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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers that will be referred to by their Roman numerals in the text.

I Nõlvak, H., Truu, M., Tiirik, K., Oopkaup, K., Sildvee, T., Kaasik, A., Mander, Ü., Truu, J. (2013). Dynamics of antibiotic resistance genes and their relationships with system treatment efficiency in a horizontal subsurface flow constructed wetland. *Sci. Total Environ. 461–462*, 636–644.

II Tiirik, K., Nõlvak, H., Oopkaup, K., Truu, M., Preem, J.-K., Heinaru, A., Truu, J. (2014). Characterization of the bacterioplankton community and its antibiotic resistance genes in the Baltic Sea. *Biotechnol. Appl. Biochem. 61*(1), 23–32.

III Tiirik, K., Nõlvak, H., Truu, M., Peeb, A., Kõiv-Vainik, M., Truu, J. (2021). The Effect of the Effluent from a Small-Scale Conventional Wastewater Treatment Plant Treating Municipal Wastewater on the Composition and Abundance of the Microbial Community, Antibiotic Resistome, and Pathogens in the Sediment and Water of a Receiving Stream. *Water*, *13*, 865.

Author's contribution

Paper I: The author is responsible for developing methodology and performing microbiological analyses (about 50%).

Paper II: The author performed most of the microbiological analyses (about 90%), participated in the data analyses (about 50%) and is responsible for writing the manuscript (about 60%).

Paper III: The author performed most of the microbiological analyses (about 90%), is responsible for the data analyses (about 80%) and for writing the manuscript (about 50%).

ABBREVIATIONS

ABC	ATP-binding cassette type efflux complex/pump
ARB	antibiotic resistant bacteria
ARG	antibiotic resistance gene
ATP	adenosine triphosphate
CARD	comprehensive antibiotic resistance database
CIA	co-inertia analysis
CRE	carbapenem-resistant *Enterobacteriaceae*
CW	constructed wetland
ESBL	extended-spectrum β-lactamase
EU	European Union
HGT	horizontal gene transfer
HSSF CW	horizontal subsurface flow constructed wetland
HT-qPCR	high-throughput quantitative polymerase chain reaction
LECA	light expanded clay aggregates
MATE	multidrug and toxic compound extrusion type efflux complex/pump
MC	mesocosm
MDR	multidrug resistance
MFS	major facilitator superfamily type efflux complex/pump
MGE	mobile genetic element
MLS	macrolide-lincosamide-streptogramin
MRSA	methicillin-resistant *Staphylococcus aureus*
NGS	next-generation sequencing
PCA	principal component analysis
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PE	population equivalent
qPCR	quantitative polymerase chain reaction
RND	resistance nodulation division type efflux complex/pump
RT-PCR	reverse transcriptase polymerase chain reaction
SARG	structured antibiotic resistance genes database
SMR	small multidrug resistance type efflux complex/pump
VRE	vancomycin-resistant enterococci
WGS	whole genome sequencing
WWTP	wastewater treatment plant

1. INTRODUCTION

The modern era of antibiotics started in the first half of the 20th century, and since then, making deadly infections curable, antibiotics have saved millions of lives. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat because all the antibiotics ever developed are susceptible to resistance (Ventola, 2015). The overuse and misuse of antibiotics has rapidly increased the development of the antibiotic resistance in microbes, and it is one of the most difficult challenges of the 21st century that poses a threat to modern medicine and food safety (Guitor *et al*., 2020). It is believed that the world is on the edge of a "post-antibiotic era", where treatable infections and routine surgery would become deadly (Hamad *et al*., 2019). Recent studies show that over 33 000 people die every year due to infections caused by antibiotic resistant bacteria (ARB) in the European Union (EU) and 700 000 globally (OECD, 2019).

The abundance of the prokaryotes (bacteria and archaea) in the world is currently estimated to be ~10^{30} cells (Flemming & Wuertz, 2019), and most of these microorganisms are not pathogenic. Numerous microorganisms produce antibiotics to gain a growth advantage and to defend against competing organisms, but antibiotics can also act as messenger molecules in microbial communities (e.g., in quorum sensing) (Berkner *et al*, 2014); therefore antibiotic resistance is not restricted to only pathogenic bacteria.

While antibiotic resistance is a major and growing public health concern, its surveillance and circulation in combinations of anthropogenic and environmental settings is remarkably limited. There are a number of studies of antibiotic resistance in environmental bacteria, suggesting that clinically significant antibiotic resistant bacterial strains often originate from the natural environment, including soil and water habitats (Almakki *et al*., 2019). These environmental ARB can disseminate antibiotic resistance genes (ARG) to human pathogens, which in turn can propagate ARGs to further recipients. Hence, the identification of sources of ARGs, their distribution in both the anthropogenic and natural environment, and an analysis of anthropogenic factors involved are necessary for the development of a strategy for combating antibiotic resistance (Osińska *et al*., 2020).

Municipal wastewater treatment is one of the major routes by which ARGs from anthropogenic settings are introduced into natural ecosystems. In addition, as ARGs are mainly located on mobile genetic elements (MGE), the high density of microbes in wastewater treatment plants (WWTP) could provide an optimum environment for horizontal gene transfer (HGT) of ARGs between environmental bacteria and human pathogens (Karkman *et al*., 2018). In contrast to nutrients (and some pathogens), there are no official limits of ARG amounts released to the natural environment via WWTP effluents.

Aquatic environments, including surface and groundwater bodies, receive ARG rich effluents from WWTPs, runoff from agricultural activity and other human

inputs, and provide suitable settings for ARGs dissemination and the horizontal exchange of ARG carrying MGEs (Marti *et al*., 2014). Treated wastewater usually contains lower amounts of ARB and ARGs than raw wastewater. However, the discharge of treated wastewater can still increase the amount of ARGs in the aquatic environments downstream of WWTPs (Cacace *et al*., 2019). A load of antibiotic residues, ARB and ARGs may be transported to groundwater, rivers, and finally to the sea (Siedlewicz *et al*., 2018), and ARG carrying microbes can be transferred back to humans through direct (e.g., swimming, contaminated drinking water) or indirect (e.g., seafood) contact with the environment (Zheng *et al*., 2021). Still, little is known about the nature and mechanisms behind the transport, transfer and accumulation of ARGs in these interconnected aquatic systems.

2. AIM OF THE STUDY

The main aim of this thesis was to describe the dissemination pathway of the antibiotic resistance genes originating from the effluent of wastewater treatment plants (WWTP) through the primary receiving waterbody to the final receiving waterbody (Baltic Sea).

The specific aims were:

- to estimate the proportion and concentration of antibiotic resistance genes (ARG) in the effluents of different types of municipal wastewater treatment systems (small-scale activated sludge WWTP and constructed wetland);
- to assess the impact of the effluent of a small-scale WWTP treating municipal wastewater on the abundance and composition of the antibiotic resistome in the receiving stream and river;
- to determine the abundance of ARGs, often connected to anthropogenic impact, encoding resistance to major antibiotic classes (tetracycline, macrolide, sulfonamide, β-lactams, aminoglycoside) in different parts of the Baltic Sea and compare the antibiotic resistomes of studied aquatic environments.

3. LITERATURE REVIEW

3.1. Antibiotic resistance

By strict definition, antibiotics are low molecular weight substances produced by microorganisms that kill or slow the growth of other microorganisms but cause a little or no damage to the host (Davies, 2006). Antibiotics are one class of antimicrobials, a larger group of substances with natural, semisynthetic or synthetic origin which includes all agents that act against all types of microorganisms – bacteria (antibacterial), viruses (antiviral), fungi (antifungal) and protozoa (antiprotozoal) (Rothrock *et al*., 2016). Therefore, all antibiotics are antimicrobials, but not all antimicrobials are antibiotics. For clarity, the term antibiotic is principally used in broadened form which includes natural as well as synthetic and semi-synthetic antibacterial agents.

Antibiotic resistance is the ability of microorganisms to overcome the effect of antibiotics designed to kill them and it is one of the biggest threats to global health today (WHO, 2021). Resistance is described by either phenotypic (e.g., growth patterns) or genotypic (e.g., presence and/or expression of resistance genes) characteristics of bacteria and can be categorized according to origin (natural versus acquired resistance) or type (single, multiple, or cross-resistance) (Davison *et al*., 2000).

3.1.1. Use of antibiotics

Antibiotics are widely used to treat infectious diseases of humans, animals and also plants. At the beginning of the modern antibiotic era, synthetic compounds were used as antimicrobials before the discovery of natural antibiotics. In 1904, Paul Erlich believed that chemical compounds could be synthetized to selectively target disease causing microbes, and that idea led him to conduct a large-scale screening routine to find a cure to syphilis which was untreatable at the time (Aminov, 2010). Hundreds of arsenic derivates were synthetized and in 1909, a breakthrough was made with the discovery of arsphenamine (which was later marketed under the name Salvarsan) (Zaffiri *et al*., 2012). That systematic screening approach became the cornerstone of drug research in the pharmaceutical industry, and in 1930, it led to the discovery of sulfa drugs (Bentley, 2009). Sulfanilamide, which was a precursor to the active drug, was marketed under the name Prontosil and was extensively used by soldiers during World War II (Durand *et al*., 2019). Many constantly modified derivatives of sulfonamides, the oldest class of synthetic antibiotics in use today, are still a viable treatment option. Two other classes of synthetic antibiotics in extensive clinical use nowadays are the quinolones and oxazolidinones (Aminov, 2010).

