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Effects of biocides and metals on antibiotic resistance | Chandan Pal

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Effects of biocides and metals on antibiotic resistance

A genomic and metagenomic perspective

Chandan Pal

SAHLGRENSKA ACADEMY
INSTITUTE OF BIOMEDICINE

UNIVERSITY OF
GOTHENBURG

Effects of biocides and metals on antibiotic resistance: a genomic and metagenomic perspective

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ABSTRACT

Background and aim: There is a concern that biocides and metals can co-select for antibiotic resistance. The aim of this thesis is to enhance our understanding of the roles of antibacterial biocides (e.g. antiseptics, disinfectants) and metals (e.g. copper, zinc) in developing, promoting and maintaining antibiotic resistance in bacteria.

Methods: In **paper I**, published studies on resistance genes to antibacterial biocides and metals were compiled and used as the basis to develop a database (BacMet) of such genes. In **paper II**, 2522 completely sequenced bacterial genomes and 4582 plasmids were studied for resistance genes and their co-occurrences. In **paper III**, 864 metagenomes from human, animal and external environments were studied for resistance genes, taxonomic compositions and mobile genetic elements. In **paper IV**, marine microbial biofilms growing on surfaces painted with copper- and zinc-based anti-fouling paint were studied using phenotypic assay (i.e. culturing of bacteria on agar containing antimicrobials) and metagenomic sequencing.

Results and discussion: The BacMet database (**paper I**) was used to characterise biocide/metal resistance genes in genomes, plasmids and metagenomes in **papers II-IV**. In **paper II**, co-occurrences of resistance genes were studied to identify biocides and metals, such as mercury and quaternary ammonium compounds, with the potential to co-select for resistance to certain classes of antibiotics. Co-occurrences of resistance genes to both antibiotics and biocides/metals were highly prevalent in bacterial isolates from human and domestic animal sources, and in genera comprising many pathogens. In general, plasmids were predicted to provide limited opportunities for biocides and metals to promote horizontal transfer of antibiotic resistance through co-selection, whereas ample possibilities existed for indirect selection via chromosomal biocide and metal resistance genes. In **paper III**, air and environments subjected to pollution from pharmaceutical manufacturing were identified as under-investigated transmission routes for antibiotic resistance genes. The high genetic and taxonomic diversity of external environments suggests that they could serve as sources of unknown resistance genes with the potential to be transferred to pathogens in the future. In **paper IV**, it was found that antifouling paints enriches RND efflux systems conferring cross-resistance, as well as integron-associated integrases and ISCR transposases but not known mobile antibiotic resistance genes, thus providing limited support for selection of mobile antibiotic resistance.

Conclusions: Overall, this thesis provides tools to study co-selection of antibiotic resistance, and enhances our knowledge of risk scenarios and the underlying genetic basis. It identifies compounds, environments and taxa with identified opportunities for co-selection, thereby also provides a basis for risk-reducing actions. It also identifies point sources and reservoirs for resistance genes with a possibility to be transferred to human pathogens. Finally, the work in this thesis also highlights that copper and zinc-based antifouling paint has the potential to co-select for antibiotic resistance via cross-resistance mechanisms.

Keywords: Antibiotic resistance, biocide, metal, co-selection, metagenomics

SAMMANFATTNING PÅ SVENSKA

Bakgrund och syfte: Det finns en oro för att biocider och metaller kan co-selektera för antibiotikaresistens. Syftet med denna avhandling är att öka förståelsen för hur antibakteriella biocider (t ex antiseptika, desinfektionsmedel) och metaller (t ex koppar, zink) påverkar utveckling, främjande och upprätthållande av antibiotikaresistens hos bakterier.

Metoder: I delarbete I gjordes en sammanställning av publicerade studier rörande resistensgener mot biocider och metaller, vilket användes som grund för att utveckla en databas (BacMet) innehållande sådana gener. I delarbete II, karaktäriserades 2522 fullständigt sekvenserade bakteriella genom och 4582 plasmider med avseende på resistensgener och deras samförekomst. I delarbete III, karaktäriserades 864 metagenom från människa, djur och externa miljöer med avseende på resistensgener, taxonomisk sammansättning och mobila genetiska element. I delarbete IV studerades marina mikrobiella biofilmer som växt på ytor målade med koppar- och zink-baserad båtbottenfärg genom odling på agarplattor innehållande metaller och antibiotika samt sekvensering av deras metagenom.

Resultat och diskussion: BacMet-databasen användes för att karaktärisera biocid- och metallresistensgener i genom, plasmider och metagenom i delarbete II-IV. I delarbete II studerades samförekomst av resistensgener för att identifiera biocider och metaller med potential att co-selektera för resistens mot vissa typer av antibiotika, till exempel kvicksilver och kvartära ammoniumföreningar. Samförekomsten av resistensgener mot både antibiotika och biocider/metaller var mycket utbredd bland bakterier isolerade från människor och djur samt i bakteriella släkten som omfattar många patogener. Plasmider verkar i allmänhet ge begränsade möjligheter för biocider och metaller att främja horisontell överföring av antibiotikaresistens genom co-selektion, medan kromosomala biocid- och metallresistensgener har stor potential att indirekt selektera för antibiotikaresistens. I delarbete III identifierades luft och miljöer som utsätts för läkemedelsföroreningar som möjliga spridningsvägar för antibiotikaresistensgener, och dessa bör undersökas vidare. Den genetiska mångfalden i yttre miljöer antyder att de skulle kunna fungera som källa för hittills okända resistensgener med potential att överföras till patogener i framtiden. I delarbete IV konstaterades det att båtbottenfärger anrikar för RND efflux-system som genom korsresistens även kan skydda bakterier mot antibiotika. Integraser och ISCR-transposaser anrikades också, men däremot inte kända mobila antibiotikaresistensgener, vilket ger begränsat stöd för selektion av mobil antibiotikaresistens.

Slutsatser: Denna avhandling tillhandahåller verktyg för att studera co-selektion av antibiotikaresistens genom metaller och biocider, och ökar vår kunskap om riskscenarier och bakomliggande genetiska orsaker. Den identifierar substanser, miljöer och taxa med möjligheter för co-selektion och utgör därmed en kunskapsgrund för riskreducerande åtgärder. Avhandlingen identifierar punktkällor och reservoarer för resistensgener med möjlighet att överföras till humanpatogener. Slutligen visar avhandlingen att koppar- och zink-baserad båtbottenfärg har potential att co-selektera för antibiotikaresistens via korsresistensmekanismer.

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This thesis will be publicly examined on 31 January 2017 at 09:00
for the degree of Doctor of Philosophy (PhD)

LIST OF PAPERS

This thesis is based on the following articles and manuscripts.

Paper I. Chandan Pal, Johan Bengtsson-Palme, Christopher Rensing, Erik Kristiansson, DG Joakim Larsson. (2014) **BacMet: antibacterial biocide and metal resistance genes database.** *Nucleic Acids Research*; 42:D737-43. DOI: 10.1093/nar/gkt1252

Paper II. Chandan Pal, Johan Bengtsson-Palme, Erik Kristiansson, DG Joakim Larsson. (2015) **Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential.** *BMC Genomics*; 16:864. DOI: 10.1186/s12864-015-2153-5

Paper III. Chandan Pal, Johan Bengtsson-Palme, Erik Kristiansson E, DG Joakim Larsson. (2016) **The structure and diversity of human, animal and environmental resistomes.** *Microbiome*; 4:54. DOI: 10.1186/s40168-016-0199-5

Paper IV. Carl-Fredrik Flach, Chandan Pal, Carl-Johan Svensson, Erik Kristiansson, Marcus Östman, Johan Bengtsson-Palme, Mats Tysklind, DG Joakim Larsson. (2016) **Does anti-fouling boat paint select for antibiotic resistance?** *Submitted*

For a complete list of papers of the author please visit
http://chandanpal.weebly.com/publications

Published and submitted work not included in this thesis

1. Pal C, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson DGJ, Hobman JL. (2017) **Metal resistance and its association with antibiotic resistance**. *Advances in Microbial Physiology*. Volume 70; ISBN: 9780128123867. Accepted for publication.

2. Bengtsson-Palme J, Hammarén R, Pal C, Östman M, Björlenius B, Flach C-F, Fick J, Kristiansson E, Tysklind M, Larsson DGJ. (2016) **Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics**. *Science of the Total Environment*, 572:697-712. DOI:10.1016/j.scitotenv.2016.06.228

3. Hammarén R, Pal C, Bengtsson-Palme J. (2016) **FARAO – The Flexible All-Round Annotation Organizer**. *Bioinformatics,* 32 (23):3664-6. DOI: 10.1093/bioinformatics/btw499

4. Bengtsson-Palme J, Boulund F, Edström R, Feizi A, Johnning A, Jonsson VA, Karlsson FH, Pal C, Pereira MB, Rehammar A, Sanchez J, Sanli K, Thorell K. (2016) **Strategies to improve usability and preserve accuracy in biological sequence databases.** *Proteomics*, 16(18):2454-60. DOI: 10.1002/pmic.201600034. *(Co-authors listed alphabetically)*

5. Bengtsson-Palme J, Hartmann M, Eriksson KM, Pal C, Thorell K, Larsson DGJ, Nilsson RH. (2015) **Metaxa2: Improved Identification and Taxonomic Classification of Small and Large Subunit rRNA in Metagenomic Data**. *Molecular Ecology Resources,* 15(6):1403-14. DOI: 10.1111/1755-0998.12399.

6. Hao X*, Li X*, Pal C*, Hobman JL, Rosen BP, Larsson DGJ, Zhu YG, Rensing C. (2016) **Widespread resistance to arsenic in bacteria is influenced by its use in Protists to kill bacterial prey**. *Submitted*. (*- *co-first authors*)

CONTRIBUTIONS TO PAPERS

I have contributed to the following parts of each paper included in this thesis:

Paper I — Conceived the idea and designed the study, performed the literature review and collected all the sequence data and metadata for resistance genes for the database, curated the database contents, designed database schema, implemented the database, designed and implemented the database website, and wrote the draft version of the manuscript.

Paper II — Conceived the idea and designed the study, collected and organised all datasets and metadata used in the study, performed bioinformatic and statistical data analysis, contributed to the interpretation of the results, and wrote the draft version of the manuscript.

Paper III — Designed the study, collected and organised all datasets and metadata used in the study, performed the bioinformatic and statistical data analysis, contributed to the interpretation of the results, and wrote the draft version of the manuscript.

Paper IV — Assisted in sample collection and culturing of bacteria from marine biofilms in the lab, performed the bioinformatic and statistical data analysis, contributed to the interpretation of the results and writing of the draft version of the manuscript.

ABBREVIATIONS

AMR	Antimicrobial Resistance
ARDB	Antibiotic Resistance Genes Database
ARGs	Antibiotic Resistance Genes
BLDB	Beta-Lactamase DataBase
BMRGs	Biocide and Metal Resistance Genes
BRGs	Biocide Resistance Genes
CARD	Comprehensive Antibiotic Resistance Database
CVMP	Committee for Medicinal Products for Veterinary Use
EMA	European Medicines Agency
EIONET	European Environment Information and Observation Network
ESBL	Extended Spectrum Beta-Lactamase
FDA	Food and Drug Administration
FDR	False Discovery Rate
HGT	Horizontal Gene Transfer
HMM	hidden Markov model
HMP	Human Microbiome Project
ISCRs	Insertion Sequence Common Regions
KPC	*Klebsiella pneumoniae* Carbapenemase
MIC	Minimum Inhibitory Concentration
MRGs	Metal Resistance Genes
MGEs	Mobile Genetic Elements
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NDM	New Delhi metallo-beta-lactamase
NGS	Next Generation Sequencing
PAN	Pesticide Action Network
PSK	Post-segregational killing
PCR	Polymerase Chain Reaction
QACs	Quaternary Ammonium Compounds
RND	Resistance/nodulation/division
rRNA	Ribosomal Ribonucleic Acid
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SSU	Small sub-units
TBT	Tributyltin
WGS	Whole Genome Sequencing
WWTPs	Waste Water Treatment Plants

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Bacteria and Antibiotics

Bacteria – the first life on earth

Bacteria were one of the first life forms to appear on earth (Boundless, 2016). They can survive in almost any environment, ranging from extremes such as hot springs, hydrothermal vents in the deepest ocean, or on the Antarctic ice cap, all the way to our kitchen table. There are tens of thousands of different types of bacteria that live in or on the human body. It is estimated that in the human intestine, there are ten times more bacterial cells than human cells in our bodies (Willey et al., 2011). Most of these bacteria are harmless or even beneficial, helping in digesting food or performing other metabolic activities, but there are also a few potentially harmful bacteria (i.e. pathogens) that can cause infections given suitable conditions. When we acquire an infection from such harmful bacteria, antibiotics are often critically important for treating it.

Pre-antibiotic era

In the pre-antibiotic era, there were few options for treating bacterial infections. For example, honey was used as topical treatment of infected wounds, some medicinal plants and beneficial plant-sap such as Yarrow tea were used to treat upper respiratory infections (Tesch, 2003), leaves and flowers of Snapdragon were used to treat burns, infections and haemorrhoids (Al-Qura'n, 2003), and Celery seeds were used to treat arthritis and urinary tract infections (Butters et al., 2003).

Metals and different inorganic chemicals were also used, for example: potassium iodide, arsenic-, magnesium-, tellurium- and mercury-oxides were used to treat syphilis and leprosy (Frazer and Edin, 1930); gold-based compounds, such as sanocrysin and krysolgan, were used to treat tuberculosis (Kayne, 1935; Shaw, 1999); silver sutures were used to repair vaginal tears after childbirth (Sewell, 1993); silver nitrate were given to prevent gonorrhoeal eye infections in new-borns (Forbes and Forbes, 1971), and so on.

Antibiotics – wonder drugs

In 1923, the discovery of antibiotics and its clinical implementation in the 1940s (Fleming, 1929; Chain et al., 1940) revolutionised the history of medicine. It has been estimated that antibiotics extended the average human life for about ten years (McDermott and Rogers, 1982). For the last seven decades, antibiotics have probably been saving hundreds of millions of lives, and still, save many lives every day.

Antibiotics are specifically acting drugs that work against bacteria and sometimes certain parasites but not on viruses or fungi. Most importantly, they are selected or designed to have as little effect as possible on the human body. When we use antibiotics they bind to targets within or on bacterial cells, and inhibit their growth or kill them, ideally without harming human cells. Clinically used antibiotics are most often derived from naturally occurring compounds synthesised by bacteria or fungi (e.g. penicillin), or they can be strictly synthetic (e.g. sulfonamides, quinolones). The latter are sometimes referred to as chemotherapeutics, but here (and in many other contexts) chemotherapeutics acting on bacteria are included within the group of antibiotics.

The antibiotics are classified based on their mechanism of action, chemical structure or spectrum of activity. Those targeting the bacterial cell wall (e.g. penicillin), the cell membrane (e.g. polymyxins) or interfering with enzymes (e.g. sulfonamides) are usually bactericidal (killing), whereas those targeting protein synthesis (e.g. tetracyclines) are usually bacteriostatic (inhibiting growth). Broad-spectrum antibiotics act against a wide range of bacteria whereas narrow-spectrum antibiotics are effective against more specific types of bacteria, such as sub-groups of Gram-positive or Gram-negative bacteria.

1.2 Antibiotic resistance

Developing completely new classes of antibiotics (as opposed to variations on existing antibiotics) is very challenging. It is easy to find chemicals that kill bacteria, but not substances that could be used as medicines. In fact, the most recent discovery of a novel antibiotic class was teixobactin in 2015 (Ling et al., 2015) which took 28 years since daptomycin was discovered in 1987 (Silver, 2011). While there are a few new antibiotics currently under development, we don't know when and if they will become usable as medicines.

1.2.1 Antibiotic resistance mechanisms

Bacteria develop resistance mechanisms to protect themselves from antibiotics (Tenover, 2006). The following resistance mechanisms are common (Figure 1): (a) **decreased uptake**: reduction of membrane permeability, which restricts access of antibiotics into the cells (e.g. resistance to tetracyclines and quinolones); (b) **enzymatic inhibition/inactivation** of the antibiotic (e.g. resistance to beta-lactams by beta-lactamases); (c) **rapid efflux** of the antibiotic out of the cell (e.g. resistance to tetracyclines and macrolides); (d) **target alterations**: mutation of the cellular structure (receptor) that the antibiotics target (e.g. resistance to oxacillin and methicillin by mutating the *mec*A gene, mutations in DNA gyrase resulting in resistance to ciprofloxacin); and (e) **acquisition of an alternative metabolic pathway** to those inhibited by antibiotics (e.g. resistance to sulfonamides).

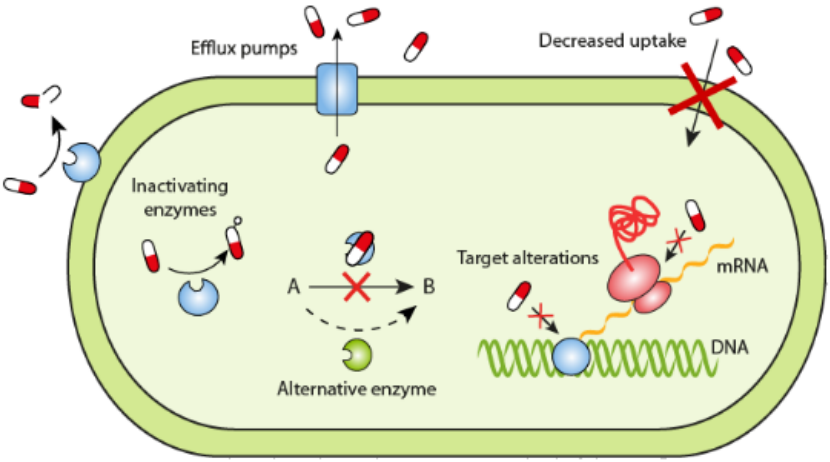

Figure 1. Antibiotic resistance mechanisms (reproduced from Gullberg et al., 2014)

1.2.2 Antibiotic resistance development

Some bacterial species are inherently resistant to certain antibiotics, typically because they have an impermeable cell wall or lack the target of the antibiotic. Resistance can also be acquired. In assessing the problem of antibiotic resistance, the emergence of resistant bacteria raises a concern of to what extent they can also spread. The first step in the emergence of resistance involves genetic changes in the bacteria. There are two ways this can happen. Sensitive bacteria can either become resistant through mutations of pre-existing DNA, or through the uptake of entire genes, for example on plasmids, or a combination of both. In principle, resistance only needs to emerge once in a bacterial species, and then the acquired resistance can be disseminated across bacterial populations and geographical borders over time. For example, NDM-1 beta-lactamases were discovered in New Delhi in India in 2008, and are now found across continents (Johnson and Woodford, 2013; Walsh et al., 2011; Kumarasamy et al., 2010).

