).

Antibiotic resistance genes were probably subjected to horizontal gene transfer even before antibiotic era (Allen et al, 2010; D’Costa et al, 2011). Evidence for selection and evolution of resistance genes before the antibiotic era exist (D’Costa et al, 2011). β-lactamases rose over 2 billion years ago and were already present on plasmids over million years ago. Many precursors of antibiotic resistance genes have evolved through ancient rather than recent evolution due to the selection caused by massive antibiotic use (Aminov, 2009).

The evolution of resistance genes can be divided into two periods, the pre-antibiotic and antibiotic. In the pre-antibiotic period the evolution of the genes was slow and did not involve
strong selection or horizontal transfer. Heavy use of antibiotics since the 1940's accelerated the evolution of antibiotic resistance due to strong selection pressure. At some point resistance genes got captured by mobile genetic elements, which disseminated through horizontal gene transfer into commensal and pathogenic bacteria (Aminov, 2009). The main mechanisms behind antibiotic resistance prevalence and spread are selection pressure favoring the resistant microbes and horizontal gene transfer of the resistance genes, which is often induced by antibiotics (Aminov & Mackie, 2007). Plasmids have been common in bacteria already before the antibiotic era, however resistance determinants in plasmids were still rare. Today plasmids are one of the most important vectors of resistance genes (Davies & Davies, 2010).

1.3 Emergence of antibiotic resistance and the rise of superbugs

There is a strong correlation between antibiotic consumption and antibiotic resistance (Goossens et al, 2005). Both sulfa drugs and penicillin did not thrive for long before clinically relevant resistant pathogens emerged. Tetracyclines were discovered in 1948 and five years later the first resistant Shigella strain was isolated. Already in 1955 the first multidrug resistant (MDR) Shigella was detected, but MDR strains were still rare. Few years after the proportion of MDR Shigella had already risen to 10% showing the remarkable pace of antibiotic resistance spread (Chopra & Roberts, 2001). Resistance can arise even against fully synthetic antibiotics like broad-spectrum fluoroquinolones. Horizontal gene transfer has played a major role in the evolution and transmission of the resistance genes and has given the rise of extended spectrum β-lactamase (ESBL) enterococci in community and hospital environments (Davies & Davies, 2010).

The most serious threat worldwide is the rise of superbugs, such as methicillin Staphylococcus aureus (MRSA). Superbugs are commensal and pathogenic bacteria that have acquired multiple resistance genes. Some strains have in addition acquired increased virulence and enhanced transmissibility (Alekshun & Levy, 2007). Many pathogens associated with epidemics of human disease have acquired multiple resistance determinants due to the massive antibiotic use. These MDR strains are causing serious problems in healthcare system worldwide. The World Health Organization (WHO) and recent G7 summit in Germany (2015) raised a concern about antibiotic resistance and according to WHO we are heading back to pre-antibiotic era if serious actions are not made (World Health Organization, 2014).

1.4 Antibiotic resistance in the environment and the anthropogenic impact

Antibiotic resistance is not restricted to pathogenic or commensal microbes, but is ubiquitous in the environment. Environmental microbiome is considered to be the natural reservoir of potential antibiotic resistance genes and has more diversity and novelty than ever expected (Wright, 2010; Lin et al, 2015). Antibiotic resistance exists in ancient permafrost (D’Costa et al, 2011), deep terrestrial subsurfaces (Brown & Balkwill, 2009), in agriculture and animal husbandry (Heuer et al, 2011) and wastewaters (Auerbach et al, 2007; Czekalski et al, 2014; Rizzo et al, 2013). Since most of the antibiotics originate from soil microbes, it is obvious that soil is a huge reservoir for antibiotic resistance (Walsh & Duffy, 2013). CTX-M β-lactamases originate from environmental Klebsiella species (Humeniuk et al, 2002) and quinolone resistance genes from Klebsiella pneumoniae (Baquero et al, 2008) showing the clear link from the environment to the clinic.
Antibiotics have been used in animal farming and aquaculture as growth promoters, for prophylaxis and for treatment of infections (Baquero et al, 2008; Roca et al, 2015; Cabello, 2006). The use and misuse of antibiotics in the clinic, community and animal farming has resulted in the emergence and spread of resistant microbes through selection caused by the antibiotics. Even sub lethal antibiotic doses select and enrich antibiotic resistance in the environment (Andersson & Hughes, 2012). The impact of increasing use of antibiotics can be seen in archived soil samples where the resistance gene abundance increases from the 1940s to present (Knapp et al, 2010).