Penicillin was the first natural antibiotic to be discovered in 1928 by Alexander Fleming when the *Penicillium* fungus contaminated a forgotten culture

plate in his laboratory. However, penicillin was not developed for clinical use until the late 1930s (Durand *et al*., 2019). The majority of antibiotic classes in clinical use today originate from the phylum Actinobacteria, from which 80%, in turn, are derived from the soil-dwelling genus *Streptomyces* (Barka *et al*., 2015).

During the so-called "golden age" of antibiotics from the 1950s to 1970s, most of the current antibiotic classes in use were discovered, and despite recent commercialization for some, no new antibiotic classes have been found after the 1980s (Durand *et al*., 2019). Also, regardless of the vast number of antibiotics discovered, less than 1% of them have held practical value in medicine (Reddy *et al*., 2011). In 2019, the average total (community and hospital sector combined) consumption of antibacterials for systemic use in the EU was in the range of 9.5–34.1 defined daily doses (DDD) per 1000 inhabitants per day (ECDC, 2020) and during the period of 2010–2019, a statistically significant decrease in consumption was observed for the EU overall. In addition to human medicine, antibiotics are also widely used in agriculture, aquaculture, horticulture and food preservation (e.g., nisin – E234).

3.1.2. Antibiotic resistance

The main problem with antibiotic therapy is that after a new antibiotic is introduced, resistance to it will eventually arise (Aminov, 2010) (Figure 1), including last-resort antimicrobials used in life-threatening, multidrug resistant (MDR) infections such as methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*, vancomycin-resistant enterococci (VRE) and carbapenem-resistant *Enterobacteriaceae* (CRE).

In clinical settings, resistant bacterial infections decrease available treatment options and increase mortality compared with those caused by susceptible bacteria (Boolchandani *et al*., 2019). Achievements in modern medicine so common today, such as major surgeries, organ transplantation, treatment of preterm babies and chemotherapy for treating cancer, would not be possible without effective treatment against bacterial infections (Laxminarayan *et al*., 2013).

Antibiotic resistance is a natural phenomenon that predates the selective pressure of clinical use of antibiotics (D'Costa *et al*., 2011). As the majority of antibiotics are produced by environmental microbiota, most of the antibiotic-producing strains also carry genes that encode resistance to the antibiotics they are producing, and these genes are usually located in the same gene cluster with the antibiotic biosynthesis pathway genes (Allen *et al*., 2010). Furthermore, most environmental bacteria that do not produce antibiotics themselves also harbor multiple resistance determinants (Cox & Wright, 2013). The role of ARGs in environmental bacteria is not only to provide defense against competitors and natural antibiotics, but ARGs also take part in other processes such as the modification and utilization of antibiotics as a food resource, detoxification of metabolic intermediates and signal trafficking (Martínez, 2008).

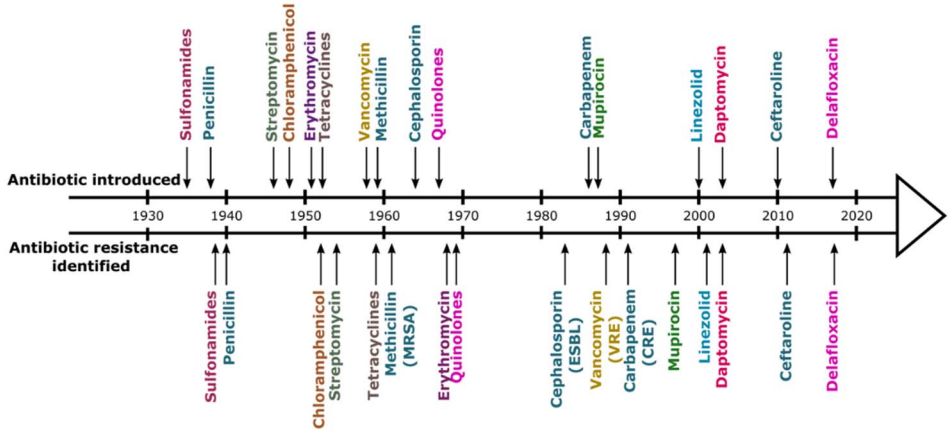

Figure 1. A timeline showing key events in antibiotic resistance development.

Naturally occurring (intrinsic) antibiotic resistance is widespread in bacteria. Still, anthropogenic factors, such as over- and misuse of antibiotics or the development of new multidrug resistant strains in hospitals and agricultural settings, are rapidly increasing the proportion of antibiotic resistance in natural environments. Antibiotic resistance precursor genes can evolve to new resistance mechanisms if they come to contact with a high concentration of antibiotics in the environment. Hence, antibiotics may act as selective agents but also as accelerator agents in resistance evolution (Marti *et al*., 2014). Many ARGs were originally located in the chromosome of non-pathogenic bacteria. Still, since the beginning of the modern antibiotic era, ARGs are increasingly found on MGEs in pathogens (Wright, 2010). So rapid dissemination of ARGs via horizontal gene transfer (HGT) is promoted. High amounts of ARGs are likely to persist in the environment because ARGs are often co-selected by heavy metals and other biocides, which increases the level of the natural background of ARGs (Czekalski *et al*., 2015; Henriques *et al*., 2016; Knapp *et al*., 2017).

Many ARGs present today in pathogenic bacteria originate from homologs that have evolved over hundreds of millions of years in either the naturally antibiotic producing bacteria or their competitors (Martínez, 2012). For example, an ESBL encoding *blaCTX-M* gene, often found in clinical pathogens, has shown to be similar with chromosomally encoded β-lactamases from *Kluyvera* spp., a typical environmental bacterium (Marti *et al*., 2014). Resistance to synthetic chemotherapeutic agents, such as quinolones and sulfonamides, may be available in the form of chromosomally encoded variants amongst the diverse bacterial domain, and can be rapidly dispersed upon the release of novel synthetic drugs (Sánchez-Osuna *et al.,* 2019).

The most attention of studying the resistance mechanisms in bacteria over the past decades has been focused on the pathogenic bacteria. Still, in many cases, these studies provide minimal information about the origins and further spreading pathways of antibiotic resistance (Wright, 2010). For understanding the resistance on a global scale, the concept of the antibiotic resistome has been

introduced. The antibiotic resistome is the collection of all the ARGs in bacteria and archaea (D'Costa, 2006), including ARGs in pathogens, antibiotic-producing microorganisms, cryptic embedded genes (which may or may not be expressed) in microbial chromosomes and also precursor genes that could evolve into ARGs (Wright, 2007).

3.1.3. Antibiotic resistance mechanisms

In principle, there are three main antibiotic targets in bacteria – the cell wall or membrane surrounding the bacterial cell, the machineries that make the nucleic acids DNA and RNA, and the machinery that produces proteins (Figure 2A). Since these targets are absent or different in human cells, the antibiotics usually do not harm our cells and are specific for bacteria.

The antibiotic resistome includes intrinsic and acquired resistance mechanisms (Figure 2B). Intrinsic resistance consists of mechanisms that have evolved as a general response to toxic molecules: the SOS response to DNA damage (Podlesek & Žgur-Bertok, 2020), MDR conferring efflux pumps (Schindler & Kaatz, 2016), chromosomally encoded inactivating enzymes such as β-lactamases (Lima *et al*., 2020) and entry barriers such as the outer membrane of Gram-negative bacteria (Acosta-Gutierrez, *et al*., 2018). Acquired resistance consists of mechanisms that evolve as countermeasures to particular antibiotics, often through HGT: compound-specific efflux pumps, expression of non-sensitive targets, and enzymes that modify targets or the antibiotic molecules (Surette & Wright, 2017).

The majority of antibiotics bind with a high affinity specifically to their targets, hence preventing the normal function of the target. Resistance can be achieved through changing the structure of a target so that the target is still able to carry out its normal function, but the antibiotic could not efficiently bind to it (target protection and target modification mechanisms; Figure 2B) or bypass the original target by producing additional low affinity targets (Peterson & Kaur, 2018). Antibiotic modification/degradation is also a commonly used strategy for converting an antibiotic ineffective, especially in the case of aminoglycoside antibiotics and chloramphenicol. The best example of antibiotic degradation is the resistance to β-lactam antibiotics, which is typically conferred by antibiotic-hydrolyzing enzymes known as β-lactamases (Peterson & Kaur, 2018).

In general, Gram-negative bacteria are intrinsically more resistant to antibiotics than gram-positive bacteria due to differences in their cell wall structure (Du *et al.*, 2018). The envelope of Gram-negative bacteria consists of an inner membrane, an outer membrane, and a peptidoglycan layer in the periplasm between the two membranes (Figure 3). Hydrophilic antibiotics (e.g., β-lactams, aminoglycosides, and glycopeptides) enter Gram-negative bacteria by diffusing through outer membrane porin proteins. Downregulating or replacing porins with more selective channels reduces the permeability (Figure 2B) of the outer membrane of the Gram-negative bacteria and therefore limits entering of the antibiotic into its cell (Blair *et al*., 2014).