It is evident that selection pressure from antibiotics plays a major role in both emergence and transmission of resistance (Hastings et al., 2004; Roberts and Mullany, 2009). Over the years the massive use and misuse of antibiotics and the resulting selection pressure has led to a toxic shock to bacterial communities, thus favouring those bacteria that were able to mutate towards resistance and those acquiring different resistance genes.

Human and animal use of antibiotics

Antibiotics were initially developed for the treatment of infectious diseases in humans. Later, the uses of antibiotics extended to animals and plants. A good correlation has been observed between human antibiotic use and the frequency of resistance development in clinical isolates (Goossens et al., 2005). Thus, without any reasonable doubt, human use of

antibiotics and the selection pressure it provides directly to the bacterial communities residing in and on our bodies is an important (probably the most important) driver behind the resistance development. The use of antibiotics is, however, not limited to the clinics, but antibiotics are also used widely in veterinary medicine and on an international level as growth promoters for meat production (Laxminarayan et al., 2013). While antibiotics are not allowed as growth promoters in the EU (European Commission, 2005), the US Food and Drug Administration (FDA) estimated that almost 80 percent of total antibiotics used in the US are fed to farm animals (FDA, 2010). Eighty-five percent of those antibiotics used in farm animals are for non-therapeutic feed addition (Sapkota et al., 2007). A strong correlation has been observed between the use of antibiotics in animals and the corresponding resistance in *Escherichia coli* isolates from animals, similar to the correlation found for human use (Chantziaras et al., 2014). There are serious concerns that resistance genes and resistant pathogens developed in animals will subsequently spread to humans (Bager et al., 1997). This can be possible by direct contact with animals or indirectly via the food chain, water, air, and manured and sludge-fertilized soils.

Introduction of antimicrobial agents into clinical medicine has increased antibiotic resistance

On average it has taken about 8 years since the introduction of a new class of antibiotics until resistance has evolved in one or several of the targeted pathogens (Schmieder and Edwards, 2012). There are numerous studies that support that the introduction of antibiotics in modern era has increased antibiotic resistance in bacterial isolates. For example, Shoemaker et al. (2001) assessed phenotypic resistance and presence of certain set of resistance genes in *Bacteroides* species from human colon samples collected over three decades. They showed an increase in bacterial strains carrying the gene *tet*Q (confers resistance to tetracycline) from about 30% to more than 80%, as well as an increase in bacterial strains carrying the genes *erm*F and *erm*G (both confers resistance to erythromycin) from <2% to 23% during the same period. Similarly, Tadesse et al. (2012) conducted a retrospective study of *Escherichia coli* isolates recovered from human and food animal samples during 1950–2002 to assess historical changes in antimicrobial drug resistance. They showed an increase in the proportion of resistant isolates in each decade since the introduction of new antimicrobial agents into clinical medicine (Figure 2). In another study, Houndt and Ochman (2000) assessed the antibiotic resistance in *Salmonella enterica* and *Escherichia coli* strains isolated from humans, as well as domesticated and wild animals over a century (between 1885 and 1987). They found all strains from pre-antibiotic era were susceptible to the selected set of antibiotics, whereas 20% of strains from antibiotic era showed high-level resistance to at least one antibiotic.

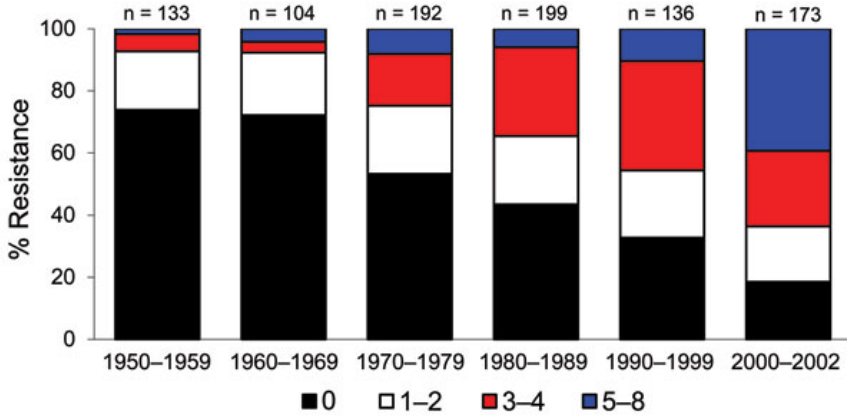

Figure 2. Antimicrobial drug resistance in Escherichia coli from humans and food animals in the United States between 1950 to 2002. Colour caption represents numbers of drug classes which isolates were resistant to (reproduced from Tadesse et al., 2012).

The role of external environments in the antibiotic resistance problem

The external environment plays important roles in the development of antibiotic resistance (Wellington et al., 2013; Larsson, 2014a; Berendonk et al., 2015). One is that it serves as a transmission route for pathogens, including both resistant and non-resistant bacteria. This is particularly important in many low-income countries where sufficient water and sewage infrastructure are lacking and there is contamination of water resources with faecal bacteria from animals and humans. Such lack of infrastructure is likely to boost resistance development severely in these regions (Graham et al., 2014).

The other role of the environment is as a source for (novel) resistance determinants (Wellington et al., 2013; Bengtsson-Palme and Larsson, 2015). Antibiotic resistance genes, although less common in areas with low human impact, are widely distributed in different environments (**paper III**). Interestingly, antibiotic resistance genes existed long before humans started to produce antibiotics. Resistance genes can be found in 30,000 years old permafrost (D'Costa et al., 2011) and in glaciers (Segawa et al., 2013) that have not been affected by human activities. This is perhaps not surprising since most of the antibiotics used are derived from naturally-produced compounds. Noteworthy, it is only during the last 75 years that pathogens have started to accumulate resistance genes on a larger scale and clear signs of exchange of antibiotic resistance genes have been observed between environmental bacteria and clinical pathogens (Forsberg et al., 2012). Thus, harmless bacteria, in and around us, likely constitute an important reservoir for antibiotic resistance determinants that under the right conditions could be horizontally transferred to pathogenic bacteria. However, in what environments and under what conditions such transfer events occur is an under-investigated area. It is however believed that a selection

pressure promoting resistant strains could facilitate this process (Perry and Wright, 2014). Indeed, much of the used antibiotics passes through the body in a chemically unaltered form, and therefore also ends up in the environment, potentially providing a selection pressure on the microbial communities present there as well. Antibiotics may also reach the environment directly through discharges from pharmaceutical manufacturing sites (Larsson et al., 2007; Larsson, 2014b).

To identify and assess complete risks associated with antibiotic resistance in the environment and its contribution to risk scenarios to human health, the role of external environments as a point source and dissemination route for resistant bacteria cannot be ignored (Ashbolt et al., 2013). Quantitative data such as abundance and diversity of resistance genes is crucial to assess such risk (Berendonk et al., 2015) but is largely lacking for most external environments. Usually, high abundances of resistance genes in a particular environment reflect either direct or indirect selection for resistance genes in that environment (**paper III**), or a possible contamination with antibiotic-resistant bacteria from other sources, and hence risks for their transmission (**paper III**; Zhu et al., 2013). In addition, it has been suggested that environments with a high diversity of resistance genes that are rarely present in the human microbiome are potential sources and possible transmission routes for resistance genes to be transferred human pathogens (Bengtsson-Palme and Larsson, 2015). Taken together, such environments with both high abundance and diversity of resistance genes can act as potential 'hotspots' for resistance development, maintenance and/or transmission. In **paper III** of this thesis, we identified such 'hotspots' by characterising not only resistance genes but also mobile genetic elements and taxonomic compositions in 864 metagenomes with high sequence depths (over 10 million sequences per sample), all generated on Illumina sequencing platforms, from 13 different types of environments.

Recently, using culture-independent techniques, Durso et al. (2012) characterised thirty-two agricultural, human-associated and environmental metagenomes and found ARGs in the human gut and agricultural metagenomes more often compared to marine and Antarctic samples. More recently, Nesme et al. (2014) and Fitzpatrick & Walsh (2015) have estimated the relative abundance of ARGs in different environments, including humans and animals, using metagenomic datasets with highly variable sequencing depths generated on 454, Sanger or Illumina sequencing platforms. However, in these three studies above, small sample sizes, low sequencing depths, non-stringent criteria for detection of resistance genes and comparisons of datasets generated on different sequencing platforms made it difficult to generalise results. In **paper III** of this thesis, we aimed to fill those technical gaps, and obtain broad and general view of the resistome (i.e. reservoirs of resistance genes) by characterising a broad types of external environments, humans and animals for abundance and diversity of resistance genes to antibiotics and other co-selective agents such as biocides and metals, as well as mobile genetic elements and bacterial taxa.

1.3 Biocides and metals can co-select for antibiotic resistance

Use of a member of an antibiotic class can co-select for resistance to another class of antibiotics with a totally distinct mode of action (Andersson and Hughes, 2010). In addition to antibiotics, metals and antibacterial biocides may provide such selection pressure and co-select for antibiotic resistance (SCENIHR, 2009). Co-selection is considered as one of the reasons that it is difficult to reverse antimicrobial resistance once it has been established in a bacterial population. Stopping the use of one particular antibiotic does not reverse resistance if the gene(s) encoding the resistance mechanism to that antibiotic are physically linked to resistance genes against other antibiotics (Enne et al., 2004). Co-selection of antibiotic and metal resistance in bacteria has been widely observed, which is mainly caused by cross- or co-resistance mechanisms (see section 1.3.1 below).

Biocide resistance was first recognised nearly 80 years ago by Heathman et al. (1936), who identified chlorine resistance in *Salmonella typhi*. Metal resistance in bacteria was detected relatively late (in 1960; Moore, 1960) when mercury-resistant *Staphylococcus aureus* was isolated from wounds, but retrospective studies demonstrated the presence of metal resistances (particularly mercury, copper and arsenic) likely long before humans walked the earth. Several decades ago, plasmid capturing and sequencing demonstrated that resistance genes to metal and antibiotics were linked especially on plasmids, because of antibiotic-metal resistance co-selection (Nakahara et al., 1977). In that study, out of 787 *Pseudomonas aeruginosa* isolates, 99.8% were metal resistant, with most (99.5%) showing resistances to more than one compound when tested against a range of metals, such as arsenic, cadmium, lead and mercury (Nakahara et al., 1977). Mutation-based cross-resistance to antibiotic compounds was first reported by Zybalski and Bryson in the 1950s (Zybalski and Bryson, 1952), where cross-resistance to antibiotics by toxic agents was studied (but the mechanism was not elucidated). In the 1970's, indications of heavy metals indirectly selecting for antibiotic resistance by co-selection was also highlighted (Koditschek and Guyre, 1974). Today, the widespread use of biocides and metals in different areas has caused increased concern about the potential for indirect co-selection of resistance genes against antibiotics (for example in a dedicated report from the Scientific Committee on Emerging and Newly Identified Health Risks of European Union) (SCENIHR, 2009; 2010).

1.3.1 Principles of co-selection mechanisms

Co-selection can occur via one of the following mechanisms (Figure 3): **(a) co-resistance**, when resistance genes that confer resistance to different antibiotics, biocides or metal compounds are physically located on the same genetic element such as plasmid or in the same cell (e.g. *mer*A and KPC beta-lactamase; Baker-Austin et al., 2006, **paper II**); **(b) cross-resistance**, when a single resistance gene or mechanism (e.g. efflux pump, overexpression of a gene) confers resistance to multiple antibiotics, biocides and/or metals

(e.g. *mdr*L confers resistance to metals, such as zinc, cobalt and chromium, biocides, such as quaternary ammonium compounds and ethidium bromides, and antibiotics, such as erythromycin, josamycin and clindamycin; (Chapman, 2003); and **(c) co-regulatory resistance**, when multiple resistance genes that confer resistance to different toxic compounds, such as antibiotics, biocides and metals, are controlled by a single regulatory gene (e.g. *czc*R regulates the expression of the CzcCBA efflux pump, which induces resistance to zinc, cadmium and cobalt. The *czc*R gene also co-regulates resistance to antibiotics such as carbapenems by repressing the expression of the OprD porin, thus blocking the antibiotics to enter the bacterial cell via porins (Perron et al., 2004).

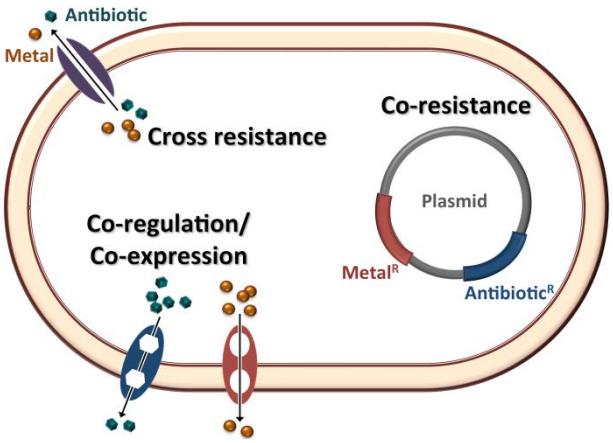

Figure 3. Principles of co-selection mechanisms (reproduced from Pal et al., 2017)

1.3.2 Studies reporting potential for co-selection of resistance between biocides/metals and antibiotics via co- and cross-resistance

There are numerous studies where biocides or metals have been reported to promote antibiotic resistance via co-resistance (i.e. co-occurrence of resistance genes) (Table 1). For examples, resistance gene to copper (*tcr*B) linked to resistance genes to erythromycin, tetracycline and vancomycin have been found (Amachawadi et al., 2011; Hasman and Aarestrup, 2002); The genes *oqx*AB (encode a efflux pump), conferring resistance to triclosan, chlorhexidine, acriflavine, QACs and fluoroquinolones, were shown to be co-located with beta-lactamases ($bla_{\text{CTX-M}}$), the copper (*pco)* and silver (*sil)* operons on the same plasmid (Fang et al., 2016), thus showed a potential for co-selection; and so on. Sandegren et al. (2012) described a multi-drug resistance plasmid isolated from a nosocomial outbreak from a Swedish hospital in Uppsala, shown to carry genes conferring resistance to a range of antibiotics (beta-lactams, aminoglycosides, tetracyclines, trimethoprims and sulfonamides), biocides (QACs) and metals, including copper, silver and arsenic (see Figure 4). More recently, in addition to bacterial isolates from animal sources, the plasmid-

mediated colistin resistance gene *mcr*-1 was also found together with resistance genes to a range of metals and antibiotics in clinical isolates (Campos et al., 2016). There are many more such examples of such multi-resistance plasmids that carry resistance genes to antibiotics, biocides and metals, isolated from various sources. Despite this, there is no comprehensive picture of which biocides and metals that are most likely to co-select for resistance towards specific classes of antibiotics. Research aimed to identify such general patterns might help us in better understanding and managing the threat of metals and biocides in antibiotic resistance development. Possibly, the increased knowledge on the detailed structure of bacterial genomes and the different mechanisms involved in co-selection could facilitate this type of research. In **paper II** of this thesis, resistance genes to antibiotics, biocides/metals and their co-occurrences, thus potential for co-selection, were characterised in 2522 completely sequenced bacterial genomes and 4582 plasmids.

Table 1. Selected studies showing co-occurrence of resistance genes to biocide/metal and antibiotics (co-resistance potential) (Adapted from Pal et al., 2017)

Biocide/Metal	**Antibiotic**	**Reference**
Copper	Erythromycin and tetracycline	Amachawadi et al., 2011
Copper	Erythromycin and vancomycin	Hasman and Aarestrup, 2002
Arsenic, copper, mercury, silver and tellurium	Chloramphenicol, kanamycin and tetracycline	Gilmour et al. 2004
Cadmium and zinc	Methicillin	Cavaco et al., 2011
Cadmium, cobalt, copper, lead, mercury, nickel, tellurium, zinc and QACs	Chloramphenicol, kanamycin, streptomycin, beta-lactam, sulfonamide and erythromycin	Zhai et al., 2016
Arsenic, copper, silver and QACs	Beta-lactam, macrolide, sulfonamide, tetracycline and trimethoprim	Sandegren et al., 2012
Copper and silver	Beta-lactam and fluoroquinolone	Fang et al., 2016
Copper, mercury and silver	Colistin, ampicillin, sulfonamide, tetracycline, streptomycin and chloramphenicol	Campos et al., 2016
Acriflavine, benzalkonium chloride, chlorhexidine and ethidium bromide	Kanamycin, gentamicin, tobramycin and amikacin	Yamamoto et al., 1988

Besides the co-selection via co-occurrences of resistance genes, cross-resistance mechanism also plays a major in co-selection. There are numerous studies that have demonstrated this (Table 2). For instance, Conroy et al. (2010) showed that some efflux pumps such as GesABC can act as a drug efflux system together with its first described role in gold efflux; triclosan was reported to have the potential co-select for ciprofloxacin resistance via overexpression of a multidrug efflux system (MexCD-OprJ) in *Pseudomonas aeruginosa* (Chuanchuen et al., 2001); chlorhexidine was reported to have the potential to co-select for erythromycin and novobiocin resistance via chromosomal efflux pump AbeS (Srinivasan et al., 2009); efflux systems encoded by the *tet*A(L) gene can confer cross-resistance to tetracycline and cobalt (Cheng et al., 1996); Webber et al. (2015) showed that exposure to biocides can cause mutations in *rpo*B genes, resulting in cross-resistance to ciprofloxacin in *Salmonella enterica* isolates; Mata et al. (2000) reported a multidrug efflux pump that encoded by the *mdr*L gene that can confer cross-resistance to zinc, cobalt, cadmium and benzalkonium chloride, as well as antibiotics such as erythromycin and clindamycin in *Listeria monocytogenes*. In **paper IV** of this thesis, cross-resistance mechanisms based on efflux systems were investigated under the stress of copper and zinc-based antifouling paint in marine settings.