Antibiotic resistance genes are now considered an environmental pollution and severe measures to prevent their further spread should be taken (World Health Organization, 2014; Roca et al, 2015). Better understanding of reservoirs as well as dissemination of antibiotic resistance genes (ARGs) is needed to fight the resistance threat in the clinic and the environment.

The hotspots for environmental bacteria to mix and exchange genetic material with pathogenic bacteria are mostly man made, such as sewage, wastewater treatment plants, animal farms and aquaculture (Baquero et al, 2008; Wellington et al, 2013). These genetic reactors are the main human impacted environments where resistance emerges and disseminates.

Urban wastewater treatment plants (UWTPs) are a significant source of antibiotic resistance pollution (Table 1) (Rizzo et al, 2013). Sewage from households and hospitals contains antibiotics causing selective pressure for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Martinez, 2009). Bacterial biofilms and stress caused by antibiotics and other pollutant compounds promote horizontal gene transfer in wastewaters (Aminov, 2011).

### Table 1. Examples of studies quantifying antibiotic resistance genes in urban wastewater treatment plants or environments impacted by UWTPs

<table>
<thead>
<tr>
<th>Sampling locations</th>
<th>Country</th>
<th>ARGs quantified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>River downstream from UWTP</td>
<td>USA</td>
<td>sul1, tetW</td>
<td>(Pruden et al, 2012)</td>
</tr>
<tr>
<td>Sediment impacted with wastewater</td>
<td>Switzerland</td>
<td>sul1, sul2, tetB, tetM, tetW, qnrA</td>
<td>(Czekalski et al, 2014)</td>
</tr>
<tr>
<td>Influent, effluent, biosolids, activated sludge</td>
<td>USA</td>
<td>tetG, tetQ</td>
<td>(Auerbach et al, 2007)</td>
</tr>
<tr>
<td>River influenced by UWTP</td>
<td>Spain</td>
<td>blaTEM, blaCTX-M, blaSHV, qnrA, qnrB, qnrS, tetO, tetW, sul1, sul2, ermB</td>
<td>(Marti et al, 2013)</td>
</tr>
<tr>
<td>Influent, biosolids</td>
<td>USA</td>
<td>tetO, tetW, sul1</td>
<td>(Munir et al, 2011)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>China</td>
<td>blaOXA-1, blaOXA-2, blaOXA-10, ampC, blaTEM-1,blaIMP</td>
<td>(Yang et al, 2012)</td>
</tr>
<tr>
<td>Sludge</td>
<td>USA</td>
<td>sul1, sul2, ermB, ermF, tetO, tetW, tetC, tetG, tetX,</td>
<td>(Ma et al, 2011)</td>
</tr>
<tr>
<td>River water and sediments influenced by UWTPs</td>
<td>UK</td>
<td>Int1</td>
<td>(Amos et al, 2015)</td>
</tr>
</tbody>
</table>
Aquaculture is the fastest growing food industry in the world and probably will continue to grow in the future (Cabello, 2006; Miranda et al, 2013). The high fish densities and increased stress weaken the fish immune system and further promote the need of antibiotics. Increased use of antibiotics selects for resistance among environmental, and commensal and pathogenic fish bacteria making the resistance problem even worse (Cabello, 2006). One of the major concerns is the development of antibiotic resistant reservoirs from where the resistance can emerge and transfer to pathogenic bacteria. Bacteria resistant to several antibiotics, including the clinically important ones, exist in aquaculture. Resistance genes transfer horizontally between environmental aquaculture bacteria and human as well as veterinary pathogens (Cabello, 2006; Miranda et al, 2013). However the prevalence of resistance in fish pathogens and the transfer routes of the resistant bacteria in the environment and food chain on global scale remain largely unknown and need further studies.