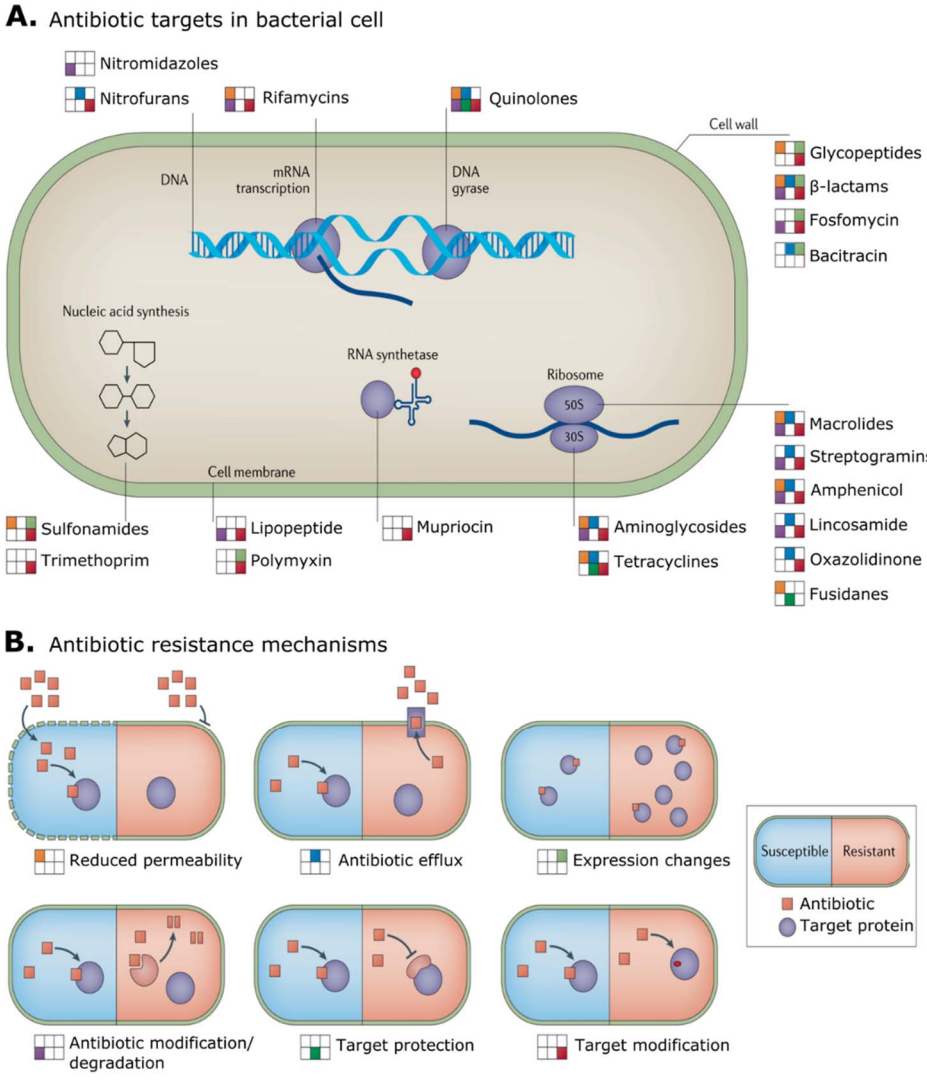

Figure 2. The grouping of antimicrobials by target site in bacterial cell (**A**). Antimicrobial resistance mechanisms acting against the antimicrobial class are depicted in grid left of the antimicrobial class name according to the layout shown in part **B**. (**B**) The mechanisms of antimicrobial resistance in resistant organisms (right, in orange), depicted in comparison with susceptible organisms (left, in blue). To the left of each labelled mechanism is the legend annotation position used in part **A**. The figure is modified from Boolchandani *et al*., 2019.

Microbial efflux pumps that transport many antibiotics out of the cell are found in almost all bacterial species and are major contributors to the intrinsic resistance in Gram-negative bacteria (Figure 3). Efflux pumps can also confer high levels of resistance to previously clinically useful antibiotics when they are over-expressed. Some efflux pumps have narrow substrate specificity (e.g., the Tet pumps that confer tetracycline resistance), but many are MDR efflux pumps that transport a wide range of structurally distinct substrates (Blair *et al*., 2015). In general, drug-specific efflux pumps are readily transmissible since they are usually located on plasmids, whereas MDR efflux pumps are usually chromosomally encoded and are not easily donated to other organisms (Schindler & Kaatz, 2016).

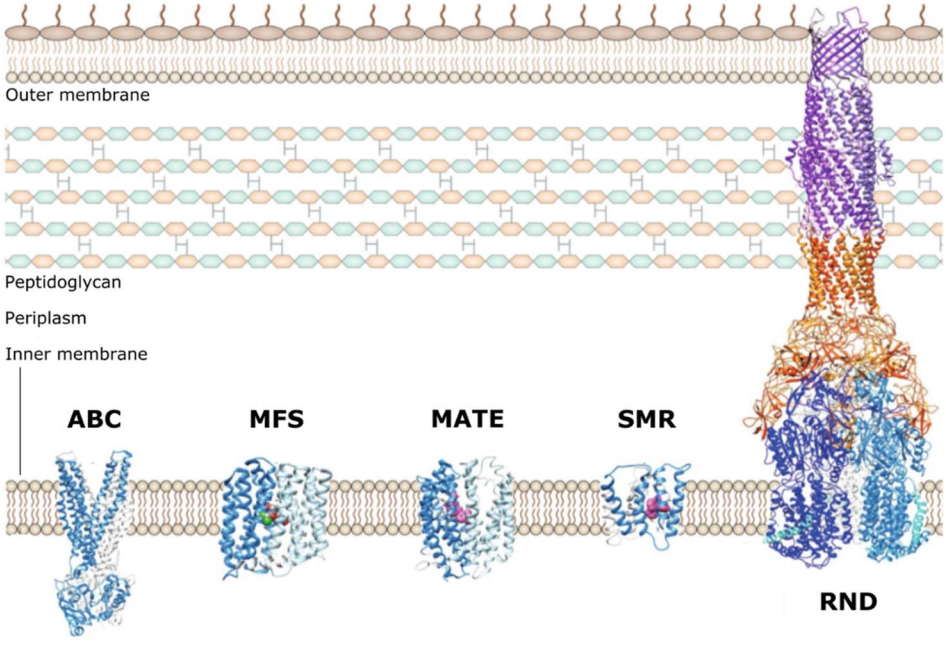

Figure 3. Structures of multidrug resistance (MDR) conferring transporter families, including the ATP-binding cassette (ABC), major facilitator superfamily (MFS), multi-drug and toxic compound extrusion (MATE), small multidrug resistance (SMR), and resistance nodulation division (RND) families. The figure is modified from Du *et al*., 2018.

MDR efflux pumps can be divided into two main groups – primary transporters, which use the energy of ATP (adenosine triphosphate) binding and hydrolysis for efflux, and secondary transporters, which are powered by the electrochemical potential of the membrane (Blair *et al*., 2014). ATP-binding cassette (ABC) family transporters are members of the first group and second group includes the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation division (RND) family and multidrug and toxic compound extrusion (MATE) family. In Gram-negative bacteria, all the MDR transporters are located in the inner membrane. The RND superfamily MDR transporters

mostly join with their partner proteins to form tripartite pumps (Figure 3), which bind substrates at the inner membrane and periplasm to efflux them to the cell exterior, when in contrast, members of the other families of MDR transporters usually function as independent units in the inner membrane to translocate substrates across the membrane bilayer (Du *et al.*, 2018).

RND family of MDR proteins were once thought to be exclusive to Gram-negative organisms, however, genes encoding proteins with structural characteristics of RND pump monomers are found within the genomes of Gram-positive organisms in addition to the four other types of MDR efflux pumps (Schindler & Kaatz, 2016). Consequently, broad-spectrum antibiotics (e.g., tetracyclines and quinolones), that act on both Gram-positive and Gram-negative bacteria, are more extensively used in medicine than narrow-spectrum antibiotics that affect only a single group of bacteria. However, an antibiotic with limited spectrum of activity may be quite valuable for the control of specific microorganism that fail to respond to other antibiotics (for instance vancomycin, that acts against Gram-positive bacteria such as *Staphylococcu*s, *Bacillus* and *Clostridium*). Also, antibiotic resistant Gram-positive organisms are responsible for some of the most serious human infections, including MRSA and VRE.

Bacterial antimicrobial resistance is usually genetically encoded, which can occur through several mechanisms, including overexpression or duplication of existing genes and point mutations but resistance can also be obtained by the acquisition of entirely new genes via HGT (Boolchandani *et al.*, 2019). This becomes a problem when ARG from non-pathogenic bacteria are transferred to pathogenic bacteria leading to clinically significant antibiotic resistance. HGT enables resistance genes to move between bacterial cells and also between different ecosystems. There are three main mechanisms of HGT – transformation, transduction and conjugation. Natural transformation is an active mechanism for taking up free DNA from the environment, conjugation occurs through direct contact between a donor and a recipient cell and transduction involves bacteriophages for the transfer of DNA (Soler & Forterre, 2020). Integrons are frequently carried on conjugative plasmids, and they are considered the primary agents of bacterial evolution due to their role in the dissemination of ARGs, development of MDR and their ability to add gene structures into bacterial genomes (Uyaguari-Díaz *et al.*, 2018). Therefore conjugation is presumably the principal route of HGT for antibiotic resistance spread in bacterial communities.