Table 2. Selected studies showing a shared mechanism of resistance (e.g. efflux) to both antibiotics and biocide/metals (i.e. cross-resistance potential)

Biocide/Metal	Antibiotic	Reference
Cobalt, chromium, zinc and benzalkonium chloride	Erythromycin, josamycin and clindamycin	Mata et al., 2000
Cobalt	Tetracycline	Cheng et al., 1996
Gold, acriflavine and alexidine	Chloramphenicol, cloxacillin, thiamphenicol and nafcillin	Conroy et al., 2010
Acriflavine, benzalkonium chloride, cetrimide	Ciprofloxacin and moxifloxacin	Huet et al., 2008
Benzalkonium chloride, chlorhexidine, ethidium bromide and acriflavine	Ciprofloxacin, norfloxacin, ofloxacin and fradiomycin	He et al., 2004
Acriflavine	Ciprofloxacin and norfloxacin	Su et al., 2005
Triclosan, benzalkonium chloride, cetrimide	Chloramphenicol, ciprofloxacin, norfloxacin and trimethoprim	Hansen at al., 2007
Triclosan	Isoniazid	Slayden et al., 2000
Triclosan	Ciprofloxacin	Chuanchuen et al., 2001

1.3.3 Conclusive evidence for a role of biocides and metals in the overall antibiotic resistance problem is lacking

There are numerous reports on genetic co-occurrences of resistance genes to both biocides/metals and antibiotics (thus indicating a potential for co-selection) as well as studies demonstrating the presence of cross-resistance mechanisms. Some studies also show that that exposure to biocides/metals enrich the expression of efflux systems or select for genes that indirectly confer resistance to both types of compounds (Gilbert and McBain, 2003; Webber et al., 2015). However, evidence that exposure to biocides/metals consistently selects for antibiotic-resistant bacteria in real life situations, and contributes to the overall resistance problem in healthcare is still largely lacking.

There are numerous studies that have highlighted the associations of antibiotic and biocide/metal resistance in bacterial isolates. For example, Lloyd et al. (2016) showed that in mercury contaminated site, resistance to three or more antibiotics was more common in mercury-resistant bacterial isolates compared to mercury-sensitive isolates collected from the fish gut. Similarly, Cavaco et al. (2011) investigated the prevalence of zinc resistance in Methicillin-resistant (MRSA) and Methicilin-sensitive *Staphylococcus aureus* (MSSA) isolates and shown that MRSA isolates carried zinc resistance gene (*czr*) more frequently than MSSA isolates. In addition, there are studies that showed that bacteria exposed to biocide develop a reduced susceptibility to antibiotics, thus selecting antibiotic-resistant bacteria (Karatzas et al., 2008; McCay et al., 2010; Romanova et al., 2006, Loughlin et al., 2002; Tattawasart et al., 1999). In contrast, there are also reports suggesting that biocide-resistant bacteria are not necessarily more resistant to antibiotics than are biocide-sensitive bacteria (Lear et al., 2006; Cottell et al., 2009; Cole et al., 2003; Anderson et al., 1997; Stecchini et al., 1992). Overall, there is no clear-cut evidence that biocide/metal-resistant bacteria would be more likely to be antibiotic-resistant compared to biocide/metal-sensitive strains. In **paper II**, we aimed to determine a generalised picture of such association of resistance in completely sequenced bacterial strains, as well as on plasmids.

Some studies have assessed the relationship to determine the influence of metals (i.e. in terms of metal contents) in the proliferation of ARGs in different environmental settings. For example, positive correlations has been observed between the concentration of certain metals and ARGs in manure and soils from multiple feedlots (Ji et al., 2012), in soils both with high metal pollution (Berg et al., 2010; Knapp et al., 2011) and comparatively less metal-polluted areas (e.g. residential) (Knapp et al., 2016). Similarly, strong positive correlations have also been observed between the presence of a selected set of biocide/metal resistance genes and ARGs in soils and faeces from dairy farms (Zhou et al., 2016; Zhu et al., 2013). In another study, a positive correlation between the concentration of copper and zinc and the level of antibiotic-resistant *Escherichia coli* isolates was observed in pig manure samples (Hölzel et al., 2012), where the phenotypic resistance of isolates followed the concentration of copper. In the majority of the above studies, the scope was to

determine the association between the levels of metal contents and antibiotic resistance genes via correlation analyses. It important to bear in mind that correlation is not causation. In some of these cases the changes in antibiotic resistance could be due to a shift in taxonomic compositions, not exposure to biocides/metals, or exposure both to biocides/metals and bacteria that tend to carry resistance genes (i.e. faecal residues) (Pal et al., 2017).

In comparison to some of the above correlation studies, other studies have investigated the effect of metals in the selection of antibiotic resistance in more controlled experimental settings, in order to determine causal relationships. In a recent study of ours, we have listed and discussed a set of correlative, and controlled concentration-response studies (Pal et al., 2017). In freshwater microcosm studies, it was found that exposure of bacterial communities to individual heavy metals such as cadmium select for multidrug-resistant microorganisms (e.g. resistant to gentamicin and tetracycline), including human pathogens (Stepanauskas et al., 2006). A controlled, field-study in agricultural soils amended with copper showed an increase in copper-resistant isolates, as well as antibiotic-resistant isolates (Berg et al., 2005) compared to untreated soils. More recently, in two controlled field studies, Hu et al. (2016a; 2016b) showed that agricultural soils amended with copper or nickel for long-term (i.e. 4-5 years) can significantly change the abundance and diversity of a range of different ARGs, as well as mobile genetic elements (MGEs). Although there are many such correlations and controlled, concentration-response studies, it is not clear whether metals select for specific resistant strains within species or if the metals mainly select for species where all members are tolerant to the antibiotic in question. This is important from the perspective of antibiotic resistance because it is primarily the selection between strains within species that has significant clinical importance (Pal et al., 2017). Many studies describe an association between the exposure of bacterial communities to biocide/metal and an increase in antibiotic resistance in various environments. Still, identifying a link all the way to the clinical outcome is difficult. Chuanchuen et al. (2001) showed that exposure of a clinically significant bacterium such *Pseudomonas aeruginosa* to the antiseptic triclosan efficiently can select for resistance to ciprofloxacin via overexpression of a multidrug efflux system (MexCD-OprJ). Using modern and historical isolates of *Staphylococcus epidermidis,* a study has suggested that long-term use of biocide such as chlorhexidine can increase the presence of a certain set of resistance genes such as *qac*A/B (Skovgaard et al., 2013). However, the study showed that biocide resistance genes were often absent in historical isolates taken before introducing biocides in practice. This has also been shown for antibiotics in many studies, where it was clear that after the introduction of antibiotic into clinical practice the proportion of resistance isolates increased (Tadesse et al., 2012) compared to isolates from the pre-antibiotic era (Hughes and Datta, 1983). Metal resistance genes were, on the other hand, often present in isolates from the pre-antibiotic era (Hughes and Datta, 1983). It is not apparent whether biocides, metals or antibiotics were mainly responsible for the selection pressure that resulted in frequent co-occurrences of resistance genes to these compounds. In **paper II** of this thesis, a

discussion was further developed to hypothesise around the actual cause of co-occurrences of resistance genes to different classes of compounds.

1.4 Antibacterial biocides, metals and their characteristics

1.4.1 Antibacterial biocides are extensively used in our daily life

Chemical agents that are intentionally used to inhibit bacterial growth or kill bacteria without being used as medicines/antibiotics are usually referred to as antibacterial biocides. They have various uses, such as in healthcare (e.g. antiseptic, disinfectant), agriculture (e.g. pesticides), food industry (e.g. preservatives), commercial industry (e.g. drinking water treatment, antifouling paints) and households (e.g. toothpaste). In our daily life, we use a variety of items, such as toothpaste, soaps, cosmetics, wipes and cleaning products, where active biocidal substances can be found. Some biocides are also used for their surfactant properties, for which the primary purpose is not their antimicrobial activity. Although biocidal compounds are usually added in comparatively low quantities to the final product, currently they represent sales in the European Union alone of approximately €10-11 billions, with market growth of 4-5% per annum (PAN Europe, 2012). The actual uses of biocidal products in different countries are not regulated, and therefore an estimation of the total amounts of biocidal products used is lacking.

1.4.2 Differences between biocides and antibiotics

Though antibiotics and biocides both can kill or inhibit the growth of bacteria, biocides have a comparably broader spectrum of activity compared to antibiotics and often act through multiple target sites within a microbial cell, including the cytoplasmic membrane, proteins, DNA, RNA and other cytosolic components (Maillard, 2002). As antibiotics always are used in or on the human body, selectivity is crucial to avoid harmful side effects. Biocides, on the other hand, are most often used outside of our bodies, and we need to be less concerned about toxic effects on human cells. Biocides are generally bacteriostatic (inhibiting bacterial growth) at low concentrations but bactericidal (killing bacteria) at high concentrations. The effectiveness of a biocide mainly depends on the exposure time and the concentration used in practice (McDonnell and Russell, 1999).

1.4.3 Metals are used in medicines and regular commodities

Some pharmaceutical products may, in addition to the principal active pharmaceutical ingredient, contain metals. For example, thimerosal (mercury-containing preservative) is still used in many vaccines including some flu shots (Ball et al., 2001). Thiazide diuretics, such as Maxzide and Dyazide, contain mercury (Velázquez et al., 1995). Some antacids such as Riopan, Gaviscon, Maalox and Mylanta that are sold in USA markets contain a high percentage of aluminium (Rogers and Simon, 1999). In medical surgery, many metal-containing products are used, such as amalgams (alloy of mercury) in dental fillings

(SCENIHR, 2008; Patterson et al., 1985) and nickel in dental bridges and in prostheses to hold bones together (Spiechowicz et al., 1984; Lyell et al., 1978). Copper intrauterine devices (IUD) for contraceptive purposes continuously release copper into the body (Kulier et al., 2007).

In our daily life, we draw advantage of many products containing metals with toxic properties. For example, antiperspirants (deodorants) and many cosmetics contain aluminium substances (Flarend et al., 2001), a few hair dyes and lipsticks contain lead substances (Al-Saleh et al., 2009), and some shampoos (e.g. Selsun Blue) contain selenium which is toxic in high doses (Diskin et al., 1979). Some household garden chemicals contain lead and arsenic (Dubey and Townsend, 2004). Copper, iron, aluminium and silver are also used in household items. Copper, zinc, cadmium and arsenic are also used as growth promoters and feed additives in agriculture and aquaculture in certain regions (Castillo et al., 2008; Li et al., 2010; Lucas et al., 1961; Nachman et al., 2013).

1.4.4 Metals are widespread – high potential for co-selection

Exposure of bacterial communities to toxic levels of metals is widespread and started even before the human history began. Recent anthropogenic activities, such as extensive mining process and industrial discharges have led to increased contamination in many environments. In an EU study (European Environment Information and Observation Network for soil - EIONET-SOIL) conducted in 2011-12, soil contamination by metals and other chemical contaminants was assessed across 38 European countries (Panagos et al., 2013). The study identified 342 thousand 'identified contaminated sites' and 2.5 million 'potentially contaminated sites'. Overall, 35% of the total contamination occurred by heavy metals, and rest by other chemical compounds, including the ones that are occasionally used as an active substance in biocides. A noteworthy aspect is also that most antibiotics and organic biocides are degradable in the environment given sufficient time (Halling-Sørensen et al, 1998; Drillia et al., 2005). In contrast, heavy metals never degrade, so they may accumulate over time in multiple environments and may represent a long-term selection pressure (Berg et al., 2005; Koplin et al., 2002). Thus, in addition to widespread uses and misuses of metals, metal contamination also creates hotspots for biocide and metal resistance development and potentially contributes to the development of antibiotic resistance via co-selection (Baker-Austin 2006; Seiler and Berendonk, 2012).

Because metals were widespread in the environment long before we started to employ metals as antibacterial agents, one may argue that the additional exposure of bacterial communities to metals through human use could play a minor role. On the other hand, when used as biocidal agents, metals often come in contact with human pathogens. If they happen to select for antibiotic resistant strains in such cases, there is a more direct risk for human health. Identifying environments that have the highest level of that potential is important. A particular case of high interest is the use of biocides and metals that are used

in antifouling paints on ship hulls where the selection pressures are anticipated to be very high. Release of copper and zinc from antifouling paints may reach to toxic concentrations, not only for the organisms trying to establish themselves on the actual surfaces of the painted hulls, but extending into the surrounding environment in areas with high boat density, such as marinas, ports and harbours (Ytreberg et al., 2010). In antifouling paints, biocides (especially heavy metals) are often used at high concentrations under the hull to reduce the growth of fouling, such as barnacles, algae or molluscs, and travel fast, leading to less fuel consumption, thus saving money. The organotin compound tributyltin (TBT) was the main choice of biocidal compound to use in cost-effective antifouling paints in 1960s-70s. Soon after TBT pollution became a serious concern for marine life (Bryan et al., 1986; Loretto and Proud, 1993), bans on TBTs started in the 1980s and it was completely banned in 2008 by the International Convention on the Control of Harmful Anti-fouling Systems on Ships of the International Maritime Organization (IMO, 2002). Since the ban of TBT, copper has replaced the use of TBT as the leading biocidal compound in anti-fouling paints (Jones and Bolam, 2007). Copper is often accompanied by zinc in many formulations of antifouling paint (Watermann et al., 2005). Several studies have indicated that these antifouling compounds have adverse effects on aquatic life (Munari and Mistri, 2007; Férnandez-Alba et al. 2002; Sobral and Widdows, 2000). However, the effect of such antifouling paints in the selection of antibiotic-resistant bacteria in marine environmental settings is virtually unknown and has not been studied so far. Therefore, in the study described in **paper IV**, we investigated whether copper and zinc containing antifouling paints can co-select for antibiotic-resistant bacteria in marine environmental settings.

1.4.5 Biocide resistance mechanisms

Bacteria can survive during biocide exposure using several mechanisms, with considerable overlap with those providing resistance to antibiotics (Chapman, 2003). The major mechanisms are as follows: **(a) reduction of outer membrane permeability**, decreasing uptake or penetration of biocides through the cell wall or cell membrane (e.g. resistance to EDTA); **(b) efflux pumps** removing the biocides out of the cell before it can act on any target sites (e.g. resistance to quaternary ammonium compounds); **(c) degradation/ enzymatic inhibition**, i.e. presence of certain enzymes that reduce the active biocide substance to an inactive form (e.g. some metal-based biocides (e.g. mercury, copper) associated with enzymatic reduction); **(d) alteration of target sites**. As biocides generally target multiple sites in a microbial cell, this specific resistance mechanism is rarely seen, with the notable exception of triclosan, which has a specific target (*fab*I in *Escherichia coli* or *inh*A in *Mycobacterium smegmatis*). Therefore, a mutation of the *fab*I gene can lead to a reduced efficacy of triclosan; finally, **(e) bacteria can form biofilms** to decrease or inhibit the effect of biocides (e.g. tolerance to hydrogen peroxide).

1.4.6 Metal resistance mechanisms

Bacteria have a few similar resistance mechanisms or principles that they use for metals, biocide as well as antibiotic resistance. In addition, there are some unique mechanisms for metals. Overall, bacterial metal resistance is based on the following main mechanisms (Hall, 2002; Lemire et al., 2013): **(a) reducing uptake** by restricting the access of metals to cell by permeability barrier (e.g. GlpF, which can reduce the rate of arsenic uptake in a cell); **(b) efflux systems** excluding excess metals out of the cell (e.g. GesABC efflux system can export gold out of the cell); **(c) extracellular sequestration** using siderophores to trap or precipitate metals at extracellular environment (e.g. CopM, which can mediate copper resistance by its sequestration in the extracellular space); **(d) intracellular sequestration** of metals in the cytoplasm or periplasmic space to prevent damaging the metal-sensitive cellular targets (e.g. CopB, which can mediate copper resistance by its sequestration in the outer membrane); and **(e) enzymatic transformation and detoxification** altering the redox state of toxic metals to a less toxic form or inactivating them (e.g. MerA, which can transform Hg^{2+} to Hg^{0}, a less toxic form of mercury).

1.4.7 Biocide and metal resistance genes database

Bacteria carry a large number of genes that code for metal and biocide resistance mechanisms. These resistance genes are located on either chromosomes or plasmids (Nies, 1999). Usually, plasmid-borne resistance genes are mobile and can move from one bacterium to another, thus bacteria carrying these genes have the potential to disseminate resistance genes from resistant strains to susceptible strains (Nies, 1999). There is currently a range of widely used databases, such as ARDB (Liu and Pop, 2009), ResFinder (Zankari et al., 2012), ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation; Gupta et al., 2014), CARD (McArthur et al., 2013; Jia et al., 2016), and Resqu (1928 Diagnostics, Gothenburg), where ARGs have been compiled in an organised way (for a broad discussion about databases see section 3.2.2). However, studies on biocide and metal resistance genes have been hampered due to lack of organised data resources on such resistance systems. One of our efforts aimed to fill that gap by building a database of biocide and metal resistance determinants. The **Paper I** in this thesis describes a new comprehensive database of such genes.