1.5 Challenges in detecting and quantifying antibiotic resistance in the environment

For the last 70 years the research on antibiotic resistance has focused mainly on pathogens. Isolating pure cultures has been and still is the most important method in clinical microbiology. Antibiotic susceptibility testing of bacteria is relatively inexpensive and does not require expensive equipment. Susceptibility testing gives important data on resistance patterns that is needed for designing treatments for patients. Clinical breakpoints for pathogenic bacteria are drawn based on susceptibility testing of different strains. Databases of clinical breakpoints (such as EUCAST, www.eucast.org) help in monitoring antibiotic resistance worldwide. Combined with molecular techniques, whole genome sequencing as an example, data from susceptibility testing can be used to find previously unknown resistance determinants acquired through mutations or horizontal gene transfer. Sequencing of whole microbial genomes gives insight about the genetic environment of the antibiotic resistance genes. Genes located on mobile genetic elements capable of horizontal transfer pose a bigger risk for resistance spread (Martínez et al, 2014). Culturing and susceptibility testing can be used for environmental bacteria (Walsh & Duffy, 2013), but only a fraction of environmental bacteria can be grown in the laboratory, so this gives only limited information about the environmental resistome.

Culture-independent methods have their advantages when working with environmental samples. PCR and quantitative PCR (qPCR) methods can be used in detecting genes from environmental DNA without the need of culturing and even in high-throughput fashion (Szczechanski et al, 2009), especially when using qPCR arrays for hundreds of genes (Zhu et al, 2013; Wang et al, 2014; Looft et al, 2012). PCR methods are relatively inexpensive and easy to perform, however some sophisticated equipment is required. The need of prior knowledge for primer design limits their use to known genes or genes with high homology to known ones. With molecular methods it is also difficult to predict the functionality of the target gene.

Metagenomics, the sequencing of the whole community DNA, can overcome the need of prior knowledge of resistance genes. It is a powerful method to detect all functional genes at the same time, including antibiotic resistance genes. Metagenomics has been used to detect antibiotic resistance in diverse environments (Chen et al, 2013; Hu et al, 2013; Li et al, 2015; Nesme et al, 2014; Yang et al, 2013; Zhang et al, 2011) and is not restricted to few a priori chosen genes but through sequencing the total community DNA can capture the whole resistome. However the annotation of antibiotic resistance genes relies on known genes in public antibiotic resistance
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gene databases (Gibson et al, 2015; Gupta et al, 2014; Liu & Pop, 2009; Zankari et al, 2012; McArthur et al, 2013). Still, in most environments antibiotic resistance genes are rare compared to other functional genes and therefore deep sequencing is needed to capture the whole diversity.

Most metagenomic sequencing platforms produce short reads that as such give only limited information about the sequenced genes. Assembling short reads into longer overlapping DNA segments (contigs) can give information about the phylogeny and genetic context of the genes. Partial or even complete genomes can be reconstructed from metagenome data (Hultman et al, 2015; Albertsen et al, 2013). This knowledge is important in ranking the risks of antibiotic resistance genes in the environment. Antibiotic resistance genes located in mobile genetic elements in pathogenic bacteria have the highest risks for human health (Martinez et al, 2014).