3.2. Spread of antibiotic resistance from anthropogenic sources to natural environment

Antibiotics and ARGs from anthropogenic sources can enter the environment through various routes (Figure 4), such as the discharge of hospital and municipal sewage, antibiotics manufacturing industry and landfill leachates of antibiotic disposal, animal husbandry, runoff from agricultural fields fertilized with manure or sewage sludge and fish farming (Ben *et al.*, 2019). This results in environments

where the natural environmental microbiome is mixed with antibiotics, ARGs and resistant bacteria from anthropogenic sources (Berglund, 2015) and it enables evolving of new resistant strains via HGT. In turn, humans may come into contact with resistant bacteria by numerous routes (Figure 4), for example consumption of crops grown using contaminated sludge or manure as fertilizer, drinking of water drawn from contaminated groundwater or surface water and swimming in marine water linked to contaminated surface water (Berglund, 2015).

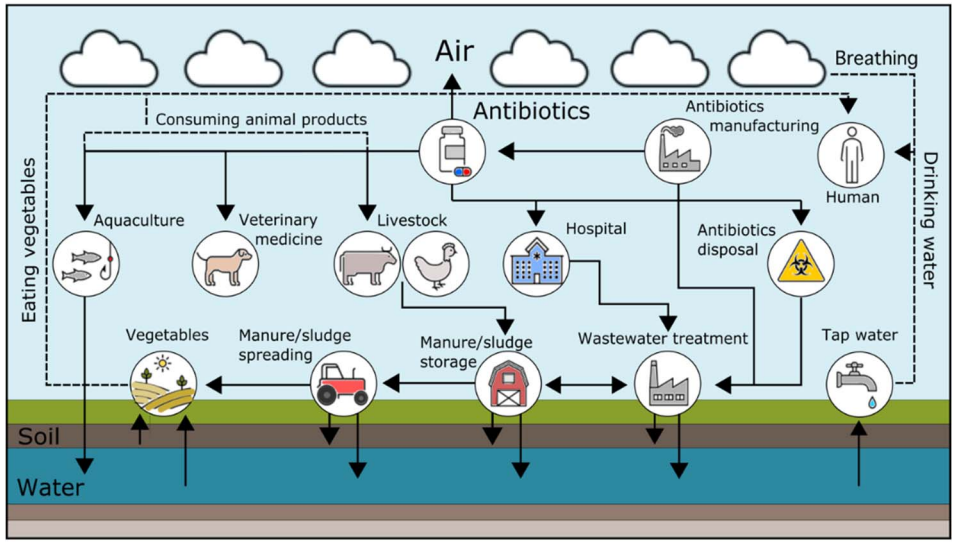

Figure 4. The network of antibiotic resistance spread routes between anthropogenic and natural environments. The figure is modified from Ben *et al.*, 2019.

Especially synthetic antibiotics, like sulfonamides and quinolones, and corresponding resistance genes can be used as human impact markers assessing natural environment quality. Sulfonamide resistance is one of the most widespread types of resistance and is very difficult to eliminate because sulfonamide resistance coding *sul1* gene is always located on a class 1 integron carrying plasmid (Poey *et al.*, 2019). Also, many *qnr* genes (e.g., *qnrA* and *qnrB*) have been found associated with class 1 integron carrying plasmids, making quinolone resistance a trait often associated with other resistance determinants co-carried on the integrons (Berglund *et al.*, 2015).

3.2.1. Wastewater treatment plants

The main goal of wastewater treatment is to remove organic compounds, nutrients and pathogens from the water, and the conventional wastewater treatment plants (WWTP) as well as alternative wastewater treatment systems such as constructed wetlands (CW) are not designed to remove micropollutants, including antibiotics, ARB and ARG (Sabri *et al.*, 2020).

WWTPs are one of the main sources of both ARB and ARGs released into the environment (Fang *et al*., 2017). As only a small amount of antibiotics is metabolized in the human body, most of the dose is excreted and reaches WWTPs with sewage. It has been shown that the selection for resistant bacteria occurs even at very low antibiotic concentrations (up to several hundred-fold below the minimal inhibitory concentration) (Gullberg *et al*., 2011), which makes it very difficult to establish a safe concentration of antibiotics in the wastewater. Moreover, as ARGs are mainly located on MGEs, the high density of bacteria coupled with high nutrient levels in WWTPs provide an optimum environment for HGT among bacteria (Karkman *et al*., 2018) which are subsequently released to the environment via the discharge of purified water from WWTPs. For example, the concentration of sulfonamide resistance encoding *sul1* gene has been reported to be in the range of 1.2×10^3–4×10^7 copies/mLin the WWTP effluent (Harnisz *et al*., 2020; Laht *et al*., 2014). Hultman *et al*. (2018) has shown that the tetracycline resistance encoding *tetM* gene was carried in different bacterial families in the WWTP influent and effluent water – members from the families *Methylophilaceae*, *Neisseriaceae* and *Rikenellaceae* harbored *tetM* in effluent water. In contrast, these families were not observed to carry the gene in the influent, suggesting possible HGT events.

Conventional WWTPs vary according to their size, from small WWTPs treating up to 2000 population equivalent (PE), medium WWTPs treating 2000 to 10,000 PE, and large WWTPs treating over 10,000 PE of wastewater (Harnisz *et al.,* 2020). While large WWTPs often harness some additional disinfection processes (e.g., ultraviolet light sterilization, chlorination, ozonation, reverse osmosis, activated carbon absorption, ultrafiltration) before treated wastewater discharge, small WWTPs usually do not. Consequently, it has been suggested that the highest abundances of ARGs are found in effluents of small (discharge ≤ 300 m^3/day) WWTPs, which are also the most numerous types in some regions, including in vicinity of the Baltic Sea (Harnisz *et al*., 2020).

Treated wastewater is most often discharged to streams and rivers, which can contribute to further dissemination of both ARB and ARGs among environmental bacteria and this creates a potential threat to the health of humans and animals using water resources as ARGs and ARB carried into the environment can be transferred again into these organisms (Osińska, *et al*. 2020). Freshwater bodies accommodate natural collection of bacteria that may allow sewage derived ARGs (and the MGEs they are located on) to persist and eventually return to human and animal pathogens, as the same wastewater receiving waterbody may serve as a drinking water reservoir (Czekalski *et al*., 2015).

Constructed wetlands (CWs) are wastewater treatment systems that apply a combination of chemical, physical and microbiological processes for water purification. CWs are increasingly used as alternatives to traditional WWTPs due to their low cost, low maintenance, and good wastewater purification efficiency (Button *et al*., 2016). The treatment performance of CWs is mainly based on the combined action of microbes and filter material which may be complemented by plants (Truu *et al*., 2009). Different types of CWs are often combined in sequence as hybrid systems to enhance wastewater treatment efficiency. CWs have been

shown to be efficient in reducing not only organic contaminants and nutrients in wastewater but also antibiotics, ARB and ARGs (Fang *et al*., 2017; Pazda *et al*., 2019).

3.2.2. Agriculture and mariculture

Studies have shown a direct relationship between antibiotic use and the emergence of resistant bacteria (Ventola, 2015). Less than ten years ago, over 75% of antibiotics used annually in EU and USA were consumed in agriculture (OECD, 2016), leading to potentially major repercussions to food safety and human health as antibiotics, ARB and ARGs can be transferred to humans with food. However, the use of antibiotics for growth promotion has been banned in all the EU countries since 2006 and in USA since 2017. The 2018 EU Regulation on veterinary medicines prohibits the prophylactic use of antibiotics in groups of animals, restricts metaphylactic use of antimicrobials in animals, and provides for the possibility to restrict the use of certain antimicrobials (e.g., carbapenems are not allowed to use in livestock) to human use only (OECD, 2019).

Despite the efforts to reduce antibiotic consumption in the agricultural sector, the agricultural use of antibiotics still severely affects the environmental microbiome as up to 90% of the antibiotics given to livestock are excreted in manure and then widely dispersed through fertilization into groundwater and surface runoff (Ventola, 2015). Manure composting before land application is recommended to control potential crop contamination with fecal pathogens, but since composting is not effective at eliminating all bacteria, excess ARB and ARGs may still be present (Jacobs *et al*., 2019), and in some cases, ARG concentration could even rise during manure composting (Nõlvak *et al*., 2016). Also, the widespread practice (albeit less so in the Baltic Sea region) of irrigation of crops with treated wastewater is a high potential pathway for the introduction of antibiotics and ARGs into the agroecosystem (Ben *et al*., 2019).

Aquaculture farms have been suggested to be hotspots for ARG enrichment and transfer due to the prophylactic and therapeutic use of antibiotics (Cabello *et al*., 2013). As coastal fish farms often use an open cage system, water transfers freely from the farms to the surrounding water and eventually to the sediment (Muziasari *et al*., 2016). Oxytetracycline, sulfonamide-trimethoprim combination and florfenicol are used to treat fish infections and are also important in human medicine (Muziasari *et al*., 2017). As the quinolone resistance encoding *qnrA* gene is located on the plasmids of environmental microbes and is originated from a water-dwelling bacterium, it is suggested that direct selection for the plasmid containing the resistance gene may happen in antibiotic contaminated habitats such as the fish farms (Martinez, 2014). In the Northern Baltic Sea, Muziasari *et al.* (2017) showed that ARGs encoding resistance to tetracycline, sulfonamide, trimethoprim, and aminoglycoside (an antibiotic that has never been used at the Finnish fish farms) were enriched in the farm sediments and were abundant and persistent in the sediments for several years but were not detected in sediments at a distance of 200 m from the fish farms.