1.5 Horizontal Gene Transfer and Mobile Genetic Elements

The role of horizontal gene transfer (HGT) in the transmission of ARGs has been widely elucidated. HGT can occur mainly in three different ways – conjugation (i.e. gene transfer by means of plasmids), transduction (i.e. gene transfer mediated by bacteriophages) and transformation (i.e. the uptake of free DNA from the environment) (von Wintersdorff et al., 2016). Mobile genetic elements such as plasmids, integrons, transposons and bacteriophages are the important vehicles in the first two processes (Frost et al., 2005).

However, the conjugation process is generally considered as the main mechanism for HGT of ARGs in various environments (Huddleston, 2014). Thus, for a risk assessment, it is important to understand to what extend resistance genes in bacteria in the environment can disseminate by HGT, which allows bacterial populations to rapidly adapt to a strong selective pressure from antibiotics or other xenobiotic compounds. In **paper II** of this thesis, we investigated to what extent plasmids with both types of resistance genes (antibiotics and biocides/metals) also carry conjugation systems, thus are self-conjugative.

1.5.1 Types of MGEs and their contributions toward co-selection

Plasmids in co-selection

Plasmids may provide advantages to the host bacteria, for example providing the means to cope with stress-related conditions. They usually carry a wide variety of genes that encode different traits and help cells to survive in tough conditions, for example, the presence of antibiotics or heavy metal stress. The appearance of these genes on plasmids in the majority of cases was thought to be connected with mobile genetic elements - transposons and integrons. Plasmids from the pre-antibiotic era harboured resistance genes to a range of different metals such as copper, mercury, arsenic and tellurium but not silver (Hughes and Datta, 1983). However, most of the plasmids from the pre-antibiotic era were devoid of ARGs. Interestingly, the plasmids that we see today often harbour resistance genes to multiple compounds. An example of such a plasmid is presented in Figure 4, isolated during an ESBL-outbreak at Uppsala university hospital in Sweden (Sandegren et al., 2012). The plasmid carries a range of ARGs, as well as multiple metal resistance genes and a biocide resistance gene – *qacEdelta*1, which confers low-level resistance to mainly quaternary ammonium compounds. Hence, when a sensitive bacterium acquires such a multi-resistance plasmid, they may become resistant to all of those compounds. Thus, it is evident that mobile genetic elements, such as plasmids, play important roles in co-selection of resistance.

Because of bacterial sequencing projects, or specific plasmid sequencing projects, we presently know the complete DNA sequence of around 5000 plasmids (as of Oct 2016). Many of these sequences have already been subjected to various types of analysis. From them, we can infer some global characteristics of the genetic constitution of plasmids. Here in this thesis, we took advantage of such large plasmid sequence resources available in the GenBank database to study co-selection via co-occurrences of resistance genes on 4582 completely sequenced plasmids (**paper II**). We also investigated genes responsible for mobility (i.e. conjugation systems) of resistance plasmids on these plasmids and linked the frequency of the co-occurrence on plasmids.

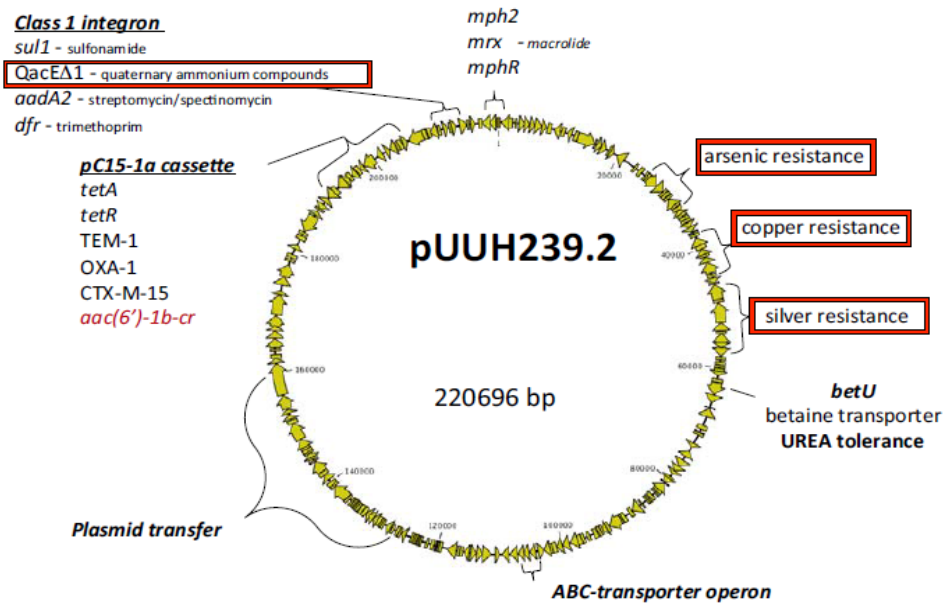

Figure 4. Multi-resistance plasmid pUUH239.2 containing resistance genes to antibiotics, biocides and metals (adapted from Sandegren et al., 2012)

In addition to resistance genes, toxin-antitoxin systems are widely found on plasmids of the host bacteria and often have roles in bacterial pathogenicity (Pandey and Gerdes, 2005). Resistance plasmids often use such toxin-antitoxin systems to ensure their persistence after host cell division. When plasmids carry toxin-antitoxin systems they can eliminate plasmid-free bacterial cells after the replication event via post-segregational killing (PSK) mechanism (Gerdes et al., 1986). It occurs when a plasmid is absent in a daughter cell after the cell division, the unstable antitoxin is degraded and the stable toxin kills the daughter cell. This way it provides a competitive advantage to the plasmid-bearing cells over plasmid-free ones, thus maintain plasmid in the host population (Gerdes et al., 1986). Thus, plasmids with resistance genes to both antibiotics and biocides/metals (i.e. with co-selection potential) together with toxin-antitoxin systems would likely be more persistent, also in the absence of selection pressure, than would resistance plasmids lacking a toxin-antitoxin system. However, it is not yet known how often resistance genes and toxin-antitoxin systems co-occur on plasmids. Thus, in **paper II**, we investigated to what extent toxin-antitoxin systems are present on plasmids that also carry resistance to both biocides/metals and antibiotics.

Integrons

Integrons are another type of MGEs that play a major role in antibiotic resistance dissemination between bacteria. They are considered as one of the most active resistance gene capture platforms (Mazel, 2006). Integrons themselves are not mobile and their dissemination is usually dependent on transposons or plasmids (Deng et al., 2015; Iliya, 2012). Based on GenBank, around ten different classes of integrons are known. Among them, integron classes 1 and 2 are well-studied compared to other classes (Deng et al., 2015), and are commonly linked to ARGs (Mazel, 2006; Gillings et al., 2014). There are studies that reported other classes of integrons (e.g. classes 3 and 4) in association with ARGs but their presence in clinical context has not been widely reported (Deng et al., 2015; Gillings et al., 2014). In **paper II-IV** of this thesis, we investigated how integron-associated integrases (i.e. the markers for integrons) are distributed and at what abundance across sequenced bacteria, plasmids, as well as in microbial communities from human and animal and external environments. In addition, in **paper II**, we also investigated how often such integron-associated integrases co-occur with resistance genes to antibiotics and biocides/metals on plasmids, thus reflect the potential for integron-driven co-selection of resistance.

ISCR elements

About ten years ago, a new class of transposable elements was discovered, termed ISCRs (Insertion Sequences Common Regions), which mobilise DNA adjacent to their insertion site by rolling circle replication (Toleman et al., 2006). An ISCR is a sequence of DNA that is able to move within the chromosome, between chromosomes and plasmids and between bacterial genomes by inserting itself into the new place in the genome. ISCR elements are often considered as a superior gene-capturing and mobilisation system than transposons and integrons (Toleman et al., 2006). Thus when a resistance gene is located on ISCR elements, it can move between bacteria easily via HGT. ISCR elements are often found in close association with horizontally acquired genes such ARGs (Toleman et al., 2006).

Currently, the ISCR family consist of around 20 members (http://www.cardiff.ac.uk/research/explore/research-units/antibacterial-agents-and-genetics-of-resistance). The genetic contexts of the ISCR classes 1 to 8 are much more well-defined than many other classes (Toleman et al., 2006; Ilyina, 2012). More recently, ISCR15, ISCR18, ISCR19, ISCR22 and ISCR23 have been reported (Iliya, 2012; Toleman, 2008). In this thesis, in parallel with integron-associated integrases, ISCR transposases (i.e. markers for ISCR elements) were investigated across sequenced bacterial genomes, plasmids, as well as in microbial communities from human and animal and external environments. In addition, in **paper II**, we also investigated how often such ISCR transposases can co-occur with resistance genes to antibiotics and biocides/metals on plasmids, thus reflect the potential for ISCR element-driven co-selection of resistance.

1.6 Genomics - as a tool to study co-selection

Advancement of next generation sequencing platforms has allowed the study of resistance genes in bacterial chromosomes and plasmids at large-scale due to rapid cost-effective sequencing. Before the high throughput sequencing era, many studies had shown the presence of resistance systems on plasmids in different organisms, especially from clinical environments, while the existence of chromosomal resistance systems was not disclosed for a long time due to technical limitations and high sequencing costs of complete genomes compared with plasmids (Silver and Phung, 1996). However, recent advancement of genome sequencing technologies has dramatically increased the number of completely sequenced bacterial genomes and plasmids. Still, the identification and characterisation of antibiotic, biocide and metal resistance systems on a global scale remain largely unexplored. The co-resistance potential of metals or antibiotics can be predicted bioinformatically via identifying resistance genes that occur together on bacterial chromosomes or plasmids, or other mobile genetic elements such as integron gene-cassettes and transposons. Therefore, in **paper II** of this thesis, we took the opportunity to study co-selection (via co-occurrence of resistance genes) on a broader scale than previously done.

1.7 Metagenomics and bioinformatics – tools to study resistance to antibiotics, biocides and metals

Only a small fraction of the total microbial diversity that exists in nature can be cultivated in the laboratory using standard microbiology methods (Amann et al., 1995). Metagenomics is considered as a powerful culture-independent technique to identify the microbes present in a community and their genetic potential to perform different functions, including tolerance to antibiotics, metals and biocides. A few years ago, our research group was the first to apply shotgun metagenomic sequencing to search for antibiotic resistance genes in microbial communities (Kristiansson et al., 2011). Developing methods to search microbial communities, as well as genomes and plasmids, for metal and biocide resistance genes is a major part of the present thesis.

In metagenomics, DNA from all (or nearly all) organisms in a complex environmental or clinical sample is extracted, fragmented and then a subset of this total DNA is either sequenced using modern next generation sequencing technologies and analysed bioinformatically, or cloned into a suitable host (vector) to screen for an acquired functional potential such as resistance to a chemical (functional metagenomics). Functional metagenomics has already identified several novel antibiotic resistance genes against beta-lactams, tetracyclines, aminoglycosides and bleomycin (Riesenfeld et al., 2004; Donato et al., 2010; Mori et al., 2008; Allen et al., 2009).

Modern next generation sequencing (NGS) technologies such as the Illumina HiSeq produce massive amounts of sequence data. Advanced bioinformatics tools can be applied

to analyse these large amounts of data and extract the biological information in them. For example, resistance genes can be identified and quantified by matching each generated short DNA read against databases of known resistance genes for antibiotics (Resqu database; 1928 Diagnostics, Gothenburg) or biocides/metals (**paper I**). It is also possible to assemble the short DNA reads into larger continuous sequences (i.e. contigs) to identify the genetic contexts of the resistance genes, especially the co-resistance possibilities on plasmids or other mobile genetic elements, and possibly find genetic links between metal/biocide resistance genes and antibiotic resistance genes, or different mobile elements. PCR-amplified barcoding regions (e.g. the 16S rRNA gene) can also be analysed from the metagenomic dataset to study the taxonomic composition of a microbial community.

2. HYPOTHESIS AND AIMS

Hypothesis: *Antibacterial biocides and metals can co-select for antibiotic-resistant bacteria.*

Overall aim: To enhance our understanding of the roles of antibacterial biocides (e.g. antiseptics, disinfectants) and metals (e.g. copper, zinc) in developing, promoting and maintaining antibiotic resistance in bacteria.

Specific aims:

1) To compile a database of known antibacterial biocide and metal resistance genes **(paper I)**

2) To identify which specific biocides and metals have the potential to co-select for resistance to certain classes of antibiotics by co-resistance mechanisms by identifying co-occurrence of resistance genes. **(paper II)**

3) To identify environments and bacterial taxonomic groups where the potential for co-selection for antibiotic resistance by biocides and metals are highly prevalent. **(paper II)**

4) To estimate to what extent plasmids with co-selection potential carry conjugative systems (i.e. self-conjugative), thus the potential to move between hosts across species and environments. **(paper II)**

5) To identify environments that could act as reservoirs, sources and dissemination routes of resistance genes to pathogens by investigating the abundance and diversity of antibiotic-, biocide- and metal-resistance genes, and mobile genetic elements found in different environments including humans and animals **(papers II and III)**

6) To investigate whether antifouling paint containing copper and zinc can co-select for resistance to antibiotics in marine bacterial communities **(paper IV)**

3. MATERIALS AND METHODS

"Those are my principles, and if you don't like them... well, I have others."

-- Groucho Marx

3.1 Methods overview

Much of this thesis work was primarily based on bioinformatics analyses, taking advantage of already published studies on resistance genes and DNA sequences of plasmids, bacterial genomes and microbial communities from human, animal and external environments. In **paper IV**, field studies were conducted in marine environments. Phenotypic assays employed included culturing of bacteria from marine bacterial biofilms on agar containing antimicrobials to assess the effects of a selection pressure from metals (especially copper and zinc) and antibiotics. The DNA of complex microbial communities was sequenced by modern next generation sequencing technologies (Illumina) and bioinformatics techniques were applied to analyse the large volumes of sequence data.

- In **paper I**, published studies on resistance genes to antibacterial biocides and, metals were used as the basis to develop a database of such genes.
- In **paper II**, 2522 completely sequenced bacterial genomes and 4582 plasmids were studied for resistance genes and their co-occurrences.
- In **paper III**, 864 metagenomes from human, animal and external environments were studied for resistance genes, taxonomic compositions, and mobile genetic elements (integron-associated integrases and ISCR transposases).
- In **paper IV**, marine microbial biofilms growing on surfaces painted with copper and zinc-based antifouling-paint were studied using phenotypic assays (culturing of bacteria on agar containing antimicrobials) and metagenomic sequencing.

3.2 Database development strategy and used database resources

The rationale behind developing the BacMet database was the lack of a dedicated, well-curated database of resistance genes to antibacterial biocides and metals. The ultimate goal was to use the newly developed database in subsequent large-scale analyses of bacterial genomes, plasmids and metagenomes - not just for our own work but also as a freely available resource for the entire scientific community.

In **paper I** of this thesis, we developed a database resource (BacMet) that includes two databases on genes conferring resistance to antibacterial biocides and metals. The first database contains genes with experimentally verified resistance function, while the second database contains genes that are predicted to have a resistance function based on similarity to known resistance genes with an assumption that obtained sequences have similar (nearly similar) functions due to high sequence similarity. The 'experimentally verified database' was developed based on available data, collected mainly from literature covered in the PubMed database resource using a variety of search terms related to biocide and/or metal resistance and by going through reference lists of those papers. The criteria used to include genes in the experimentally verified database were - (a) removal/mutation or insertion/overexpression of a gene into genome or into an inserted plasmid results in an increased or decreased susceptibility to the biocide/metal, respectively; or (b) insertion of a plasmid lacking the gene of interest showed increased susceptibility compared with insertion of the same plasmid carrying the gene; or (c) genes that are part of an operon, where the operon is experimentally confirmed to be involved in resistance, but where evidence for a resistance function of each individual component gene is still lacking. Additionally, the metadata for each experimentally confirmed resistance genes was collected from a range of other resources including NCBI GenBank and non-redundant protein databases, UniprotKB, the Transporter Classification Database and the original literature. The metadata for each resistance genes includes the protein sequence, source organism, genetic location (either chromosomal or plasmid), list of compounds that the gene confers resistant to and brief information on the listed compounds, gene description including information on cross-resistance to antibiotics if available, and other relevant information including link to external databases for more information.

The rationale behind developing the other database (i.e. the BacMet predicted database) was that the resistance genes may differ between species and/or occur in different forms that are not (yet) experimentally investigated. For plasmid-borne resistance genes, this might not a major issue since the mobile genes are highly conserved and can move between species, hence identical or very similar copies of the mobile resistance genes are often found in different species. However, if we screen genomes or metagenomes against a database of only experimentally verified sequences using strict criteria, we might miss many resistance genes that are present on chromosomes in different species that carry different forms of a resistance gene. On the other hand, if we relax the criteria to search for resistance genes in genomes or metagenomes, we might detect false-positives that have a conserved section (e.g. a domain) similar to the resistance gene but have a different function than conferring resistance. Therefore, the protein sequences of the resistance genes (both plasmid-borne and chromosomal) in the BacMet experimentally confirmed database was used as the basis to obtain highly conserved sequences with the potential to confer resistance to biocides/metals using similarity searches against NCBI non-redundant protein database. To do that, for resistance genes reported to be found on plasmids, a fixed sequence identity cut-off of 90% was applied. However, for chromosomal genes, instead of

applying a fixed sequence identity cut-off the annotation of the BLAST hits against each experimentally confirmed gene was examined manually. In this process, only similar sequences were accepted down to a sequence identity cut-off where NCBI annotation indicated a protein likely to have a different function than conferring resistance including conserved hypothetical protein sequences.

It is worth mentioning that some metals, such as cobalt, copper, manganese, iron, nickel, molybdenum and zinc, are essential for the physiological stability of bacteria and essential for growth (Bruins et al., 2000). They are associated with a wide range of metabolic functions as coenzymes or cofactors, catalysts, and structural stabilisers of enzymes and DNA-binding proteins. Usually, they are required in trace amounts but at high concentrations, they have adverse effects and become toxic to microorganisms. On the other hand, some metals, such as mercury, arsenic, lead, cadmium, bismuth, silver, aluminium and tellurium, have no biological roles and are often toxic to organisms even at very low concentrations (Bååth, 1989). Since all metals become toxic at elevated concentrations and thus can exert a selection pressure, the scope of the BacMet database was to include resistance genes to not only toxic metals but also genes providing resistance/tolerance to essential metals.