Functional metagenomics, the cloning and expression of environmental DNA in a laboratory host, can overcome the limits of PCR and metagenomic sequencing in detecting mostly known resistance genes. In functional metagenomics environmental DNA is cloned in large fragments (10 – 200 kb) into a laboratory host e.g. *Escherichia coli* and the susceptibility of the host to different antibiotics is tested. Clones with resistance phenotype are screened for the antibiotic resistance determinant by sub-cloning, mutagenesis or *in-silico* analysis, which can be laborious and time consuming. Cloning and expressing the genes in the host can be difficult and are the main disadvantages of functional metagenomics, although they can be solved to some extent by using other hosts than *E. coli*. Proteomics combined with functional metagenomics is promising new way to overcome the tedious screening of potential clones. Using proteomic tools the expressed proteins can be identified in high-throughput manner and by comparing to a strain without the cloned DNA, the putative new resistance determinants identified (Fouhy et al, 2015).
2. AIMS OF THE THESIS

Antibiotic resistance seems to be ubiquitous in the environment. The use and misuse of antibiotics has resulted in multi resistant bacteria, pollution of the environment with antibiotic resistance genes and the possible return of the pre antibiotic era with limited possibilities to treat bacterial infections. In my thesis I studied how anthropogenic pollution affects the antibiotic resistance abundance in the impacted environments and how antibiotic resistance genes have spread on global scale. Two example environments, aquaculture and wastewater, with high anthropogenic impact were chosen for study sites. Using publicly available metagenomic data from both human impacted and “pristine” environments the global dispersal patterns of antibiotic resistance were modeled.

Aquaculture introduces fish commensal bacteria and occasionally antibiotics to the sediment beneath the farms and causes eutrophication and oxygen depletion altering the bacterial community. In I my aim was to study the antibiotic resistance abundance and persistence in the sediments beneath the fish farms and the effects on the surrounding environments by sampling sediments in the close proximity to the farms.

Urban wastewater treatment plants are considered to be hotspots for antibiotic resistance and horizontal gene transfer. In II and III I investigated how different urban wastewater treatment plants eliminate resistance genes from wastewaters and what is the amount of resistance genes still ending up in the environment. By screening hundreds of genes I wanted to deepen my understanding on the ARG dynamics in the UWTP and on the environmental effect of ARG release to the environment.

Antibiotic resistance genes are known to spread and transfer efficiently in bacterial communities. In IV using metagenomic sequencing data my aim was to understand the global patterns of horizontal transfer of antibiotic resistance gene between different environments and if biogeography or ecology affects the dispersal of these genes.
3. SUMMARY OF THE METHODS

3.1 Samples and data used in this study

3.1.1 Aquaculture

Sediment samples were collected in the summer time during 2006–2009 from aquaculture sites in Northern Baltic Sea in the Turku Archipelago, Finland and in August 2007 from Stockholm Archipelago, Sweden. The sampling locations and the sampling procedures are described in detail in I.

3.1.2 Urban wastewater treatment plants

The urban wastewater treatment plants were sampled in 2010–2011 for II and III. For II 24 h composite samples were collected from raw wastewater and final effluent waters from three UWTPs in Tartu, Estonia and Helsinki, Finland over a one-year period from December to December. For III the UWTP in Helsinki, Finland was sampled on four seasons over one year. Water samples from raw wastewater and final effluents were 24 h composite samples. In addition dried sludge was studied on all seasons. The sediments near the final effluent discharge pipe were sampled once on Summer 2011. More detailed description can be found from II and III.

3.1.3 Metagenomes

The metagenomic data was collected from public databases (IMG, MG-RAST and CAMERA) and all metagenomes were assigned geographic location with GPS coordinates and habitat annotation based on the environmental metadata available. Samples from same environment and geographical location were pooled to represent that environment in that geographical location. More detailed description of the data retrieval and processing is available in IV.
### 3.2 Methods used in this study