3.2.3. State of antibiotic resistance in receiving waterbodies

While antibiotic resistance is a major and growing public health concern, the surveillance of it in environmental settings is remarkably limited. Aquatic environments, including surface and groundwater bodies, receive effluents from WWTPs, runoff from agricultural activity and other human inputs, and provide ideal settings for the dissemination of ARGs (Marti *et al*., 2014).

Even though treated wastewater usually contains considerably lower amounts of ARGs than raw wastewater, the discharge of treated wastewater can still increase the amount of ARGs in the aquatic environments in the vicinity or downstream of WWTPs (Cacace *et al*., 2019). The load of antibiotic residues, ARB and ARGs may be transported to groundwater, rivers, and finally to the sea (Siedlewicz *et al*., 2018). Still, not much is known about the trends and mechanisms of transport, transfer, and accumulation of ARGs in these aquatic systems.

Free-living waterborne bacteria can travel and carry their characteristics, including ARGs, furthest but most of the environmental bacteria living in aquatic ecosystems are arranged in biofilms, which are surface-associated highly structured aggregations of bacteria that live in an organized community that facilitates their survival and dispersal (Marti *et al*., 2014). In a water environment, biofilms are attached to pebbles, plants, tree branches and even sediment (Reichert *et al*., 2021), and they are able to incorporate planktonic microorganisms (including ARB) and substances (including ARGs) into their matrices (Engemann *et al*., 2008). It has been suggested that biofilms may contribute to the evolution of antibiotic resistance due to high cell density, close proximity, and accumulation of MGEs within biofilms (Yan & Bassler, 2019). HGT mediates the flow of ARGs between the microorganisms in biofilm and planktonic environmental bacteria (Wu *et al*., 2019) and with the maturing of biofilms, bacteria can also be released back into the water (Zhang *et al*., 2018).

3.2.3.1. Antibiotic resistance in freshwater environment

Freshwater availability is one of the major problems the world is facing today, and approximately 1/3 of the drinking water requirement of the world is obtained from surface waters (Edokpayi *et al*., 2017). Chlorination of drinking water can inactivate and decrease ARB but is not able to remove ARGs (Furukawa *et al*., 2017).

Releasing treated wastewater into natural streams and rivers introduces a point source pollution in the environment and it has been shown that the diversity and structure of the microbial community and resistome of the receiving waterbody downstream from the WWTP effluent is significantly altered (Mansfeldt *et al*., 2020; Sabri *et al*., 2020). However, at least partial stream water bacterial community structure recovery has been reported with increasing distance from the WWTP discharge point (Price *et al*., 2018; Proia *et al*., 2018; Pascual-Benito *et al*., 2020; Lee *et al*., 2021). Several processes may contribute to the decrease of ARGs downstream from the WWTP effluent discharge point, including dilution

by additional water inflows via groundwater and/or tributary inputs, biological degradation (e.g., predation, photodegradation, lower temperature and various other environmental conditions unfavorable to wastewater bacteria) and cell sedimentation (Lee *et al*., 2021). Public exposure to wastewater-originating antibiotic resistance might be most significant only over short range (few kilometers) from points of discharge, especially in the presence of additional water inflow, as ARG levels have been shown to decrease rapidly over 2–5 km distance (Price *et al*., 2018; Lee *et al*., 2021). Lee *et al.* (2021) showed that abundances of *sul1* and *ermB* genes, which are both indicators of anthropogenic activities, increased one order of magnitude downstream of the WWTP effluent (10^3 to 10^4 and 10^2 to 10^3 copies/mL, respectively) compared to upstream location and decreased gradually to the state similar to upstream over 2 km distance.

It has been suggested that many ARGs carrying bacteria and pathogens reside in the particulate fraction of WWTP effluent that settles into the riverbed sediment and becomes an inherent antibiotic resistance reservoir (Brown *et al*., 2019). Yu *et al*. (2020) found that the particles brought by the effluent into the stream settle downstream close to the WWTP discharge point, thus also affecting the distribution of ARGs in the streambed. The increase in abundance of opportunistic pathogens like *Pseudomonas aeruginosa* and *Acinetobacter baumannii* as well as enterococci in receiving water and sediments downstream of the WWTP discharge has also been reported (Brown *et al*., 2019).

Lakes are an important freshwater source, as they contain nearly 90% of the surface freshwater worldwide, but they also serve as the convenient sink for the discharge of domestic and industrial wastes. Moreover, lakes have the potential to store and accumulate ARGs to a greater extent than streams and rivers, because the discharged pollutants are not rapidly transported further away from the contamination site and since the water residence time in lakes is much longer, the retention time of contaminants is also longer (Czekalski *et al*., 2015).

According to Czekalski *et al*. (2015), the relative abundance of sulfonamide resistance encoding *sul1* gene in Swiss lakes was in the range of 0.0015–0.2% and *sul2* reached up to 0.0034%. The abundance of *sul1* gene was best explained by the presence of WWTPs in the lake catchment area, whereas the abundance of *sul2* gene appeared to be strongly related to long water retention time in lakes. Yang *et al*. (2017) showed similar results in lakes where the relative abundance of *sul1* gene was in the range of 0.005–0.2% and *sul2* in the range of 0.0005–0.03%.

Consequently, the fate and spread of ARGs in receiving waterbodies can depend on the waterbody characteristics but also on the ARG hosts' lifestyle and can be very different in water and sediment phases. Still, the information about the effect of WWTP effluents on the antibiotic resistome quantity and structure, and its relationships with chemical conditions, bacterial and archaeal community abundance, and structure, as well as pathogen prevalence in the receiving waterbodies, is still quite limited.

3.2.3.2. Antibiotic resistance in marine environment

The marine environment is considered to be the ultimate sink for sewage and other anthropogenic activities, and therefore, marine sediments may act as a sink but also as a secondary source of contaminants that poses potential danger for aquatic organisms (Siedlewicz *et al*., 2018).

The coastal zone can be as wide as 1400 km along some coasts and less than a kilometer along others, with an average width worldwide of about 50 km, comprising about 8% of the surface of the ocean (Walker *et al*., 2019). Almost all the waste carried by surface water of a continent enters the coastal zone through estuaries, which are waterbodies with a free connection to the sea, where the water salinity is diluted with fresh water from a river. Riverine runoff, WWTPs, and aquaculture are major pollution sources of antibiotics and ARGs in estuarine and coastal environments. In low-income countries, poorly treated or untreated wastewater is often directly discharged into the ocean, which triggers the widespread occurrence of antibiotics and ARGs in coastal environments (Zheng *et al*., 2021). Once rivers flow into the ocean, the abundance of antibiotics and ARGs usually decreases due to seawater dilution; however, even at low concentrations, antibiotics have shown to pose selective pressure on microbes and enrich ARGs in coastal waters (Duarte *et al*., 2019). ARGs can be transferred to humans through direct (e.g., swimming) or indirect (e.g., seafood) contact with the environment (Zheng *et al*., 2021) and also pose a health threat to marine animals. Long-term exposure to antibiotics shapes the gut bacteria, immune function, growth, reproduction, and digestion in marine organisms (Walker *et al*., 2019). Leonard *et al*. (2015) showed that 0.12% of the *E. coli* isolates in the coastal bathing water of England and Wales were resistant to cephalosporins, indicating that the recreational exposure of ARB in seawater is underestimated. It is possible that wildlife that are able to move long distances, such as birds and fish, play an important role in the global spread of ARB and ARGs (Chen *et al*., 2020) – for example, aquatic birds have been shown to disseminate tetracycline and β-lactam resistance determinants.

Possible contamination sources of the open ocean waters beyond the coastal zone include atmospheric fallout, oil spills and dumping of hazardous wastes and sewage from the ships (Walker *et al*., 2019) and particles discharged in the upper mixed layer may eventually sink so far that they can no longer be resuspended and thus become part of the sediment of the deep ocean waters.

3.3. The state of antibiotics and ARGs spread in the Baltic Sea region

The Baltic Sea is simultaneously one of the largest brackish water areas and also one of the most polluted seas in the world due to its limited water exchange (water residency time is 30 years), shallowness (average depth is 50 m and more than 1/3 is shallower than 30 m), and large catchment area (1.7 million km^2) (HELCOM,

2017). Densely populated coastal areas (16 million people) and intensive industry and agriculture (including farming, animal husbandry and aquaculture) produce large amounts of waste, including antibiotics and their metabolites, ARB and ARGs (Siedlewicz *et al*., 2018). In Germany, Denmark and Poland, 60–70% of the Baltic Sea's catchment area consists of farmland, while in Finland, Russia, Sweden and Estonia, between 65% and 90% of the catchment area is made up of forests, wetlands and lakes.

There are over 3100 conventional WWTPs in the Baltic Sea watershed, medium sized WWTPs (2001–10,000 PE) being the most numerous type in most of the area, generating about 4 million tons of dry solids from sewage sludge annually. The average runoff to the Baltic Sea from the catchment area is 10 L/s/km^2. According to a rough estimate, WWTPs release about 1800 tons of pharmaceuticals per year to the Baltic Sea (UNESCO & HELCOM, 2017), and it is believed to be the main source of pharmaceuticals introduced into the marine environment. The Baltic Sea ecosystem is particularly sensitive to pharmaceutical pollution because of its low biodiversity and many species are experiencing increased physiological stress due to the brackish water environment. In the Baltic Sea region, pharmaceuticals emissions from manufacturing facilities are generally assumed to be very low compared to inputs occurring during the consumption phase.