3.2.1 Resistance genes can have other functions besides resistance

Metal resistance and/or homeostasis are common processes in many bacteria. Often, a large number of genes linked to tolerance/resistance mechanisms in a genome are found in some bacteria. One notable example is the bacterial strain *Janthinobacterium sp. HH01*, where almost 2.4% (170 chromosomal genes) of the total genes in the genome are linked to resistance mechanisms towards biocides/metals and antibiotics (Hornung et al., 2013).

Some genes involved in resistance mechanisms have been reported to be involved in metabolic processes. For example, DNA helicases *ruv*B and *rec*G have roles in DNA repair but also are involved in chromate resistance in *Pseudomonas aeruginosa PAO1* (Miranda et al., 2005); superoxide dismutases (*sod*A and *sod*B) have roles as basic cell defence in bacteria under oxidative stress but also are involved in resistance mechanisms to selenium and antibacterial peroxides (Bébien et al., 2002). Thus, some genes appear to be present in many bacterial genomes, where they have roles in different physiological activities, while at the same time they also contribute to increased resistance/tolerance to biocides/metals and antibiotics. Such genes have been included in the BacMet database. Thus, when such genes are detected in genomic or metagenomic data, one should reflect on that they might be there for a very different reason than protecting the bacteria against these types of compounds. There are some very similar cases that have been observed for ARGs. For example, plasmid-encoded beta-lactamases acquired by pathogens through HGT might originally have been penicillin-binding proteins involved in the synthesis of peptidoglycan (Kelly et al., 1986; Massova and Mohashery, 1998); In *Providencia stuartii*, chromosomal 2′-

N-acetyltransferase is involved in the modification of the bacterial peptidoglycan; however it also can inactivate gentamycin (Payie et al., 1995). While the BacMet database lists only genes that are known to confer a resistance phenotype (experimental database), depending on the context the same genes might have other functions as well.

Genes involved in the regulation of resistance genes also contributes to resistance, but indirectly. These regulatory genes have limited role in co-selection of antibiotic resistance via co-resistance but rather through co-regulation. For example, *bae*SR system increases multidrug and metal resistance by inducing the *acr*D and *mdt*ABC drug efflux systems and are critically required for survival under tungsten stress, and *bae*R overexpression confers resistance to novobiocin and deoxycholate, as well as to beta-lactams (Nishino et al., 2007; Appia-Ayme et al., 2011). The BacMet database has listed such genes in the experimentally confirmed database. However, resistance phenotype conferred by these regulators is highly dependent on expression level and the context, thus a resistance phenotype should not be inferred only by their presence in a genome.

3.2.2 Considerations for using databases of resistance genes

The annotation information present in many sequence databases is poor and therefore, using a high quality and well-curated database is highly recommended for bioinformatic annotation of resistance genes (Bengtsson-Palme et al., 2016a). A poor choice of the database can therefore indirectly affect the conclusions from a study. Comparisons between studies become difficult when we use different databases to screen for antimicrobial resistance genes (Bengtsson-Palme et al., 2016a). This is largely due to database type (e.g. experimentally validated and/or predicted sequences present in the database), comprehensiveness (e.g. number of resistance genes present in a database) and mobility potential (e.g. database contain only mobile or chromosomal resistance genes) of sequences present in different databases.

Antibiotic Resistance Genes Database (ARDB) was one of the earliest databases to screen for ARGs in genomic and metagenomic sequences but the database was not updated after 2009 (Liu and Pop, 2009). The Comprehensive Antibiotic Resistance Database (CARD) which uses an ontology-based classification system of the ARGs was developed in 2013 and actively updated (McArthur et al., 2013; Jia et al., 2016). It is possibly the most comprehensive resource for ARGs information currently available. The ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) is another important resource for resistance genes to antibiotics occasionally used by users (Gupta et al., 2014). MEGAres, a database of antibiotic resistance genes to 22 different antibiotic classes, have recently been developed (Lakin et al., 2016). Though some of the above databases are based on thorough curation and are actively maintained, they do not clearly separate experimentally verified and predicted gene entries. Furthermore, it is unclear if the genes in these databases have been found on MGEs or only have been detected on chromosomes. Other small and specialised

resources on beta-lactamase resistance genes exist. For example, the Lactamase Engineering Database (LacED; (http://www.laced.uni-stuttgart.de/) and beta-lactamases resources hosted by Lahey clinic (http://www.lahey.org/studies/) and Beta-lactamase Database (BLDB; http://bldb.eu/) that contains many classes and sub-classes of beta-lactamases with corresponding structures, functions and their kinetics. Some of these ARG databases are occasionally used for the annotation of ARGs in bacterial genomes as well as in metagenomic studies. Resfams, which is another highly specialised curated ARG database, is based on a set of collected reference sequences from CARD database, the LacED, and beta-lactamase database hosted by Lahey Clinic. Resfams contains protein families and associated hidden Markov models (HMMs), based on sequences with confirmed antibiotic resistance function and organised by ontology (Gibson et al., 2015).

If the main purpose of a study is to identify potential risk for dissemination of antibiotic resistance or metal resistance between bacteria, a database of only mobile or acquired (i.e. horizontally transferable) ARGs is often desired. For that purpose, Resqu (1928 Diagnostics, 2013) database was used for identifying experimentally verified horizontally transferable ARGs in sequencing data in **papers II-IV** in this thesis. Alternatives to this database are the ResFinder (Zankari et al., 2012) and Resistance Determinants Database (RED-DB; http://www.fibim.unisi.it/REDDB/). These two databases contain ARGs related to the mobile resistome. However, it is unknown whether these mobile resistance genes are experimentally verified or not. The additional advantage of using Resqu database is that in addition to mobile ARGs, Resqu database also contains gene markers for MGEs, such as integron-associated integrases (*int*I) and ISCR transposases. However, currently the database is not actively updated (last update Nov 2013) compared to ResFinder or CARD databases, thus a thorough update of the Resqu database is on our wish list.

In contrast to databases for ARGs, the availability of specialised public resources is much smaller for biocide and metal resistance genes. In **paper I**, for the first time, we developed a database that contains manually curated entries of experimentally validated resistance genes to antibacterial biocides and metals, as well as predicted resistance genes to such compounds. The database can be used to search for known biocide and metal resistance genes in any DNA sequences. In this thesis, the BacMet database (**paper I**) was used in the studies described in **papers II-IV** to characterise the resistance genes to antibacterial biocides and metals in bacterial genomes, plasmids and metagenomes. Since the BacMet database contains only resistance genes to biocides/metals, whereas the CARD database contains resistance genes to antibiotics, to shed light on cross-resistance, genes present in both BacMet and CARD databases were considered as resistance genes with cross-resistance potential. Such a resistance gene dataset with cross-resistance potential was used only in the study described in **paper IV**.

I acknowledge that genotypic prediction of resistance relies on highly curated databases of known resistance determinants, and thus neither we can identify mechanisms that have yet

to be defined, nor does it take into account the context and expression level of genes. Therefore, other approaches such as phenotypic susceptibility testing are still necessary to confirm the presence or absence of resistance genes detected via genotypic prediction, and to detect new mechanisms of resistance.

3.3 Sample collection & genomic and metagenomics datasets used in this study

In **paper II**, a dataset of 2522 publicly available completely sequenced bacterial genomes (from 565 different bacterial genera), comprising 1926 plasmids, were retrieved from the NCBI bacterial genomes database (Acland et al., 2014). Since, the fully sequenced genomes contained less than half (42%) of all completely sequenced plasmids available in the NCBI plasmids database, the sequences of 1926 plasmids that were found among the complete bacterial genomes were expanded to 4582 (hosted by 313 different bacterial genera) to obtain a more comprehensible set of completely sequenced plasmids.

In **paper III**, in total, we studied 864 metagenomes representing 13 different environments based on the metadata information collected from original literature describing the metagenomes, and the MG-RAST and Human Microbiome Project (HMP) (Peterson et al., 2009) repositories. Environmental metagenomes were retrieved from the MG-RAST repository. In total, 358 publicly available shotgun metagenomes were collected from external environments representing soil (n=200), water (n=45), sediment (n=60), mine (n=7), wastewater/sludge (n=32) and a Beijing smog event (n=14). Our previous work in environments polluted by discharges from pharmaceutical manufacturing suggested that environments with very high levels of antibiotics are reservoirs of resistance genes (Kristiansson et al., 2011; Bengtsson-Palme et al., 2014). Hence, these are risk environments that might play a role in antibiotic resistance development (Larsson, 2014a). However, in the MG-RAST database repository (Meyer et al., 2008), no deeply sequenced metagenomes exist from such environments. Therefore, 11 sediment samples collected from an Indian river and two lakes polluted by pharmaceutical production were also included in this study. Additionally, 145 metagenomes from animal sources were retrieved from the MG-RAST. In addition to the environmental and animal-associated metagenomes, 350 metagenomes from different body sites of healthy adults including the gastrointestinal tract (n=100), oral (n=100), skin (n=50), airways (n=50) and the urogenital tract (n=50) were retrieved from the HMP repository. All these shotgun metagenomes were generated by Illumina sequencing technology with a sequencing depth of at least 10 million reads per metagenome, which allows detection and quantification also of less common resistance genes, taxa and MGEs.

In **paper IV**, as part of the field experiment, biofilms were established on surfaces painted with copper- and zinc-based antifouling paints by submerging plastic panels approximately

one metre below the water surface in a local marina on the Swedish west coast. The biofilm samples were collected from the panel surfaces after 2.5-4 weeks, and were studied using phenotypic assays (culturing of bacteria on agar containing antimicrobials) and metagenomic sequencing.

3.4 Whole genome and metagenome sequencing

Antibiotic resistance is a phenotypic characteristic of bacteria that can be detected using traditional microbiology techniques - antibiotic sensitivity testing performed in broth or in agar. Culture-based approaches usually take 24-48 hours for fast-growing bacteria such as *Escherichia coli*, and may take up to several weeks for slow-growing bacteria such as *Mycobacterium tuberculosis* (Kent and Rubica, 1985). The phenotypic resistance is traditionally assessed via counting of colony forming units, or merely studying biomass formation in liquid media, after exposing bacteria to selective agents such as antibiotics. The tests basically reveal the presence or absence of a resistance property of bacterial strain at the tested concentration of the antimicrobial agents. Since a majority of resistant pathogens are well characterised, culture-based investigation of antibiotic resistance works very well for the majority of those that can be cultured under laboratory conditions using selective media. However, the culture-dependent technique may be labour-intensive and time-consuming. In addition, often the genes responsible for phenotypic traits of resistance and their abundance remain unknown with this technique. Another potential limitation is that for culturing bacterial isolates prior knowledge about selective media is needed. Otherwise, culturing of the bacterial isolates on a range of different non-selective media for few times might result in losses of antibiotic resistance potential (Ludwig et al., 2012). There is a range of techniques that may be used to determine the genetic basis of antibiotic resistance phenotypes. For example, whole genome sequencing (WGS) approaches are widely used to obtain genotypic information. In addition, molecular detection techniques such as standard PCR (e.g. isolates are tested for the presence of certain resistance gene by PCR amplification). Since, only a fraction of bacteria from a microbial community (e.g. human gut) can be cultured using standard culture-based laboratory techniques in the lab the quantitative PCR (qPCR) are also often used to not only detect a 'limited' set of resistance genes but also determining their expression level both in individual bacterial strains and microbial community. However, to study complete resistance gene profiles (i.e. resistome) in a microbial community, shotgun metagenomics approach is frequently used to overcome some the limitations of culture-dependent sensitivity testing and amplification-based methods such as qPCR. A more detailed description of each of these techniques is provided below.

3.4.1. Whole-genome Sequencing

Since the first complete sequencing of the bacterium *Haemophilus influenza* in 1995, the number of completed genomes of bacteria has increased rapidly, especially for the last 7 to 8 years due to the advancement of next-generation sequencing technologies and lower sequencing costs. Currently, using whole genome sequencing (WGS), it is possible to completely sequence a bacterial genome and identify its resistance genes in less than 12 hours. Studies have suggested using WGS as a screening tool in infectious disease therapy due to low sequencing costs and the ability of WGS to accurately predict phenotypic resistance (Zhao et al., 2015; Tyson et al., 2015). Tyson et al. (2015) used a WGS approach to identify resistance genotypes for 76 multidrug-resistant *Escherichia coli* strains, showing that resistance genotypes were highly correlated with resistance phenotypes. Similarly, in another study by Zankari et al. (2013), out of 197 bacterial isolates from pig manure, phenotypic resistance could be predicted for 99.7% of the isolates by genotypic prediction via WGS, demonstrating the potential of WGS-based techniques to replace phenotypic indicators of resistance. Thus, the vast amount of data generated by WGS technologies can be used in a wide range of research and clinical applications. However, it should be noted that such phenotypic predictions of resistance based on sequence data does not work in every context. For example, *Pseudomonas aeruginosa* isolates often carry no single acquired resistance gene but are resistant to a range of different antibiotics due to their intrinsic resistome capability via genes encodes for basic functions of the cells (Fajardo et al., 2008). In **paper II** of this thesis, we used data derived from WGS projects and similarity searches using known resistance genes to find genes that are highly similar to known resistance genes, and thus likely have similar functions as resistance genes, in bacterial genomes and plasmids retrieved from NCBI bacterial genomes and plasmids databases, respectively.

3.4.2 Standard PCR and Quantitative PCR (qPCR)

Standard PCR is able to determine the presence of specific resistance genes in bacterial isolates and has improved clinical diagnosis by providing results within hours. PCR methods are relatively inexpensive, easy to perform, culture-independent and have high sensitivity. However, for bacterial communities, qPCR approaches are regularly used to quantify the abundance of resistance genes where the technique can be used to study from one up to a few hundred resistance genes in parallel. Thus it is often considered as a cheap and attractive tool to quantify ARGs in bacterial community. For example, qPCR approaches have been used to quantify ARGs in bacterial community from a variety of environments, including wastewater treatment plants (Karkman et al., 2016; Mao et al., 2015), Feedlot lagoon wastewater (Smith et al., 2004), air from cattle feed yards (McEachran et al., 2015), soils (Graham et al. 2016; Knapp et al. 2011), groundwater (Böckelmann et al., 2009), aquaculture (Muziasari et al., 2014, Tamminen et al., 2011), pharmaceutically polluted sites (Rutgersson et al., 2014), swine farms (Zhu et al., 2013) and

glaciers (Segawa et al., 2013). However, the major issue with qPCR techniques is that the approach only targets well-studied resistance genes, where prior knowledge of designing sequencing primers is essential. In addition, amplification bias, false-negative results due to inhibition in PCR and false-positive results due to nonspecific amplification could be major issues with PCR and qPCR. Thus, although qPCR is highly sensitive and can detect resistance genes at very low abundances, even in high-throughput fashions (Zhu et al., 2013; Looft et al., 2012), it has some major limitations and remains a largely non-explorative approach. Thus, to obtain a complete resistance profile of a bacterial community, metagenomics plays a major role in this area.

3.4.3 Shotgun metagenomics

Metagenomics is often used as a powerful technique to identify and describe "who is there?" (i.e. microbes present in a microbial community) and "what are they capable of doing?" (i.e. total genetic potential) including resistance potential. Shotgun metagenomics involves extraction and random sequencing of DNA directly from complex samples, including the DNA of uncultivable bacteria. In the studies described in **papers III** and **IV**, genomic DNA was extracted from the collected environmental samples, and was subjected to shotgun metagenomic sequencing on Illumina HiSeq NGS platform at high depth (between 14-72 million reads per sample).

Although metagenomics is widely used technique to study 'potential' resistance genes in microbial communities, it has several laboratory-associated limitations and technical challenges (see below).

1. One of the major limitations of the metagenomics approach to study antibiotic resistance is that the obtained DNA sequences from a community cannot provide the information on whether a resistance gene detected is expressed under a given environmental condition or disease state.

2. The choice of DNA extraction techniques can influence the results because of the differences in extraction and lysis efficiency, which presents a challenge to representing all organisms present in a community (Wesolowska-Andersen et al., 2014). Therefore, extraction techniques may provide a partial representation of the actual community. It is also possible that DNA will be extracted from only highly abundant genomes in the community.

3. Furthermore, it is possible that DNA sequences obtained from the metagenomics sequencing can actually be free DNA or dead bacterial cells in a community.

Besides these conceptual limitations and laboratory-associated technical challenges, additional challenges exist for analysis of metagenomic data (see section 3.5.1).

Next generation sequencing technologies have developed dramatically since their introduction in the market in 2005. Since then NGS technologies have excelled the genomic and metagenomic research to a new level. In many metagenomic projects studying antibiotic resistance utilising the 454 pyrosequencing platform, typically only few hundred thousand reads per sample were generated (Kristiansson et al., 2011; Nesme et al., 2014; Durso et al., 2012). Limited sequencing depth often affects the sensitivity to estimate both the abundances and diversity of resistance genes in the sample. Thus, it is worth mentioning that in terms of measuring specific gene abundance, metagenomics is considered as less sensitive compared to qPCR, particularly when the sequencing depth is small for a sample (i.e. only a few hundred thousand up to a few million reads per sample). The 454 sequencing technology can only detect very common (i.e. highly abundant) resistance genes (Kristiansson et al., 2011; Nesme et al., 2014). However, with the introduction of the Illumina technology, the sequence depth obtained at affordable prices is constantly increasing and often reach 10-50 million reads per sample (**papers III** and **IV**; Bengtsson-Palme et al., 2016b), thus provides the opportunity to identify many low abundant resistance genes in a bacterial community. However, we acknowledge that even in such high sequencing depth, some low-abundant resistance genes remain undetected in the studies based on shotgun metagenomic, even when selective culturing can be used to show their presence (Bengtsson-Palme et al., 2015a). In **papers III** and **IV**, we took advantage of publicly available sequence data generated on the high-throughput Illumina sequencing platform to measure the abundances of resistance genes in bacterial communities from multiple environments.