<table>
<thead>
<tr>
<th>Method</th>
<th>Study</th>
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<tr>
<td>Sediment sampling</td>
<td>I, III</td>
</tr>
<tr>
<td>Wastewater sampling</td>
<td>II, III</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>I, II, III</td>
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<tr>
<td>Primer design</td>
<td>I</td>
</tr>
<tr>
<td>PCR optimization</td>
<td>I</td>
</tr>
<tr>
<td>PCR</td>
<td>I, II, III</td>
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<tr>
<td>Quantitative PCR optimization</td>
<td>I, II</td>
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<tr>
<td>Quantitative PCR</td>
<td>I, II, III</td>
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<tr>
<td>Bioavailability measurements</td>
<td>I</td>
</tr>
<tr>
<td>Metagenomic data retrieval</td>
<td>IV</td>
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<td>Metagenome assembly</td>
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<td>Metagenome gene calling</td>
<td>IV</td>
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<tr>
<td>Functional gene annotation</td>
<td>IV</td>
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<td>Antibiotic resistance gene annotation</td>
<td>IV</td>
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<tr>
<td>Marker gene annotation</td>
<td>IV</td>
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<tr>
<td>Statistical analysis</td>
<td>II, III, IV</td>
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<tr>
<td>Network analysis</td>
<td>IV</td>
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</tbody>
</table>
4. RESULTS AND DISCUSSION

To get a deeper understanding on the anthropogenic effect on antibiotic resistance persistence and abundance, in this thesis antibiotic resistance genes were quantified in aquaculture and wastewater environments using qPCR and a high throughput qPCR assay. Aquaculture sites were sampled on several years and the effect of fish farming and antibiotic use on the resistance abundance and persistence was studied (I). Wastewaters were studied in different urban wastewater treatment plants in Estonia and Finland (II). The UWTP in Helsinki, Finland was further monitored for one full year and the impact of wastewater release on Baltic Sea sediments was also quantified with a qPCR array targeting almost 300 genes (III). Additionally, using publicly available metagenomes, I tested the famous Baas-Becking hypothesis ‘Everything is everywhere, but the environment selects.’ The dispersal and possible HGT of ARGs was assessed using network analysis (IV).

4.1 Aquaculture

The presence, quantity, dissemination and persistence of several tetracycline resistance genes were studied in four different aquaculture sites in Finland and Sweden, all located in the Baltic Sea. Tetracycline resistance gene abundances were elevated in all aquaculture sites studied (I). All seven tetracycline resistance genes were detected from aquaculture sites. No genes were detected in any of the control sites outside the farms, showing the local impact of the aquaculture on the sediment bacterial community (Figure 2). Therefore aquaculture does not pose a direct threat on the surrounding environment on resistance gene spread. However resistance genes can transfer horizontally between bacteria from aquaculture, and commensal and pathogenic fish bacteria (Cabello, 2006), since antibiotic resistant bacteria and resistance genes often occur at aquaculture sites (Dang et al, 2007; Akinbowale et al, 2007; Nonaka et al, 2007; Miranda et al, 2003). Although my results did not show dissemination of the genes (I), persistent antibiotic resistance in the aquaculture sites might further move to the food chain and threaten human health.

The farmed fish are occasionally medicated with antibiotics. To address the possible selection pressure favoring tetracycline resistance in the sediments the tetracycline concentrations in the sediments were measured with HPLC and biosensor. The biosensor responds to several tetracyclines including tetracycline and oxytetracycline. The HPLC assay was configured for tetracycline and oxytetracycline. However, all samples were below the detection limit in the sediments (biosensor: 300 ng/g sediment wet weight for tetracycline, HPLC: 66 and 25 ng/g sediment wet weight for tetracycline and oxytetracycline, respectively) (I).

Tetracycline resistance genes were detected at aquaculture sites through 2006 – 2009, even though antibiotics were not found from the sediments. These resistance genes persisted in the aquaculture sites without a clear selection pressure (I). Even low doses of antibiotics can select for antibiotic resistant organisms (Andersson & Hughes, 2012) and resistance genes can have low fitness costs or even fitness increase in the absence of selection (Enne et al, 2005; Luo et al, 2005). The persistence of the ARGs can also be explained by a constant influx from an external source. Possible sources can be the fish commensal and pathogenic bacteria (Cabello, 2006), wastewater or animal farming effluents (Dang et al, 2008), fish hatchlings (Rhodes et al, 2000) or the fish feed (Seyfried et al, 2010; Kerry et al, 1995).
Figure 2. Presence and quantification of seven tetracycline resistance genes in aquaculture sites and control sediments. A) Sediments collected from FIN1 farm site and the control sediments collected from 200 m intervals from the farm. B) FIN2 farm and Swedish farm sites and control sites. The mean copy numbers and standard deviations of \textit{tetM}, \textit{tetC}, \textit{tetA} and \textit{tetH} genes were calculated from three technical replicates and normalized with 16S rRNA gene copy numbers in the sediment community DNA. Figure adopted from I.
4.2 Urban wastewater treatment plants