According to the HELCOM report, based on the data obtained in 2003–2014, all of the monitored pharmaceuticals in the category of antimicrobial agents (antibiotic, antifungal, antiviral, antiparasitic, disinfectant, antiseptic) and antidote, 11 out of 30 (37%) substances are detected in environmental samples (water, sediment or biota) (HELCOM, 2017). Sulfamethoxazole (a sulfonamide antibiotic) was the most frequently detected antimicrobial substance and it was detected in 12 out of 140 (9%) seawater samples and in 50% of tested sediment samples. Clarithromycin (a macrolide antibiotic) was detected in two out of 126 water samples. From 2001 to 2014, ~2.3 tons of sulfonamide, 0.6 tons of trimethoprim, 1.2 tons of oxytetracycline, and 0.04 tons of florfenicol were used in fish farming in Finland and since the Baltic Sea has no tides and water circulation is very slow the fish farming waste impacts directly the sediments beneath the farms (Muziasari *et al*., 2017).

The information about the spread of ARB and ARGs in the Baltic Sea catchment area as well as in the Baltic Sea itself is quite sporadic and fragmented. It has been shown that the abundance of individual ARGs in the WWTP effluents in the Baltic Sea catchment area is in the range of 10^2–10^8 copies/mL (Börjesson *et al.,* 2010; Laht *et al*., 2014; Harnisz *et al.,* 2020; Osinska *et al.,* 2020), and tends to be higher during winter (Osinska *et al*., 2020) and in small and medium WWTP effluents (Harnisz *et al*., 2020). The antibiotic resistome proportion in the effluents of large scale WWTPs in Sweden has been shown to be in the range of 5–10% per 16S rRNA reads, with macrolide-lincosamide-streptogramin (MLS) class resistance genes being reduced and aminoglycoside, tetracycline, sulfonamide and β-lactam resistance genes proportion increased during wastewater treatment (Bengtsson-Palme *et al*., 2016). The MLS class resistance genes are

also shown to be more abundant in the sewage sludge (compared to WWTP influent and effluent), whereas sulfonamide resistance genes are most abundant in the final WWTP effluent (Karkman *et al*., 2016). Kotlarska *et al.* (2015) showed that up to 37% of *E. coli* isolated from raw wastewater samples from the WWTPs in the shore of Gulf of Gdansk, southern Baltic Sea, were resistant to at least one of the tested antimicrobial agents and the resistance rate increased to 47% at the marine outfalls.

In WWTP effluent receiving freshwater bodies in the Baltic Sea catchment area, the individual ARG abundance range is commonly 10^2–10^5 copies/mL (Nõlvak *et al.,* 2018; Osinska *et al*., 2020). In the Lake Mälaren water sample from a small yacht harbor, the proportion of antibiotic resistome was 15.7% per 16S rRNA reads, dominated by bacitracin, multidrug and trimethoprim resistance types and showing highest individual abundance for *sul1* gene (Nõlvak *et al*., 2018). The abundance of ARB downstream of WWTPs in Polish rivers is shown to be 30 to 2.3×10^3 CFU/mL (Osinska *et al*., 2020). Recently, Khan *et al*. (2019) has studied antibiotic resistance in the Svartån river in Örebro, Sweden, based on cultured resistant bacteria to qualitatively monitor 84 ARGs, conferring resistance to all major antibiotic classes, and detected 43 ARGs in the receiving river water compared to 22 in upstream from the WWTP. Lai *et al*. (2021) found that the relative abundance of ARGs in the Swedish rivers was three to five times higher in the downstream river water compared to upstream from the WWTP (0.013% vs 0.0024% and 0.0081% vs 0.0024%) and ARGs were more diverse in more urbanized regions.

The information about the Baltic Sea antibiotic resistome is even more sporadic than the estimates concerning the catchment area. It is shown that most of the *Staphylococcus*-like strains (64–97%) isolated from seawater and sand of Ustka Beach, southern Baltic Sea, are resistant to ampicillin, oxytetracycline and penicillin, while less than 30% were resistant to gentamicin, neomycin and streptomycin (Skórczewski *et al*., 2014) and among all enteric bacteria, the highest percentage (79–96%) of strains are resistant to clindamycin and penicillin (Mudryk *et al.,* 2016). Muziasari *et al.* (2016) showed that even though the genes encoding resistance to sulfonamide/trimethoprim and tetracycline were relatively abundant in the sediments below fish farms (0.002% and 0.12%, respectively), outside of the farms the sulfonamide/trimethoprim resistance genes were not detected, and the relative abundance of tetracycline resistance genes was 0.001%. Thus, it is likely that these resistance genes were introduced to the sediment via fish farming using antibiotics. There seems to be a background resistome in the Baltic Sea sediments consisting mainly of genes encoding resistance to efflux pumps which confer resistance to chloramphenicol (0.048%) and multidrug (0.02%), which are also previously shown to be dominant in the background resistome of sea sediments (Muziasari *et al.,* 2016).

3.4. Antibiotic resistance spread monitoring methods

Isolating pure bacterial cultures combined with antimicrobial susceptibility testing, which determines how well specific bacteria can grow in the presence of antimicrobials, is historically one of the most important methods in clinical microbiology (Boolchandani *et al*., 2019). Antimicrobial susceptibility testing is useful for studying phenotypic resistance of bacteria. Still, it is of low throughput and metagenomic studies from patient samples have shown that in some cases, bacteria detected in culture may not be actually responsible for disease symptoms that they are accounted for (Rudkjøbing *et al*., 2016). To date, only 1% of bacteria can be cultured by current techniques, which has inevitably created a need to learn more about the unculturable species and their functions (Bodor *et al*., 2020).

PCR (polymerase chain reaction) has become a popular method for detecting ARGs in environmental samples because it is sensitive, provides fast results, gives direct information about the analysed genes and does not need the prior cultivation of bacteria (Luby *et al*., 2016). Quantitative PCR (qPCR) also provides quantitative information about the abundance and RT-PCR (reverse transcriptase PCR) about the expression of the targeted ARGs, in addition to the benefits of PCR, in real-time using fluorescent dyes. However, PCR-based assays are limited to known genes or to genes with high homology to known ones (Karkman *et al*., 2018). Also, target gene quantification results from environmental samples depend on several factors, such as the method and quality of DNA extraction, the subsequent presence of inhibitory substances (i.e., humic acids, organic contaminants) in the extracted microbial community DNA, the qPCR chemistry used and amplification efficiency achieved and the overall quality of the resultant datasets (Nõlvak *et al*., 2012a). High throughput qPCR (HT-qPCR) is a relatively rapid and convenient method for analyzing hundreds of ARGs at the same time, but at a downside, it is not possible to optimize individual assays during a run and so all assays would experience the same qPCR cycling conditions (Waseem *et al*., 2019). This is crucial because specific primers might require different annealing temperatures.

The major advantage of microarray technology is that thousands of ARGs can be profiled in one run. However, microarrays suffer batch-to-batch variability and are generally less sensitive and less specific than HT-qPCR, and microarray data also needs additional validation by qPCR (Waseem *et al*., 2019).

Metagenomics, along with the sequencing of the whole resistome, can overcome the need for prior knowledge of resistance genes. However, the annotation of ARGs is still relying on known genes in public ARGs databases (Karkman *et al*., 2018). It has also been suggested that while these studies are useful to obtain a general view of the most abundant ARGs, they are not effective for detecting genes with a low abundance (Pärnänen *et al*., 2019). Still, constant advances in sequencing technologies have increased the data available on microbial genomes, and continually decreasing costs have made sequencing a viable tool for antimicrobial resistance surveillance. The major technological revolutions in whole genome sequencing (WGS) after the first generation sequencing (whole genome

shotgun sequencing, e.g., capillary based Sanger sequencing technology) were next-generation sequencing (NGS; high throughput sequencing, e.g., Illumina) and the third generation of sequencing (single molecule long-read sequencing, e.g., Nanopore). A known limitation of NGS technologies is the need for a PCR amplification step, which creates a bias in read distribution and ultimately affects the coverage (Fanning *et al*., 2017). To address this limitation, the third generation sequencing technologies were designed where single DNA molecules are directly sequenced. A drawback in Nanopore sequencing is the relatively high sequencing error rate (Heikema *et al*., 2020).

Besides sequence-based metagenomics, functional metagenomics is a powerful sequence-unbiased and culture-independent approach for characterizing antibiotic resistomes (Boolchandani *et al*., 2019) which has enabled the discovery of several new antimicrobial resistance mechanisms and the genes held responsible. On the downside, a gene has to be functional outside its native microbial host and confer the same phenotype to be identified by functional metagenomic selections.

Today, qPCR and NGS technologies are the most widely used methods for studying antibiotic resistance in the environment. However, since every method has its limitations, the best way to describe the resistome would be to simultaneously employ several techniques to avoid technical bias and give a comprehensive view of the subject.