3.4.4 Functional metagenomics

Functional metagenomics involves cloning and expression of unknown large fragments (often 2-100 kb or even longer) of community DNA in a bacterial host such as *Escherichia coli*, followed by phenotypic testing of the host, in this context susceptibility testing to a range of different antibiotics. A major advantage of using functional metagenomics is that novel genes may be discovered without any apparent sequence/structural similarity to known resistance genes. Thus, it can overcome the limits of PCR-based methods and shotgun metagenomic sequencing approaches, where primarily known resistance genes can be identified. Several studies have applied functional metagenomics approach to discover many novel ARGs from a range of different environments (Allen et al., 2009; Hatosy and Martiny, 2015; Pehrsson et al., 2016; Riesenfeld et al., 2004; Donato et al. 2010). However one of the major issues with the functional metagenomic approach is that the cloning and expression of the inserted DNA fragment/gene in a host bacterium could be difficult. Since *Escherichia coli* is still the most preferred cloning host used in metagenomics, genes that do not express in *Escherichia coli* is often missed in this step. Thus, expanding the clonal host range might improve the situation to some extent. Additionally, in the cloning process the amount of inserted DNA in the host is often limited (typically <100 kb). Thus, some

potential resistance genes remain undetected. If the insert size of the DNA fragment is short, we can only identify single-gene resistance mechanisms, as only one short stretch of DNA is assayed at a time. Therefore, if its function is dependent on another gene (for example, a regulatory gene or a gene from a resistance operon) that is absent in the inserted fragment DNA, the method will not detect the resistance. In addition, even if the gene is expressed in the host, the gene might not be functional in the host bacteria (i.e. *Escherichia coli*) used for cloning. The major advantage of using shotgun over functional metagenomics is that the shotgun approach is fast and at high sequencing depth it can identify many of the resistance genes present in a community, as well as providing taxonomic information from that community.

3.5 Genomic and metagenomic data analysis

With the increasing pace of genomic and meta'omic research using sequencing technologies, the bioinformatic tools and databases required to analyse large-scale data have also been improved over the years. Sample size (i.e. number of samples), reference sequence database size (i.e. number of sequences in a database), average sequence depth in a sample (i.e. number of reads), and read lengths generated from different sequencing platforms often put a different set of requirements for data analysis tools. For optimal performances, many such analysis tools are constantly upgrading with a variety of flexibilities during data analysis stage. In **paper III**, 9.2 Tb of metagenomic data was analysed, which is equivalent to approximately 2.5 million bacterial genomes (if the average bacterial genome size is 4 Mb). Quality filtering of such large dataset is one of the challenges and time-consuming processes we face today in the NGS era. In **paper IV**, Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), which is fast, was used for quality trimming, as well as trimming the adaptors from fastq sequences. In addition, to obtain the quality control report, FastQC quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was possible to apply on the filtered data in the same run. However, in **paper III**, for quality filtering of large numbers of shotgun metagenomes (n=864), a super-fast quality filtering tool was needed. Thus, Seqtk (Li et al., 2012), which is extremely fast compared to many other available quality filtering tools, was used for that purpose.

3.5.1 Resistance gene quantification and normalisation

Besides using high-quality databases, another important part of bioinformatics analysis for resistance genes is the software tools used to annotate or identify resistance genes in genomes and metagenomes from sequencing data. Identification of resistance genes in sequencing data predominantly depends on homology-based sequence similarity searches to already known resistance genes, using the principle that the genes that share homology often perform similar functions. There are many available bioinformatic tools, such as

Blast (Boratyn et al., 2013), USEARCH (Edgar 2010), Vmatch (Kurtz et al. 2016) and BWA (Li and Durbin 2009), are widely used to perform this. However, the accuracy of such homology-based identification of resistance genes in the sequencing data depends on both the search criteria and the databases used. In **paper II** and **III**, we used USEARCH, which is several orders of magnitude faster than BLAST; whereas in **paper IV**, Vmatch was used. In USEARCH, a minimum sequence identity threshold of 90% over the entire coverage of the query reads against a target gene in the database was used. Similarly, in Vmatch, a minimum sequence identity threshold of 90% over 20 amino acids was employed and only two mismatches were allowed. In all cases, we used short reads to search for resistance genes in shotgun metagenome data.

To identify and quantify resistance genes in a genome or metagenomic sample, another common practice is to assemble the short reads into longer contigs, followed by mapping the contigs to reference sequences of resistance genes using a similarity search tool such as BLAST. However, some reads never overlap with adequate sequence coverage, thus the assembler cannot join reads, resulting in a gene split over two or more contigs. Therefore, positive hits to resistance genes usually get missing during the assembly process. Thus, short read mapping is often considered to be better for resistance gene identification (Inouye et al. 2014; Clausen et al. 2016). Our short-read mapping approach only detected resistance genes and MGEs that were highly similar to the reference sequences in the databases.

In this context, it is worth mentioning that there are some technical challenges in the metagenomic data analysis stage to study resistance genes (see below).

1. During the sequence data generation on NGS platforms, under-sampling (i.e. not enough sequence data from a DNA sample) of metagenomic samples is a major issue when studying antibiotic resistance in microbial communities. Under-sampling leads to that not all resistance genes are detected from a community due to limited sequencing depth. However, it is often difficult to determine the sequencing depth required to detect extremely lowly abundant genes (i.e. a few copies present in a community). In addition, metagenomics has limited sensitivity. For example, it is often difficult to detect a quantitative change of a resistance gene for a certain magnitude (not necessarily a change from zero).

2. Sequencing error could arise during sequencing stage. Thus, it could produce some false-positives and false-negative results when detecting and quantifying resistance genes from metagenomic data.

3. Determining the genetic context of resistance genes by metagenomic assembly is challenging. The reason behind this is that resistance genes can occur in multiple contexts in metagenomes, and thus assembly algorithms often cannot assign the

genes to its actual genetic contexts and often break the contigs around resistance genes, resulting in multiple contigs (Bengtsson-Palme et al. 2016b; 2015a).

4. The computational resources required for metagenomic assembly provide an additional challenge, as the amount of RAM required for metagenomic assembly poses a challenge even for many computing clusters. In addition, metagenomic assembly is a time-consuming process. It can take weeks to months to assemble terabases of sequence data.

After obtaining raw gene counts from metagenomic data, normalisation is usually performed to adjust for biases introduced by variable sequencing depths in different metagenomes and variable gene lengths present in a reference database. Gene length is considered as a major issue since there is a higher probability to randomly sample a longer gene than a shorter gene by the shotgun approach. To, overcome the challenge with the gene length, in **papers III** and **IV**, the absolute genes counts obtained from the metagenomes were normalised by the length of resistance genes retrieved from Resqu and BacMet databases.

The amount of sequence data required to detect resistance genes is often ignored but can have a direct consequence in downstream analyses. In metagenomic data, the reads usually come from all the organisms present in a microbial community, not only bacteria. Thus, normalisation by the total number of sequences in samples can be misleading when comparing samples with different taxonomic composition. Since 'antibiotic resistance' is a phenomenon related to bacteria, it is often relevant to calculate the relative abundance of resistance genes counts to only the bacterial fraction of the sample. Therefore, the abundance of bacterial 16S rRNA marker genes in a sample is most commonly used to normalize resistance gene abundance in that sample, resulting in resistance gene counts per 16S rRNA. In the studies described in **papers III** and **IV**, we used reads matching 16S rRNA genes both for the taxonomic classification and characterisation within the bacterial part of the communities (see section 3.5.3), and to normalise the relative abundance of resistance genes to a measure that reflects the proportion of bacteria in the microbial communities.

Quantification of resistance genes in the majority of the studies in the field of antibiotic resistance is based on either shotgun metagenomics or quantitative PCR (qPCR). When comparing quantitative data of resistance genes between multiple environments, the choice of normalisation method often matters. However, there is not yet consensus on the best normalisation method to study the abundance of functional genes in metagenomics samples. Depending on the composition of microbial communities, the average 16S rRNA copy number may vary from 1 to 15 copies per cell with an average of 4.2 copies (Větrovský et al. 2013). Using 1690 bacterial genomes, Větrovský et al. (2013) estimated that only 15% of genomes contain a single 16S rRNA copy, while 21% of the genomes

contained two 16S rRNA copies, 3–7 copies of 16S rRNA were relatively common, while over 7 copies of 16S rRNA per genome were relatively rare. Therefore, normalisation using single-copy genes, such as *rec*A, *rpo*B, *gap*A, *gyr*B, *rpo*A or *pyr*H, has been suggested to reduce the between-sample variability, more directly reflecting the abundance of bacterial cells in a sample since such single-copy genes are usually present in one copy per genome. In our study described in **paper III** and **IV**, we compared quantitative data of resistance genes among different types of environments. However, given the complexity of the studied communities, species with both high and low 16S rRNA copy numbers per genome are represented in all communities, and therefore the average copy number per genome usually vary considerably less between metagenomes than between genomes. Given that our interpretations of differences in the relative abundance of resistance genes across environment types differ by up to several orders of magnitude, we are convinced that our biological results and conclusions would be valid regardless of normalisation method.

3.5.2 Co-occurrence analysis of resistance genes

Network analysis is becoming increasingly popular in genomic and metagenomic studies, and has been widely used to explore the interactions/associations among proteins in metabolic pathways. More recently, coexisting patterns of microbial taxa in soils (Barberán et al., 2012; Ma et al., 2016), ocean (Milici et al., 2016), activated sludge (Ju et al., 2014), oral diseases (Shiba et al., 2016) and human gastrointestinal tract (Zhang et al., 2014) were shown via network analysis.

In **paper II**, for the first time, a network analysis approach was applied to predict co-resistance potential of metals or antibiotics by identifying co-occurrence of resistance genes that occur together on bacterial chromosomes or plasmids. In that study, we conducted a large-scale genomic survey of all completely sequenced bacterial genomes and plasmids retrieved from NCBI resources and performed a network analysis of the co-occurrence patterns of resistance genes between biocides/metals and antibiotics found on the same genetic element or in the same bacterial strain irrespective of their location on chromosome or plasmid. Often studies of associations/interactions of genes obtained from network analysis form clusters of genes. Thus, edge (i.e. a link between two genes) filtering is often required for visualisation purposes. In **paper II**, the networks that we presented, were filtered to avoid such dense clustering of genes and show only common co-occurrences (i.e. found in at least ten plasmids or in ten genomes) of resistance genes found on studied plasmids and genomes. Therefore, we acknowledge that there might be more connections between resistance genes than those are shown in the network as they were not highly frequent. In **paper II**, the networks of resistance genes were explored and visualised using Cytoscape (version 3.2.0; Shannon et al., 2003), which is frequently used in network analysis.

3.5.3 Taxonomic data analysis

To investigate "who is there?" in a microbial community, determining the taxonomic composition in a metagenome is commonly used as one of the main objectives in a bioinformatics analysis of metagenomic data. To perform taxonomic analysis of barcoding genes such as the small sub-unit (SSU) rRNA genes (16S rRNA for bacteria and archaea; and 18S rRNA for eukaryotes) are often used, and taxonomy is inferred based on these extracted barcoding sequences. First, the barcoding genes are extracted from the metagenomic dataset and then a similarity search is performed for the extracted barcoding genes from the entire metagenome to a reference database of barcoding genes, such as SILVA (Quast et al., 2013) or Greengenes (McDonald et al., 2012). There are a range of specialised software tools, such as SSU-ALIGN (Nawrocki, 2009), rRNASelector (Lee et al., 2011), riboPicker (Schmieder et al., 2012), SortMeRNA (Kopylova et al,. 2012), MetaRNA (Huang et al., 2009), and Metaxa2 (Bengtsson-Palme et al., 2015b), that are commonly used to extract SSU rRNA sequences from a metagenome and assign extracted sequences to appropriate taxa. Some of these tools, such as SortMeRNA, use a kmer-based search approach using an index created from a database of marker genes. Instead of using these specialised software packages, the common similarity search tools such as Blast (Boratyn et al., 2013) and BWA (Li and Durbin, 2009) are also useful and often used for extracting 16S/18S rRNA data from metagenomes and assigning them to taxa. These general-purpose search tools use a local alignment method to compare the query sequence to a database of SSU sequences.

In the studies described in **papers III** and **IV**, metagenomic sequencing was used to explore the taxonomic compositions of microbial communities. In both of these studies, our recently developed software package Metaxa2, which outperforms many currently available software for classifying 16S rRNA genes (SSU) reads into appropriate taxa (Bengtsson-Palme et al., 2015b), was used to the extract partial sequences of SSU rRNA genes from all the metagenomes, followed by assigning extracted sequences to different taxa at different taxonomic levels – kingdom, phylum, class, order, family, genera, species and subspecies, using a built-in database of manually curated entries of SSU rRNA genes from SILVA (release 111; Quast et al., 2012) and MITOZOA (version 2.0; release 10; D'Onorio de Meo et al., 2012). The extracted raw counts for each taxonomic level were then normalised to counts per million reads to calculate the relative abundance of each taxon.

3.6 Statistical methods

To investigate "who is there?" and "what are they capable of doing?" in a microbial community, taxonomic and functional profiles (e.g. resistance genes) of metagenomes are interesting and useful information on their own. In addition, comparisons of such profiles

can be used to describe similarities and differences between microbial communities. In this thesis, we applied a range of different statistical approaches to identify differences between groups and/or environments in terms of resistance genes, mobile genetic elements or taxonomic compositions. All the statistical analyses were conducted in R (R Development Core Team, 2013).

3.6.1 Choosing appropriate statistical tests to analyse genomic and metagenomic data

After obtaining the count data of functional genes or taxa from bioinformatics analysis of genomic or metagenomic data, the next step usually is to perform a statistical test to assess the differences between experimental conditions or groups. Both parametric (e.g. t-tests, Analysis of Variance- ANOVA) and non-parametric tests (e.g. Wilcoxon, Kruskal-Wallis) can be performed depending on the characteristics of the data (e.g. sample size, ordinal or ranked data, their distribution, outliers) to test group means or medians. In **paper IV** of this thesis, differences in bacterial growth and alpha-diversity measurements between communities from unpainted and painted surfaces were tested for significance using a two-way ANOVA with time and treatment as independent variables.

Genomic and metagenomic count data are not normally distributed, and thus data transformation is frequently applied (often by square root or logarithm transformation) to change the distribution of the data so that it better fits the assumptions of standard parametric tests. However, in many metagenomic samples, individual genes or taxa counts are often zeros and thus have high variance. Thus, the individual genes or taxa are often binned into certain groups or classes, resulting in low variance, and make parametric tests appropriate for testing the differential abundance of groups or classes. In **paper IV**, we used an over-dispersed Poisson generalised linear model in an ANOVA-like design including covariates for time and treatment for differentially abundant resistance genes (Kristiansson et al., 2009). In addition, because of the non-normal distribution of count data from genome and metagenomic analysis, non-parametric tests such as Kruskal-Wallis and Wilcoxon rank-sum tests are also frequently used to determine whether the median of the observations from two or more group are the same. Many large-scale genomic (Smillie et al., 2010; Gibson et al., 2015) and metagenome studies (Le Chatelier et al., 2013; Qin et al., 2012) comparing two groups of individuals have used the Wilcoxon rank-sum test, which is a rank-based non-parametric test for comparing two groups of observations without any assumption of certain distribution. The Kruskal-Wallis test is an extension of the Wilcoxon rank sum test with more than two groups of observations. In **paper II** in this thesis, Wilcoxon rank sum tests were performed to test the difference in sizes between plasmids with and without co-selection potential, as well as between conjugative and non-conjugative plasmids.

In statistics, correlation and regression are used to determine the associations between variables. Correlation is usually used to measure the strengths of association between two variables. The value of the correlation coefficient from a correlation analysis varies between +1 and -1, where +1 is interpreted as the strongest association between the two variables. Usually, the common ways to assess correlations between variables are Pearson correlation, Kendall rank correlation and Spearman rank correlation, and they are based on different assumptions. Choosing the right correlation tests largely depends on the characteristics of the data. Pearson correlation is widely used in statistics to measure the degree of the relationship between variables with assumptions such as homoscedasticity of data (i.e. same variance in different groups). In contrast, both Kendall and Spearman rank correlations are non-parametric tests that measure the strength of dependence between two variables, and are appropriate correlation analyses when the variables are measured on a scale that is at least ordinal. In **paper III**, the association between the richness of resistance genes and taxa was assessed. Since the data did not follow any standard parametric distribution and the number of samples was high, Spearman's rank correlation was used. Similarly, partial correlations (Spearman's) between the richness of ARGs and biocide/metal resistance genes were calculated, while controlling for the effect of taxonomic richness.

Many of the statistical analyses deal with hundreds of genes and can introduce false positives. For some genes, statistical tests can have significant *p*-values (e.g. lower than 0.05) purely by chance. Therefore, to take that into account for multiple testing, false discovery rate (FDR) is controlled (Benjamini and Hochberg, 1995). Thus, the *p*-values obtained from statistical tests in **paper IV** were adjusted for multiple testing using Benjamini-Hochberg false discovery rates and an FDR$\leq$0.05 was considered statistically significant.

I acknowledge that in **paper II**, the purpose of the large-scale genomic study was simply to identify combinations of ARGs and biocide/metal resistance genes that are common in sequenced bacteria, ignoring the evolutionary pressure that might result the overrepresentation of co-occurrences of pairs of known resistance genes. I also acknowledge that, in the study described in **paper III**, we considered applying statistical tests to assess significant differences in relative abundance of resistance genes between environments. However, the metagenomes covered within each environment type (e.g. animal, human, water, soil etc.) were collected from multiple studies and thus not random and independent samples from these environment types (i.e. there could be a strong bias towards a particular animal species, a particular study etc.). This could easily lead to invalid *p*-values and over-interpretation of the data. Thus, we intentionally chose not to perform formal statistical tests to assess any significant differences in the relative abundance of resistance genes between environments.