In addition to the aquaculture antibiotic resistance genes were quantified from urban wastewater treatment plants (II, III). In II three UWTP’s in Finland and Estonia were monitored for more than a year for extended spectrum β-lactamase genes (bla\textsubscript{ctx-m-32}, bla\textsubscript{oxa-58}, and bla\textsubscript{shv-34}), sulphonamide resistance genes (sul1 and sul2) and tetracycline resistance genes (tetM and tetC) in influent and effluent waters. In III an UWTP in Helsinki, Finland, also sampled in II, was monitored for one year for almost 300 resistance genes in the influent and effluent waters and dried sludge. The impact of wastewater release on the environment was addressed by sampling

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**Figure 3.** Quantification of resistance genes in Influent and effluent waters in three UWTP’s in Finland and Estonia. Gene copy numbers are normalized with 16S rRNA gene copy numbers. The results present the whole study period without seasonal comparison. Stars denote statistical difference between the copy numbers in influent and effluent waters. *** at p<0.01, * 0.03 > p > 0.01. Figure adopted from II.
sediments near the purified wastewater discharge pipe and the resistance profile was compared to the resistance profile of control sediments without the impact of wastewater release (III).

Wastewater purification reduced bacterial numbers and the quantified antibiotic resistance genes from the influent to effluent waters by several fold. However on the relative amounts when normalized with 16S rRNA gene there was no statistically significant difference for most of the genes (Figure 3). The wastewater purification was probably unspecific in removing antibiotic resistance genes and based mainly in the reduction of bacteria (Figure 3) (II, III). Antibiotic resistance is ubiquitous in sewage and also in final effluents released to the environment (II, III). The Helsinki UWTP was the most efficient in removing ARGs (Figure 3) (II). The resistance gene abundance decreased several fold from sewage to effluent waters, and dried sludge, (III) and the antibiotic resistance profiles changed from raw influent to final effluents and dried sludge (Figure 4). The dried sludge was shown to be a major reservoir of resistance genes (III) that has also been seen in other UWTPs (Yang et al, 2014; Munir et al, 2011).

Only few genes accumulated in the sediments near the release site sediments (III). One of the enriched genes was related to clinical class 1 integrons, qacEΔ1 (Gillings et al, 2008; Gaze et al, 2011; Wellington et al, 2013). Clinical class 1 integrons have been proposed as a marker gene for anthropogenic pollution in the environment (Gillings et al, 2015), and my results support that. However, wastewater release did not have a big impact on the resistance gene abundance in the sediment bacterial community (III).

![Figure 4](image-url)  
*Figure 4.* Ordination plot from the antibiotic resistance gene (ARG) abundance profiles showing the clustering of different sample types and the change from raw inflow to the final effluents and dried sludge in the Helsinki UWTP. The release site sediments did not differ substantially from the control sediments based on the ARG profile. ARG results were normalized to relative abundances with 16S rRNA gene copy numbers. Bray-Curtis dissimilarity index was used in constructing the MDS plot. Ellipses were drawn with 95% confidence.
4.3 Metagenomes

Sequence similarity networks (SSN) were built from 97 sampled environments representing 339 metagenomes. The networks showed that ecology is a key factor in determining the clustering of metagenomes. Ecology seems to strongly affect the metagenome gene composition. The network analysis revealed that inland waters can connect otherwise geographically separate microbial communities (IV). This is in agreement with speculations about the role of aquatic environments in spreading antibiotic resistance determinants (Baquero et al, 2008). Our results also support that the Baas-Becking hypothesis, everything is everywhere, but the environment selects, applies to genes in addition to microbes for which it was originally formulated (IV).