4. MATERIAL AND METHODS

The pathway of the propagation of antibiotic resistance genes originating from the WWTP effluent through the primary receiving waterbody to the final receiving waterbody is presented in this dissertation. The three studies that the current thesis is based on, focused on the ARGs dynamics and their relationships with the system treatment efficiency in a horizontal subsurface flow constructed wetland (HSSF CW) effluent (Paper I); assessing the impact of the effluent of a small-scale WWTP on the antibiotic resistome in the receiving waterbody downstream from the WWTP effluent discharge point (Paper III); and characterizing the bacterioplankton community and its ARGs in the Baltic Sea (Paper II).

4.1. Characterisation of wastewater treatment systems and their effluent (Paper I, III)

In this study the activated sludge WWTP and unplanted hybrid CW were targeted. Both studied wastewater treatment systems were located in Nõo borough, Estonia, the center of a parish with a permanent population of about 1500 people, a primary school, and a high school. The small-scale activated sludge WWTP (Paper III) was established in 2002 and treats domestic municipal wastewater combined with the effluents of small-scale dairy and meat industries with a maximum capacity of 2100–2300 PE and mean effluent discharge rate of 290 m^3/day. The WWTP has no disinfection step for its purified wastewater effluent, which is discharged to the middle section of Nõo stream, a 9 km long waterbody (flow rate: 0.017–0.2 m^3/s, flow speed: 0.2–0.5 m/s, average depth: 0.3 m), which flows into the Elva River (average flow rate: 2.2 m^3/s) approximately 3.4 km downstream of the WWTP.

The unplanted hybrid CW system (Paper I) located on the premises of Nõo WWTP and was fed with raw wastewater pumped from the inlet of the activated sludge WWTP. The pilot system consisted of a septic tank (2 m^3), followed by six parallel vertical subsurface flow mesocosms (MC) with a total area of 6 m^2, a collection well, and 21 parallel HSSF MCs (LxWxD – 1.5×0.2×0.6 m). The three HSSF MCs used in this study were filled with light expanded clay aggregates (LECA) with 2–4 mm particle size forming the wetland media. The hydraulic loading rate was ≤20 mm/d and the wastewater retention time in the HSSF MCs was 1.2 days. The effluent of the CW was discharged in the Nõo stream. A detailed description of the experiment is given in Paper I.

Six stream and river water samples and six sediment samples were collected along the 3.7 km distance gradient of the Nõo stream and Elva River to assess the effect of the WWTP outflow to the receiving waterbody (Paper III). The stream water and sediment samples were taken from ~20 m upstream and at 0.3 km, 2.7 km, and 3.2 km downstream of the activated sludge WWTP discharge point. Two river water and sediment samples were taken from the Elva River, ~10 m

upstream of the Nõo stream inflow and downstream of the Nõo stream inflow, 3.7 km from the WWTP.

4.2. Microbial community analysis (Paper II, III)

The microbial community structure and composition were analyzed from the WWTP effluent, Nõo stream water and seawater of the Baltic Sea. Bacterial communities from four different marine sampling sites (Tallinn Bay, Narva Bay, Gulf of Riga and Gulf of Finland) (Paper II) were characterized using microbial community profiling based on the 16S rRNA gene V6 region using forward (5'-GAACGCGARGAACCTTACC-3') and reverse (5'-ACAACACGAGCTG ACGAC-3') primers (Gloor *et al.,* 2010) and Illumina® HiSeq 2000 sequencing combinatorial sequence-tagged PCR products. A detailed PCR product preparation, sequencing, sequence data preparation and taxonomic assessment description is given in Paper II. The composition of the bacterial community was classified down to genus level.

Due to significant replenishment of reference databases over the recent years, the data on bacterial community of the Baltic Sea water was reanalyzed in the current thesis. The assembled reads were processed with Mothur v.1.44.1 (Schloss *et al*., 2009) with SILVA v.138.1 (Pruesse *et al*., 2007) used as a reference database for taxonomic assignment while keeping other analysis parameters similar to original analysis (Paper II).

The microbial community of WWTP effluent and Nõo stream water as well as sediment samples (Paper III) was characterized by shotgun metagenome sequencing. Paired-end DNA sequencing libraries (2 × 150 bp) were constructed from all collected stream and river sediment samples, WWTP effluent and two stream water samples (upstream and 0.3 km downstream of WWTP) and sequenced using the Illumina® NextSeq 500 system. The DNA concentration of the other water samples was too low for metagenomic sequencing. The composition of the bacterial and archaeal community was classified down to species level using Kaiju (Menzel *et al*., 2016) v.1.7.3 and Megahit (Li *et al*., 2016) v.1.1.2 was used to assemble quality checked reads into contigs (minimum length of 300 bp). A detailed description of sequence data generation, processing and analysis is given in Paper III.

4.3. ARG resistome analysis (Paper III)

The metagenomes of WWTP effluent and two of Nõo stream water samples (upstream and 0.3 km downstream from the WWTP effluent) were analyzed with the ARGs-OAP v.2.2 (Yin *et al.,* 2018) for antibiotic resistome profiling and the proportion of antibiotic resistome in the microbial community was presented per number of 16S rRNA reads. Classification of ARGs to resistance mechanism

types was based on the annotations of the respective genes in the CARD (Comprehensive Antibiotic Resistance Database) database (v.3.0.8).

According to SARG (Structured Antibiotic Resistance Genes) database used within ARG-OAP analysis, antibiotic resistance is divided into 24 types (aminoglycoside, bacitracin, β-lactam, bleomycin, carbomycin, chloramphenicol, fosfomycin, fosmidomycin, fusaric-acid, fusidic-acid, kasugamycin, MLS, multidrug, polymyxin, puromycin, quinolone, rifamycin, spectinomycin, sulfonamide, tetracenomycin_C, tetracycline, trimethoprim, vancomycin and unclassified) and 1209 subtypes. Antibiotic resistance subtype corresponds to the specific antibiotic resistance gene (e.g., subtype sul1 corresponds to sulfonamide resistance encoding *sul1* gene).

4.4. Application of ARGs quantification (Paper I, II, III)

QPCR methodology was used in all conducted experiments to quantify bacterial and archaeal 16S rRNA genes and ARGs abundance in order to estimate the scope of antibiotic resistance potential in targeted microbial communities.

Originally, seven ARGs commonly found in aquatic environments and covering major antibiotic classes were quantified from the effluent of HSSF CW (Paper I): β-lactamase-resistance-encoding *ampC*, sulfonamide-resistance-encoding *sul1*, MLS-resistance-encoding *ermB,* fluoroquinolone-resistance-encoding *qnrS,* and tetracycline-resistance-encoding *tetA*, *tetB* and *tetM*. From the water of the Baltic Sea (Paper II) the same selection of ARGs with the substitution of *qnrS* with β-lactamase-resistance-encoding *blaSHV* gene was initially targeted. The list of targeted ARGs was expanded to fourteen for analysis of WWTP effluent and the water of the receiving stream (Paper III): aminoglycoside-resistance-encoding *aadA*, β-lactamase-resistance-encoding *blaCTX-M*, *blaTEM1* and *blaOXA2*, chloramphenicol-resistance-encoding *catQ*, fluoroquinolone-resistance-encoding *qnrS*, sulfonamide-resistance-encoding *sul1* and *sul2*, tetracycline-resistance-encoding *tetA*, *tetB*, *tetQ* and *tetW* and multi-resistance-encoding *acrB* and *mexF*. In addition, *aadA*, *sul2*, *blaCTX-M*, *blaOXA2* and *blaTEM1* resistance genes were quantified from the marine and CW samples for the current thesis.

All quantifications were performed on RotorGene ® Q (Qiagen, Foster City, CA, USA) system using reaction mixture containing 5 µL of Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), an optimized concentration of forward and reverse primers, template DNA, and sterile distilled water for a total volume of 10 µL. The detailed description of the qPCR programs, primers and optimized amplification conditions used are shown in Table 1 and in respective papers. Immediately after the qPCR assay, a melting curve analysis was performed by increasing the temperature from 70 °C to 95 °C (0.35 °C/3 s) with continuous fluorescence recording. All samples were run in triplicate, and negative controls were included in every qPCR run.

In Paper I and II, the target gene copy numbers, representing the abundance of ARGs in the tested samples, were deduced from the standard curves and

presented as gene copy numbers per milliliter (copies/mL) of water in Paper I and copy numbers per liter (copies/L) of seawater in Paper II. Antibiotic resistance encoding functional genes were normalized against bacterial 16S rRNA genes, representing the relative abundance of ARGs in bacterial communities, using amplicon-specific amplification efficiencies and Ct values, as described in Nõlvak *et al.* (2012a).

The calculation of the target gene copy numbers in Paper III and for additional ARGs amplifications for the current thesis were performed by combining amplification efficiency estimations with LinRegPCR program (Ruijter *et al*., 2009) with estimation of fold difference between a sample and multiple data points from the standard curve as described in Nõlvak *et al*. (2016) and presented as gene copy numbers per milliliter (copies/mL) of water for WWTP and CW effluents as well as stream and river water, or as copies/L for seawater. In order to evaluate the relative abundance of ARGs in the whole microbial community, all targeted ARGs were normalized against total 16S rRNA genes (bacterial + archaeal 16S rRNA).

In the current thesis, all previously quantified gene copy numbers in Paper I and II were recalculated using improved methodology.

Table 1. Characteristics of qPCR primer pairs and programs used.