3.6.2 Diversity analysis of resistance genes and taxa

Diversity is often calculated from metagenomic data to estimate the varieties of resistance genes or taxa that are found in a sample or environment. Richness is the simplest form of diversity estimation, which simply counts the number of different genes or species in a sample, if needed normalised to a given sequencing depth. Rarefaction approach takes this one step further and calculates the richness at multiple sequencing depths. In the study described in **paper III**, the richness of resistance genes, MGEs and bacterial genera were estimated using subsamples of 10 million reads from each metagenome. Similarly, in the study described in **paper IV**, we estimated the richness of bacterial genera using subsamples of 35 million reads. To estimate the alpha-diversity (i.e. diversity within each sample), the Shannon diversity index which takes into account both richness (i.e. numbers of kinds) and evenness (i.e. distributed equally or not) of taxa in a microbial community are also used (Shannon, 1948). Shannon's diversity index increases if both richness and evenness increases. Since the Shannon index incorporates both richness and evenness, it becomes difficult to compare microbial communities that differ greatly in richness. Therefore, in **paper IV**, in addition to bacterial richness, we also calculated Shannon's indices and Pielou's evenness indices.

Distribution of functional and taxonomic profiles and how much they vary between samples within an environment may be of interest from an ecological perspective. Thus, in the study described in **paper III**, the beta-diversity (i.e. diversity between samples) was estimated based on metrics considering the presence/absence data of resistance genes, MGEs and taxa (family level). To further evaluate the ecological processes that drive high/low beta-diversity of resistance genes and taxa between samples, we used the approach proposed by Baselga (2014), and divided beta-diversity into two parts – 'nestedness' and 'turnover'. The nestedness describes the variability caused by losing resistance genes from a common "core", while turnover instead describes the variability that comes from unique genes appearing in different samples. The rationale behind using such an approach is that nestedness can describe the tendency to lose resistance genes or taxa, whereas turnover instead can describe the tendency to replace resistance genes/taxa with other genes/taxa. These two processes cannot be distinguished through by only estimating the total beta-diversity. For example, assume that there is a high beta-diversity of beta-lactamases in the human gut. The high beta-diversity could be due to that some individuals carry many beta-lactamases and some few; or due to that different individuals have different sets of beta-lactamases. Therefore, in **paper III**, the measure of nestedness and turnover was used in beta-diversity analysis.

4. RESULTS AND DISCUSSION

4.1 Database contents

Paper I of this thesis describes the BacMet database, an easy-to-use bioinformatics resource of antibacterial biocide- and metal-resistance genes. The BacMet database consists of two databases - a database of manually curated and experimentally verified resistance genes, and a database of predicted resistance genes based on sequence similarity. Currently, the BacMet database contains 704 and 40556 resistance genes to antibacterial biocides/metal resistance genes in 'experimentally verified' and 'predicted databases', respectively (Table 3). The database contains resistance genes to 43 different chemical classes including 58 antibacterial biocides, 23 metals and 30 other xenobiotic compounds. To the best of our knowledge this is the first comprehensive resource of antibacterial biocides and metal resistance genes. While there are many databases for resistance genes to antibiotics, BacMet database provides, for the first time, the opportunity to study co-selection of resistance via co-occurrences of resistance genes in bacterial plasmids and genomes on a large scale. According to the user statistics data, the database has users from 84 different countries (as of January 2017). To increase usability, BacMet database provides the opportunity for the users to submit their own data related to antibacterial biocides and resistance with a condition of internal curation before submitted data included in the database.

Table 3. General statistics of the BacMet database contents (version 1.1 as of January 2017)

Database characteristics	Counts
Total compounds listed	111
Total experimentally verified resistance genes	704
Chromosomal resistance genes in BacMet experimentally confirmed database	520
Plasmid-borne resistance genes in BacMet experimentally confirmed database	184
Biocide resistance genes	260
Metal resistance genes	379
Genes with both biocide and metal resistance potential	65
Total resistance genes in BacMet predicted database (non-redundant)	40556

The BacMet database contains resistance genes to both essential metals (e.g. iron, manganese, cobalt and magnesium) and metals with toxic properties (e.g. arsenic, cadmium and mercury). In addition to the sequence data and other relevant information (e.g. compound description) for the resistance genes, BacMet provides tools for identification of biocide- and metal-resistance genes in proteins and DNA sequences including genomes, plasmids, metagenomes and metatranscriptomes. BacMet database also provides the similarity search tool – BLAST on the BacMet web-server, where user has the opportunity to characterise resistance genes to biocides/metals in user-provided sequence data. In addition to browsing the entire database for information of genes of interest, it is also possible to download the database sequences for off-line data analysis of sequence datasets.

Many novel potential resistance genes to both antibiotics and metals have been described in complex microbial communities using functional metagenomic approaches or domain-specific similarities search (Li et al., 2014; Pehrsson et al., 2016; Allen et al., 2009). Hence, there are most likely many unknown resistance genes to both antibiotics and biocide/metals that we have not been encountered yet in cultured bacteria isolated from complex microbial communities. In addition, many cultured bacteria have neither been characterised nor verified for their resistance functions (if there are any) in experimental settings. Over time, we can therefore foresee that databases, including BacMet, should expand to correspond to a growing list of known resistance genes. An update of the BacMet database is planned for 2017.

The BacMet database is not only a resource for the scientific community dealing with the risks associated with antibacterial biocide and metals. Manufacturers of metal surfaces and coatings, biocide companies and producers of food preservatives can benefit from the BacMet database as it can assist in understanding the development of tolerance mechanisms to certain products.

4.2 Potential biocides/metals for risk of co-selection

In **paper II**, network analysis was used to present new insights into the co-selection potential of antibacterial biocides and metals towards antibiotics, based on common co-occurrences of resistance genes. On plasmids, mercury and QACs were found to have higher potential for co-selection for resistance to a range of different classes of antibiotics via co-resistance (i.e. co-occurrence) mechanisms. On plasmids, resistance genes to most other biocides and metals rarely occurred together with ARGs, suggesting a limited risk scenario for co-selection of antibiotic resistance. On the other hand, resistance operons of metals such as copper, silver and arsenic were frequently occurred together on plasmids but separately from ARGs, suggesting a limited risk scenario with the transfer potential between bacteria by these metals for metal-driven co-selection of antibiotic resistance. It also suggests that these metals have a higher potential to select for resistance towards each

other, but more rarely so for antibiotics. We know that the presence of metal resistance genes (MRGs) in bacteria is not random (**paper II**). There is evidence that associate the metal exposure in different environments (due to either geological or anthropogenic activities) and presence of metal resistance genes (Zhou et al., 2016; Berg et al., 2010). Use and misuse of antimicrobial metals have also resulted in high abundance of such resistance genes in bacteria (Yazdankhah et al., 2014; Zhu et al., 2013). Besides that, there is a new hypothesis that macrophage killing and protozoan predation also act as a driver for the occurrence of resistance genes to certain set of metals (e.g. copper, zinc, arsenic) in bacteria (Hao et al., 2015; 2016; Hao et al., submitted). It is believed that macrophages and protists use metal-poisoning as a weapon to kill bacterial prey (Hao et al., 2015; 2016) and in that case often a toxic mix of metals (i.e. copper, silver, zinc and arsenic) is used by protists and macrophages. In response, to survive from such metal stress, bacteria evolve metal detoxification systems and are selected for metal resistance (Hao et al., 2016), resulting frequent occurrences of metal resistance genes in bacteria. Therefore, protozoan predation of bacterial prey could be considered as a major driver for the selection of metal resistance and co-selection of the antimicrobial resistance, and provide an argument to support why co-occurrences of copper, silver and arsenic resistance operons are frequently observed on plasmids.

For the first time, the study described in **paper II** also uncovered frequent co-localization of a gene (*cad*D) conferring resistance to cadmium and zinc, and resistance genes to aminoglycosides and macrolides, on plasmids. This genetic co-localization suggests that these heavy metals could not only to co-select for resistance towards aminoglycosides and macrolides but also promote HGT of antibiotic resistance. This is a matter of concern since after a ban on using antibiotics for growth promotion of animals in Europe and some other parts of the world, supplementation by metals such as zinc, cadmium and arsenic is commonly used for growth promotion in livestock and poultry industries (Nachman et al., 2013; Li et al., 2010). Thus, a serious consequence of the unrestricted use of these metals in animal growth promotion could potentially be a promotion of antibiotic resistance towards macrolides and aminoglycosides.

In many isolated studies where plasmids have been characterised, other co-occurrence patterns between resistance genes to biocides/metals and antibiotics have been observed but our network analysis showed that they are not frequently occurring. For example, the plasmid isolated from an outbreak in Uppsala university hospital (see Figure 4) was shown to carry resistance genes to arsenic, copper, QACs and a range of antibiotics. I acknowledge that over time, when more completely sequence plasmids become available, many other unknown but frequent co-occurrence patterns, and thus potential for co-selection, between antibiotics and biocides/metals will be uncovered.

4.3 Distribution of resistance genes and co-selection potential across environments

In **paper II**, we showed that resistance genes to antibiotics and biocides/metals occurred together more frequently in bacterial isolates from human and domestic animal sources than in isolates from other environmental sources. Similarly, when only plasmids rather than entire genomes were considered, similar patterns could be observed. These results support that bacterial isolates or plasmids from human and animal sources are at a higher risk for co-selection of antibiotic resistance. In **paper III**, we found that these are the two environments where ARGs are most common in terms of relative abundance and diversity compared to most other environments. One can argue that this is a logical consequence of the fact that microbiota from humans and domestic animals are the only two sources that we regularly and intentionally exposed to high levels of antibiotics. In contrast, resistance genes to biocides/metals were widely distributed in bacterial isolates from different environments (**paper II**), where the majority of bacterial chromosomes (85%) carried genes involved in biocide/metal resistance mechanisms, some of which were ubiquitous across all environments. When we studied bacterial communities across environments, a higher abundance and diversity of resistance genes to biocides/metals was observed compared to ARGs (**paper III**). Thus, one of the hypotheses that can be established from these findings is that exposure to antibiotics has driven the overrepresentation of co-occurrence between ARGs and biocide/metal resistance genes in bacteria from humans and animals, rather than exposure to biocides or metals. Since metals are widespread across environments (Nriagu, 1996) including humans and animals due to high use for a variety of purposes, there are ample opportunities for selection given that local concentrations are sufficiently high. Since human- and animal-associated microbiota are the two main sources, where not only commensals but also pathogenic bacteria reside, the largest potential for biocides and metals to promote clinically relevant antibiotic resistance is, therefore, in and on our bodies. An addition, the role of external environments should also be considered since the external environments can serve as potential transmission routes for pathogens (**paper III**).

4.4 Bacterial taxa with high potential for co-selection

Based on the co-occurrence analysis of resistance genes on 4582 plasmids and their host bacteria belong to 333 different genera, we found that presence of ARGs on plasmids was particularly frequent in certain set of bacterial genera such as *Providencia*, *Citrobacter*, *Klebsiella* and *Enterobacter*, where over three copies of ARGs were found per plasmids (**paper II**). In contrast, biocide/metal resistance genes were found in bacteria belong a wide number of different genera including many clinically relevant ones, such as *Escherichia*, *Staphylococcus*, *Salmonella* and *Klebsiella,* as well as some genera mostly associated with the external environment, for example *Acetobacter*, *Sinorhizobium*, *Burkholderia* and *Rhizobium*. However, co-occurrences of these two genes types – ARGs and resistance genes to

biocides/metals was largely found in clinically important bacterial genera including *Escherichia*, *Staphylococcus*, *Salmonella* and *Klebsiella* (**paper II**). This is most likely a consequence of that the pathogens have been exposed to a selection pressure from antibiotics to a much greater extent than other bacteria have.

We do not know to what extent pathogenic bacterial strains carry these plasmids with resistance genes to both antibiotic and biocides/metals, thus the potential for co-selection. However since most well-described pathogenic strains belong to these genera, there is a higher probability that plasmids from these genera will show a similar pattern of high potential for co-selection. This clearly shows the risk for biocide/metal-driven co-selection in clinical settings, where antibacterial biocides and metals are often used. It is noteworthy that many plasmids isolated (often via metagenomic assembly) from uncultivated sources were shown to carry resistance genes to both antibiotics and biocides/metals, suggesting a reservoir of uncultivable bacteria exist in microbial communities which can carry plasmids with co-selection potential.

I acknowledge that the completely sequenced plasmids available in the NCBI plasmid database unevenly represent the phylogenetic tree of bacteria. For example, 47%, 30% and 9% of the completely sequenced plasmids studied in **paper II** were isolated from bacteria belonging to phyla *Proteobacteria*, *Firmicutes* and *Spirochaetes*, respectively. However, it still covers a wide range of bacterial genera (313 genera) from mainly these three major phyla. It also represents isolation sources not only from clinically relevant bacteria but plasmids isolated from a wide a range of environmental sources, supporting our generalisations on the co-selection potential of genera comprising many pathogens.

4.5 Associations between plasmid characteristics and their co-selection potential

In **paper II**, we showed that plasmids with co-selection potential (due to the co-occurrence of resistance genes) tend to be conjugative (i.e. carry conjugations systems) compared to plasmids lacking resistance genes to different antimicrobials. We also found that the size of the plasmid has an effect on the presence of both ARGs and biocide/metal resistance genes on plasmids. Plasmids with both ARGs and biocide/metal resistance genes tended to be larger than those without this combination of genes. Among resistance plasmids, usually small ones with a size of less than 10 kb carried only ARGs, but not biocide/metal resistance genes. In contrast, among resistance plasmids, usually the ones with a size of over 20 kb more often carried resistance genes to both antibiotics and biocide/metals.

In addition, the association between the presence of toxin-antitoxin systems and resistance genes to both ARGs and biocide/metal resistance genes was also examined. Plasmids carrying resistance genes to biocides/metals were found more likely to carry toxin-

antitoxin systems than plasmids without any resistance genes (**paper II**). This is largely explained by biocide/metal resistance genes and toxin-antitoxin systems both being more frequent with larger plasmids size. However, plasmids with only ARGs did not follow a similar size distribution. Accordingly, toxin-antitoxin systems were equally common on plasmids carrying only ARGs as on plasmids carrying no resistance genes at all.

4.6 Distribution of MGEs across environments and their role in co-selection

In **paper III**, characterisation of known MGEs (especially integron-associated integrases and ISCR transposases) across environments revealed that their relative abundances and the richness were highest in the environments polluted by discharges from pharmaceutical production and in wastewater/sludge. In contrast, human and animal microbiomes carried much lower abundances of MGEs. Greater diversity and relative abundances of MGEs were observed in metagenomic samples from external environments. This was observed despite the fact that MGEs are studied in much greater depth in human pathogens due to the clinical importance of HGT of resistance genes, which in turn would be expected a biased estimation of a higher prevalent of MGEs in the human microbiome compared to external environments.

The human microbiome was dominated by the transposases ISCR2, ISCR5 and ISCR8 and integron-associated integrase class 1 (*intI*1). In contrast, in metagenomes from external environments, almost all types of investigated integrases and ISCR elements were detected in relatively high abundances. In the field study (**paper IV**), under copper and zinc stress (via antifouling paint), the most abundant integrase genes in bacterial communities from the painted surfaces in marine environments were integrases class 9 (*intI*9) and 10 (*intI*10), highlighting that the types of MGEs present in external environments are quite different from the ones that are frequently found in human and animal microbiomes (**paper III**). In the field study, we observed an overall higher relative abundance of both integron-associated integrase and ISCR transposase genes in bacterial communities from antifouling-painted surfaces compared to those from unpainted surfaces. Therefore, it suggests that bacterial community exposed to the copper and zinc-based antifouling paints can create a hotspot for mobilisation of resistance genes via MGEs.

When we studied only the completely sequenced bacterial genomes and plasmids, we found that integron-associated integrases and ISCR transposases were common on plasmids and were found on 7-10% of the plasmids carrying ARGs and/or biocide/metal resistance genes. Based on network analysis of resistance on plasmids, we also found that MGEs such as integrons and ISCR transposon elements are often found on the same plasmids as ARGs and biocide/metal resistance genes (**paper II**). Thus, selective pressures favouring maintenance of biocide/metal resistance genes within gene cassettes of integrons

may contribute to the maintenance of ARGs that are physically linked. Thereby, transposons and integrons might play a role in the process of biocide/metal-driven co-selection of antibiotic resistance. Studies, for example, Gaze et al. (2011) found that MGEs such as class 1 integrons, and *qac* resistance genes (mainly confer resistance to quaternary ammonium compounds) are common in the bacterial community exposed to antibiotics, biocides and/or detergents, suggesting opportunities for co-selection of resistance via MGEs.