4.3.1 Horizontal gene transfer of antibiotic resistance genes in the environment

Antibiotic resistance genes transfer efficiently in bacterial communities and can cross broad taxonomical boarders. To understand better the transfer of resistance genes and forces affecting their dispersal, putative horizontal gene transfer events including antibiotic resistance genes were studied with network analysis using publicly available metagenomes. The resistance genes were annotated against the Antibiotic Resistance Gene Database (Liu & Pop, 2009) and putative horizontal gene transfer events were detected by identifying blocks of nearly identical of DNA in otherwise distantly related sequences (IV). Networks were constructed and analyzed as described in IV. Antibiotic resistance genes were exceptions compared to other genes that formed ecologically homogenous clusters (IV). Tetracycline gene (tet34) was shared between inland water and host related metagenomes and made tight clusters in the network showing putative horizontal transfers between these two habitats (Figure 5). The same phenomenon was observed with chloramphenicol resistance gene found from soil and host related metagenomes. These two examples show that antibiotic resistance genes can overcome geographical and taxonomical barriers and spread between distinct environments and phylogenetically unrelated bacteria, possibly through few or several HGT events (Halary et al, 2010; Smillie et al, 2011). This might be one of the reasons for the remarkable dissemination of antibiotic resistance genes in different environments.

Figure 5. Examples of putative cross-habitat HGT events among nodes embedding A) tetracycline resistance determinants and retrieved from inland waters (blue nodes) and host (red nodes) and B) chloramphenicol resistance in host (red) and soil (yellow) derived samples.
5. CONCLUSIONS AND FUTURE PROSPECTS

In this work antibiotic resistance gene abundance, persistence and faith in the environment was studied using molecular methods and meta-analysis of metagenomes. Using two example environments I show that antibiotic resistance genes are abundant in environments with anthropogenic impact and that the genes can persist in the environment without a clear selection pressure. On the other hand UWTPs turned out to be effective in removing antibiotic resistance pollution from wastewaters. The impact of wastewater release on the sediments near the discharge sites is limited and aquacultures impact the antibiotic resistance in the sediments only locally. Using metagenomic data available in the public databases I show the different dispersal patterns of antibiotic resistance genes in the environment, possibly explaining the huge dispersal of ARGs in bacterial communities in both the environment and the clinic.

I showed that high-throughput qPCR assay is a useful tool to describe and study the antibiotic resistance profile in UWTP samples and to compare the fate of many genes at the same time. The use of only few genes gives only a limited view of the antibiotic resistance dynamics, especially in environments with high ARG diversity. High-throughput qPCR assays can be powerful in describing the resistome and to identify key marker genes that should be used for monitoring anthropogenic pollution and the emergence of antibiotic resistance in different environments. However, even with a high-throughput qPCR array with hundreds of genes, the need for prior knowledge limits its use to known genes.

Meta-analysis of metagenomic sequence data was proven to be a useful way of studying ecological questions in larger, even global scale. Metagenomic sequence data from diverse environments is available to all researchers for free and combined with cloud computing, does not need big investments. The little amount of metadata and the diverse annotation schemes used by different projects and databases makes the full scale utilization of the data still a challenge, although these issues are addressed.

In this work I have touched the surface of the antibiotic resistance problem but still many questions remain. Although the UWTPs remove the resistance genes effectively, there is a huge variety of microbes, resistance genes and mobile genetic elements in the sewage. The questions remaining include; are the genes transferred in the sewage between bacteria, what are the main mechanisms affecting the rate of transfer, which bacteria are carrying the resistance genes and what are the risks of resistance gene release to the environment for human health? In the forthcoming studies tools, such as high-throughput qPCR arrays and metagenomics, could be used in unraveling these questions.

Antibiotic resistance genes are considered an environmental pollution and their dissemination should be monitored. The methods I have used and the results presented in this thesis can be used for monitoring the reservoirs of antibiotic resistance and to predict the emergence of new resistance determinants.

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7. REFERENCES


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