4.7 Antifouling paint select for antibiotic resistance via cross-resistance

In **paper IV**, we studied marine bacterial communities to investigate whether copper and zinc-based antifouling paint can select for antibiotic-resistant bacteria. Since, biocides/metals can provide a selection pressure on bacterial communities, an enrichment of genes that encode the resistance/nodulation/division (RND) efflux systems were observed in bacterial communities from painted surfaces compared to bacterial communities from unpainted surfaces. Interestingly, nearly half (48%) of all detected resistance genes that confer resistance to biocides/metals were the genes involved in efflux systems, of which nearly 70% were RND efflux systems. We estimated almost four-fold higher relative abundances of resistance genes involved in encoding RND efflux systems in bacterial community from painted surfaces compared to bacterial community from unpainted surfaces. Metal ions have been described to induce expression of RND efflux pumps in a wide range of bacteria (Nies, 2003). In addition, the RND efflux systems have already been documented to play major roles in bacterial multi-drug resistance (Poole, 2005; Webber and Piddock, 2003), and are highly relevant to antibiotic resistance in Gram-negative bacteria (Piddock, 2006). In our study, many of the detected RND pumps were multi-drug resistance efflux systems that are capable of exporting not only metals but also antibiotics, thus providing opportunities for cross-resistance between metals, biocides and antibiotics to the bacterial host. Notably, one of the most abundant and enriched RND systems by the antifouling paint was GesAB (part of the GesABC efflux system), which has the potential to provide resistance to a wide range of antibiotics, biocides and other xenobiotics via cross-resistance, including chloramphenicol, thiamphenicol, cloxacillin, oxacillin, chlortetracycline etc (Conroy et al., 2010). We also found high relative abundance of GolT, a P-type ATPase, which can provide high-level copper resistance in the absence of CopA (Espariz et al., 2007). In paint-exposed bacterial community, except the resistance gene *vat*F, conferring resistance to macrolides, no enrichment of mobile ARGs was observed. However, phenotypically we observed tetracycline resistance isolates from the bacterial community. Therefore, the presence of high-level tetracycline resistance (phenotypically) but absence of high level classical *cop/pco* genes in the paint-exposed bacterial community could be explained by the presence of high abundance of *ges*AB genes encoding multidrug-efflux systems, and *gol*T genes. This suggests that there might be many

yet unrecognized co-selection opportunities mediated by genes enriched in the antifouling paint-exposed communities. Taken together, copper and zinc-based antifouling paint showed the potential to select for metal resistance, as well as cross-resistance to antibiotics via multidrug-efflux systems.

4.8 Limitations in different studies, research needs and final remarks

4.8.1 Studying cross-resistance mechanism is needed

In **paper II**, we dealt with completely sequence bacterial genomes and plasmids, and largely focused on co-resistance mechanisms by studying co-occurrences of resistance genes. Cross-resistance (e.g. due to efflux systems or mutation-based) usually also play a major role in co-selection but it was largely out of scope for this thesis to describe opportunities for cross-resistance among different bacteria and communities. However, we studied cross-resistance via RNA efflux systems in **paper IV** in limited depth. To obtain a complete picture of co-selection of resistance more studies on cross-resistance and co-regulatory resistance are needed.

4.8.2 Metadata is highly important in microbiome research

I acknowledge that in **paper III**, we were unable to classify the animal-associated environments into domestic and wild groups as we did in **paper II**. In **paper II**, we analysed all publicly available bacterial genomes and plasmids from various sources (including animals). There, we found that antibiotic resistance genes were more abundant in bacteria/plasmids isolated from domestic animals compared to wild animals. Therefore, the idea of sub-division of animal-associated metagenomes into wild and domestic groups based on available metadata was initially considered in **paper III** as well. However, lack of comprehensive metadata in MG-RAST for most of these animal-associated metagenomes was a critical limitation. In addition, many metagenomes retrieved from the MG-RAST were not associated with published papers, and thus tracking down important metadata information from the literature was also a major challenge. These limitations have unfortunately restricted us to label all the metagenomes collected from animal sources as being part of the same 'animal-associated' category, irrespective of their actual habitats and/or the level of antibiotic use/treatment.

4.8.3 More studies are needed on air

I acknowledge that all deeply sequenced publicly available air samples (over 10 million reads per metagenome) studied in **paper III** were derived from a single Beijing smog event

that lasted for 5 days. Therefore, there are considerable limitations on to what extent we can generalize other types of air environments, or even other cities, with different sources of bacteria. Therefore, more air metagenomes would be needed to allow broader generalisations. We included available small metagenomic datasets (i.e. only a few hundred thousand sequences per metagenome) generated on 454 sequencing platform, from US air samples that overall supported the hypothesis that air could be a more important transmission route for resistance than acknowledged so far. However, to make conclusions about risks, one would need to investigate if the bacteria carrying the resistance genes belonged to pathogenic species/strains, to what extent they were alive in the air, and assess the abundance per volume of such bacteria to be able to judge the likelihood of being exposed to an infectious dose.

4.8.4 Genetic context of resistance genes is of high interest

Determining the genetic context for ARGs and their association with neighbouring genes on a genetic element is indeed critical to shed light on the risks for spread of resistance (Bengtsson-Palme and Larsson, 2015). If the resistance genes are located in association with integrases or transposases on chromosomes, there is a risk for transfer of such resistance genes to plasmids. However, if they are located on plasmids already, the risk for spread of resistance is even higher. Performing such analyses in metagenomes is, however, not without major challenges. Some of previous metagenomic studies in our own lab (Bengtsson-Palme et al., 2016b; 2015a; 2014), looked into the genetic contexts of resistance genes via metagenomic assembly, but identified many technical challenges associated with the assembly process, especially around resistance gene regions (mainly because the assembly algorithms fail when the same gene can be surrounded by a large variety of DNA sequences). In all these studies, we were only able to identify the context of a very small fraction of the resistance genes, and probably these examples were biased towards those genes occurring only in few contexts of which one or few were with strongly dominant. Therefore, assembling the large number of metagenomes (n=864, corresponding to 9.2 Tb of sequence data) would have taken a very long time while relatively little information would, at present, have gained from that effort. Identifying the genetic contexts of resistance genes was therefore considered to be out of scope in **papers III** and **IV**.

4.8.5 Understanding the link between resistance genes and taxa is important

It is important to bring up taxonomy in relation to resistance genes in metagenomes, as resistance genes are not randomly distributed across species. Linking specific genes to a specific host species is one of the major limitations that metagenomic approaches face, which need to be overcome in the future. In **paper III**, we highlighted the recently developed epicPCR methodology (Spencer et al., 2016), which has the potential for

identifying the possible hosts of specific genes (including resistance genes) in microbial communities.

4.8.6 Co-selection potential is difficult to predict from metagenomic data

Determining the co-selection potential of biocides and metals for antibiotic resistance has been studied in the **paper II** by studying the co-occurrences of resistance genes that are found together on completely sequenced bacterial genomes and plasmids. However, using shotgun metagenomic approach to study co-selection in individual bacterial isolates and microbial community has several limitations. One of the major technical challenges is to assemble the obtained short reads from the sequencing machines into complete sequences of the genomes of the microorganisms and plasmids present in the microbial community due to insufficient sequence coverage. As a consequence, it becomes a complicated task to extract individual genome and plasmid from metagenome data. Thus, determining the true genetic context of resistance genes in a bacterial strain or plasmid found in a microbial community becomes difficult. It is worth mentioning that recently developed epicPCR (Spencer et al., 2016) and Inverse-PCR (Pärnänen et al., 2016) methodologies can overcome some of these limitations. More importantly, Inverse-PCR methodology in combination with long-read sequencing technology (for example, using PacBio) could be a potential approach to study genetic context of resistant genes, especially their presence with and/or within the mobile genetic elements. Thus, potential for co-selection capabilities could be uncover, together with the potential for transfer of such genes via HGT.

In contrast to determining the co-selection potential bioinformatically via co-occurrences of resistance genes in bacterial genomes and plasmids, prediction of co-selection potential based on cross-resistance mechanism is more challenging (Pal et al., 2017). This is largely due to that a gene or mechanism such as efflux pump that are the key for cross-resistance often has broad and largely uncharacterised substrate specificity. Therefore, assessing cross-resistance potential of a bacterial strain should only be determined by phenotypic assays.

4.8.7 Concentrations of biocides/metals are important to consider for co-selection

In general, the 'in-use' concentration of biocides, often exceed 1,000 times than that of their MIC, in order to achieve a rapid rate of killing. At such high concentration, a biocide usually interacts with multiple target sites in the bacterial cell. As a result, bacteria rarely become resistant through adaptation or other mechanisms. However, bacterial communities are often also exposed to biocides at sub-inhibitory concentration. Exposure to sub-inhibitory concentrations of biocides to bacteria over time has been shown to increase resistance to biocides as well as antibiotics in bacteria (Molina-González et al. 2014; Capita et al., 2014; Webber et al., 2015; Christensen et al., 2011; McCay et al., 2010;

Huet et al., 2008). However, there are also opposite results that show no effect of sub-lethal concentration of biocides to antibiotic resistance (Suller and Russell, 2000; Kastbjerg and Gram, 2012). It is still not known at what minimum concentration certain metals can potentially induce and drive co-selection of antimicrobial resistance. In natural environments, the concentration of metals such as copper and cadmium often reaches to concentrations that are believed to be sufficient enough to drive the co-selection (Seiler and Berendonk, 2012).

Low concentrations of biocides and metals can be found in many different environments, for example, wastewater treatment plants and hospital effluents. These environments, where antibiotics are also present, may thus act as breeding grounds for bacteria where environmental bacteria interact with clinical pathogens allowing the transfer of resistance genes between them. Therefore, it is important to understand the minimal concentrations at which antibiotics, biocides and metals can select or co-select for antibiotic resistance. Understanding which exposure levels are safe versus posing a risk will help regulatory bodies to manage the use of biocides and metals in households, commercial industry and clinics in a more sustainable way.

4.8.8 Bioavailability of metals and xenobiotics determines their biological effects

The redox state and bioavailability of metals, and xenobiotics in the environment can be altered through physical or biological processes. Study has shown that tolerance to metals is generally highest in environments, such as sediment and biofilm, where metal bioavailability is comparatively higher compared to many other environments (Wright et al., 2006). Thus, the bio-availability should be taken into account when concerning the co-selection of heavy metals, because it is the bioavailable fractions of heavy metals that impose the selection pressure on bacteria (Seiler and Berendonk, 2012).

5. CONCLUSIONS

1. A database (BacMet) has been developed that can be used to characterise bacterial DNA, including genomes, plasmids and metagenomes for resistance genes to antibacterial biocides, metals and other xenobiotic compounds. The database has already become widely used in the scientific community.

2. We showed that bacteria carrying biocide/metal resistance genes are more likely to carry an antibiotic resistance gene compared to bacteria lacking biocide/metal resistance genes.

3. Plasmids with co-selection potential (due to the co-occurrence of resistance genes to both antibiotics and biocides/metals) tend to be self-conjugative (i.e. carry conjugations systems) compared to plasmids lacking resistance genes to different antimicrobials.

4. Plasmids carrying resistance genes to biocides/metals are more likely to carry toxin-antitoxin systems than plasmids without any resistance genes.

5. Plasmids with resistance genes to both antibiotics and biocides/metals tend to be larger than those without this combination of genes. Small resistance plasmids (less than 10 kb in size) tend to carry only ARGs but not biocide/metal resistance genes. In contrast, some resistance plasmids with a size of over 20 kb tend to carry resistance genes to both antibiotics and biocide/metals.

6. A set of metals and antibacterial biocides have a higher potential for co-selection for resistance towards to certain classes of antibiotics compared to others. For example, copper, silver, arsenic, antimony, cobalt, nickel, cadmium, iron, zinc, mercury and QACs are all potential co-selectors for resistance to, e.g. sulfonamides, beta-lactams, amphenicols, tetracyclines and aminoglycosides.

7. Clinically important bacterial genera such as *Escherichia*, *Staphylococcus*, *Salmonella* and *Klebsiella* harbour plasmids with resistance genes to both biocides/metals and antibiotics, and thus a higher potential for co-selection, compared to other bacterial genera. In addition, a reservoir of uncultivable bacteria exists in microbial communities which can carry plasmids with co-selection potential.

8. Two environments were found to be of particular risk for co-selection of antibiotic resistance – microbial communities associated with human and domestic animals.

9. We identified air and antibiotic-polluted environments as under-investigated transmission routes and reservoirs for antibiotic resistance genes.

10. The high taxonomic and genetic diversity of external environments supports the hypothesis that they can serve as vast sources of unknown resistance genes, with the potential to be transferred to pathogens in the future.

11. We found limited evidence for widespread opportunities of biocides/metals to co-select for antibiotic resistance plasmids, thus promoting horizontal gene transfer. On the hand, we identified ample possibilities for these chemicals to select for antibiotic-resistant bacteria through chromosomal biocide/metal resistance genes.

12. Based on the widespread presence of biocide and metal resistance genes across environments and the clear overrepresentation of antibiotic resistance genes in bacteria from domestic animals and human sources, where antibiotic usage is large, we hypothesize that the co-occurrence of antibiotic resistance genes and biocide/metal resistance genes most often is the results of a historical antibiotic selection pressure, rather than a historical selection from metals or biocides. However, for those bacteria when resistance genes to both compounds are found together, we should acknowledge the risk that biocides and metals can contribute to the promotion of antibiotic resistance.

13. Copper and zinc-based antifouling paint can select for metal-resistant bacteria and co-select for certain antibiotic-resistant bacteria, but without enriching for known mobile ARGs. Instead, the paint promotes bacteria carrying genes providing cross-resistance, such as those encoding RND efflux systems. Given the bacterial density in biofilms, the presence of genetic elements promoting mobilisation of DNA, and the prevailing selection pressure, surfaces painted with antifouling paint, such as ship hulls, might be hotspots for the emergence and dissemination of resistant bacterial strains in the marine environment.

14. Overall, there is scientific evidence that biocides/metals select for biocide/metal resistance in certain situations, but there is so far a lack of conclusive evidence that this is contributing to a significant part of the growing clinical challenges with antibiotic resistance. This may be because their contribution is, in fact, minor or because the possible causal links have not yet been comprehensively investigated.

6. FUTURE PERSPECTIVES

1. Many recent reports and antimicrobial resistance (AMR) action plans did not tackle all the potentially relevant pathways and drivers of antimicrobial resistance in the environment (WHO, 2015; European Commission, 2011; Department of Health/Defra, 2013; O'Neill, 2016). So far, most AMR action plans did not acknowledge the role of antibacterial biocides and heavy metals in selecting and maintaining antimicrobial resistance in the environment. In the future, closer attention to such drivers for antibiotic resistance promotion is needed. In a recent review by Singer et al. (2016), some of these deficiencies of AMR action plans have been highlighted.

2. A regular update of the BacMet database is needed. The next update is scheduled in February 2017.

3. Urban air and environments polluted by discharges from antibiotic manufacturing are under-investigated as potential sources, reservoirs and/or transmission routes for resistance genes, requiring more focus. Regulations need to be in place to reduce the environmental release of antibiotics from pharmaceutical manufacturing sites, and metals and biocides from other large-scale industrial sites.

4. The genetic context of resistance genes should be better studied in bacteria to uncover potential co-selection opportunities via co-resistance. In addition, the co-occurrence of such genes on mobile genetic elements such as plasmids could indicate the potential for horizontal transfer of these genes to other bacteria.

5. Cross-resistance (including mutation-based) and co-regulatory resistance mechanisms need to be considered together with co-resistance (co-localization) for obtaining a broad picture of co-selection in future studies.

6. Antibacterial biocides should only be used for purposes where there is enough evidence of benefits, in agreement with the Swedish strategy for combatting antimicrobial resistance (Swedish Government 2016). Biocides have clear advantages in certain applications, for example as disinfection agents in health care. At the same time, there are applications with little or no apparent benefits, such as uses in antibacterial soap and antibacterial surfaces of a range of products. Many studies have shown that normal soap is enough for hand washing than antibacterial soaps as there is no added health benefit for consumers when using antibacterial soaps (Luby et al., 2004; 2005; Larson et al., 2003; 2004; Faoagali et

al., 1995). Therefore, if usage is more based on that people are afraid of bacteria, rather than documented reduced risks for infections, we should consider not using them, i.e. benefits should be weighed against risks. Recently, the US Food and Drug Administration (FDA) imposed a ban on 19 biocidal compounds including triclosan and phenol (FDA, 2016).

7. The identified potential of metals (e.g. zinc/cadmium) to co-select for antibiotic resistance (against aminoglycosides, macrolides) through common co-occurrences of resistance genes on plasmids requires close attention (i.e. restrictions on using heavy metals as growth promoters in food animals). Recently, Committee for Medicinal Products for Veterinary Use (CVMP) of European Medicines Agency (EMA) recommended withdrawing zinc oxide from food supplements in animals because of the risks for co-selection for antibiotic resistance (EMA, 2016).

8. Another question that may arise in this context is if regulatory agencies should consider banning copper and zinc in antifouling paints due to risks for promoting antibiotic resistance. My answer is – probably not, at least not for this reason alone, given the knowledge we have today. On the risk side, there is still very limited evidence that this contributes to the emergence or transmission of antibiotic-resistant pathogens (not discussing other eco-toxicological effects of copper and zinc, as that is outside of the scope of this thesis). On the benefit side, there are major savings in fuel consumption (and hence emissions) and increased speed to consider. In addition, there is still a limited alternative to the use of heavy metals in antifouling paint, and the risks with alternative chemicals need to be evaluated as well.

9. In a real world scenario, the mixture of chemicals often common in different ecosystems. Combination effects of multiple chemicals including antimicrobial compounds can drive the co-selection of antibiotic resistance. Guo et al. (2014) showed that compared to exposure to a single metal, exposure to multiple metals can increase the abundance and diversity of antibiotic resistance genes in animal gut microbiota, suggesting a critical impact of co-exposure of multiple metals for co-selection of antibiotic resistance. However, more studies are needed to fully comprehend the risks for co-selection of antibiotic resistance due to combination effects of antimicrobial compounds.

10. There are conflicting pieces of evidence to whether sub-lethal concentrations of biocides and metals co-selects for antibiotic resistance (Capita et al., 2014; Webber et al., 2015; Peltier et al., 2010; Suller and Russell, 2000). Therefore, more studies are needed to evaluate e.g. the critical concentrations of antimicrobial biocides/metals for the selection/maintenance of multidrug-resistant strains in different environments and to better understand the role of

some genes in the adaptation of bacteria to different hosts. In addition, determining sub-lethal (selective) concentrations of biocides/metals that could drive HGT of ARGs between hosts could be important to focus on in the future (Jutkina et al., 2016) to better understand the risk for dissemination of resistance genes.

11. Finally, changes in public attitude are needed. Directly or indirectly we have contributions towards antibiotic resistance problem but many of us probably feel that we have no role in either the problem or its solution. Therefore, initiatives to inform and change attitudes at the global scale are encouraged.

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