Primers	Sequence 5'-3'	Target gene	Amplicon size (bp)	Primers concent-ration (µM)	qPCR program	Reference
Arc519F	CAGYCGCCRCGGTAA	Archaeal 16S rRNA	393	0.6	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 15 s; 56°C 30 s; 72°C 30s	Espenberg *et al.*, 2016
Arch910R	GAATWGGCGGGGGRGC					
L-V6	GAACGCGARGAACCTTACC	Bacterial 16S rRNA	111	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 54°C 30 s, 72°C 30 s	Hummelen *et al.*, 2010
R-V6	ACAACACGAGCTGACGAC					
785FL	ggactacGGATTAGATACCCTGGTAGTCC[1]	Bacterial 16S rRNA	156	0.8	50°C 2 min, 95°C 10 min; 40 cycles: 95°C 15 s, 62°C 30 s, 72°C 30 s	Nõlvak *et al.*, 2012b
919R	CTTGTGCGGGTCCCCGTCAAT					
Bact517F	GCCAGCAGCCGCGGTAA	Bacterial 16S rRNA	530	0.6	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 30 s; 60°C 45 s; 72°C 45s	Liu *et al.*, 2007
Bact1028R	CGACARCCATGCASCACCT					Dethlefsen *et al.*, 2008
aadA-F	GCTGGCCGTRCATTTGTAC	*aadA*[2]	479	0.8	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 15 s, 59°C 30 s, 72°C 30 s	Nõlvak *et al.*, 2018
aadA-R	AGCACTACATTYCGCTCATCG					
acrB-F	ATATCCTACGATTGCACCGC	*acrB*	160	0.8	50°C 2 min, 95°C 10 min; 40 cycles: 95°C 15 s, 58°C 30 s, 72°C 30 s	Szczepanowski *et al.*, 2009
acrB-R	GGTACCCGTGGAGTCACTGT					
Lak2-FP	GGGAATGCTGGATGCACAA	*ampC*	67	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 60°C 30 s, 72°C 30 s	Volkmann *et al.*, 2004
Lak1-RP	CATGACCCAGTTCGCCATATC					
catQ-F	AGGTGCACTTACAGTATGACTGC	*catQ*	262	0.8		

Primers	Sequence 5'-3'	Target gene	Amplicon size (bp)	Primers concent-ration (µM)	qPCR program	Reference
catQ-R	AACGTGGGAAGTTCTCGTCATAC				50°C 2 min, 95°C 10 min; 40 cycles: 95°C 15 s, 57°C 30 s, 72°C 30 s	Stedtfeld *et al.*, 2018
ermB-F	AAAACTTACCCGCCATACCA	*ermB*	139	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 60°C 30 s, 72°C 30 s	Knapp *et al.*, 2010
ermB-R	TTTGGCGTGTTTCATTGCTT					
tetA-F2	TCAATTTCCTGACGGGCTG	*tetA*	96	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 58°C 30 s, 72°C 30 s	Börjesson *et al.*, 2009
tetA-R2	GAAGCGAGCGGGTTGAGAG					
tetB-F1	AGTGCGCTTTGGATGCTG	*tetB*	101	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 58°C 30 s, 72°C 30 s	Börjesson *et al.*, 2009
tetB-R1	TGAGGTGGTATCGGCAATGA					
tetM	GGTTTCTCTTGGATACTTAAAT CAATCR	*tetM*	88	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 60°C 30 s, 72°C 30 s	Peak *et al.*, 2009
tetM	CCAACCATAYAATCCTTGTTCRC					
tetQ-F	GCTCACATTGATGCAGGAAA	*tetQ*	153	0.8	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 15 s, 58°C 30 s, 72°C 30 s	Modified from Szczepanowski *et al.*, 2009
tetQ-R	CGTAGAAGCCCGRACAGTAA					
tetW-FC	GGGAAATTGTTCGGACAGAC	*tetW*	549	0.8	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 15 s, 57°C 30 s, 72°C 30 s	Call *et al.*, 2003
tetW-RC	AACGGATACCATCCCTGACA					
sul1-F1	CTGAACGATATCCAAGGATTY CC	*sul1*	239	0.8	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 54°C 30 s, 72°C 30 s	Heuer *et al.*, 2008
sul1-R1	AAAAATCCCATCCCCGGRTC					

Primers	Sequence 5'-3'	Target gene	Amplicon size (bp)	Primers concent-ration (µM)	qPCR program	Reference
sul2-F	CTCAATGATATTCGCGGTTTYCC	*sul2*	245	0.6	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 58°C 30 s, 72°C 30 s	Heuer & Smalla, 2007
sul2-R	AAAAACCCCATGCCGGGRTC					
UP	ACCAAYGATATYGCGGTGAT	*blaCTX-M*	101	0.6	50°C 2min, 95°C 10min; 45 cycles: 95°C 15s, 56°C 30s, 72°C 30s	Modified from Colomer-Lluch *et al.*, 2011
LP	ACATCGCGRCGGCKYTCT					
blaSHV-F	TGATTTATCTGCGGGATACG	*blaSHV*	95	0.8	50°C 2 min, 95°C 10 min; 40 cycles: 95°C 15 s, 55°C 30 s, 72°C 30 s	Haeggman *et al.*, 2004
blaSHV-R	TTAGCGTTGCCAGTGCTCG					
blaTEM1-F	CATTTYCGTGTCGCCCTTAT	*blaTEM1*	167	0.6	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 57°C 30 s, 72°C 30 s	Szczepanowski *et al.*, 2009
blaTEM1-R	GGGCGAAAACTCTCAAGGAT					
blaOXA2F	TCTTCGCGATACTTTYCTCCA	*blaOXA2*	177	0.8	50°C 2 min, 95°C 10 min; 35 cycles: 95°C 15 s, 55°C 30 s, 72°C 30 s	Szczepanowski *et al.*, 2009
blaOXA2R	ATCGCACAGGATCAAAAACC					
mexF-F1	CAGGACAAGCARTACCTGGTGGC	*mexF*	566	0.8	50°C 2 min, 95°C 10 min; 40 cycles: 95°C 15 s, 60°C 30 s, 72°C 30 s	Tiirik *et al.*, 2021
mexF-R2	AGGTARAYCTGCAGGGTGTCG					
QnrS-Ru	AAACACCTCGACTTAAGTCT	*qnrS*	169	0.4	50°C 2 min, 95°C 10 min; 45 cycles: 95°C 15 s, 52°C 30 s, 72°C 30 s	Guillard *et al.*, 2011
QnrS-Fu	GTGAGTAATCGTATGTACTTTTGC					

[1] Fluorophore is attached to the base marked in bold; sequence of artificial tail added to the primer sequence and enabling hairpin formation (Nazarenko et al., 2002) is given in lowercase.

[2] The primers amplify all *aadA* gene variants (*aadA1*-*aadA25*) except *aadA4*, *aadA5*, *aadA9*, and *aadA14*.

4.5. Statistical analysis

Spearman's rank correlation coefficient was used to evaluate the extent to which water quality parameters and wastewater purification efficiencies correlated with target gene concentrations and relative abundances (Paper I), and Pearson correlation coefficient was calculated to relate the target gene abundances and relative abundances in the stream and river water and sediment to the physicochemical parameters of the studied materials (Paper III).

Partial correlation analysis was applied to reveal the impact of temperature on the relationships between the abundance of ARGs (log-transformed) and environmental parameters (Paper I) and to relate the target ARGs abundances and relative abundances in water and sediment to the physicochemical parameters of the studied materials (Paper III).

Principal component analysis (PCA) was applied to assess the ARG data pattern in marine bacterioplankton (Paper II) and the proportions of pathogens in bacterial communities of stream and river water and sediment (Paper III). In the case of the 16S rDNA amplicon sequencing data, principal coordinate analysis (PCoA) was used to explore and visualize similarities in a low-dimensional space among bacterioplankton samples and Procrustes analysis was used to assess the overall degree of association between ordinations of bacterioplankton samples and ARGs abundances (Paper II). Additionally, in the current thesis, PCA based on the correlation matrix of the ARG abundances (log-transformed) and relative abundances was applied to assess the pattern of ARGs in different sampled media (WWTP, CW, seawater and stream water) using R version 4.0.3.

To visualize the differences in ARGs abundances (log-transformed) and relative abundances (calculated against bacterial 16S rRNA gene abundance) using different calculation methodologies, scatterplots with bidirectional error bars were composed in the current work using R version 4.0.3.

Co-inertia analysis (CIA) was applied to explore the covariance between bacterial genera (clr-transformed) and ARGs relative abundances in the bacterial community of the Baltic Sea using R v.4.0.3 package ade4 (Dray & Dufour, 2007). The proportions of 50 most abundant genera (clr-transformed) in the bacterial communities of the analyzed samples of the Baltic Sea were visualized as heatmaps (Ward-linkage method and Euclidean distance) using R v.4.0.3 package pheatmap (Kolde, 2015).

5. RESULTS AND DISCUSSION

5.1. Antibiotic resistance in wastewater treatment facility effluents

Based on the shotgun metagenome data, the proportion of the antibiotic resistome per reads of 16S rRNA genes in the prokaryotic community of Nõo activated sludge WWTP effluent was 31.9%. The resistome was dominated by bacitracin, multidrug, and sulfonamide resistance-type determinants and RND-type efflux pumps, inactivation, target alteration, and target replacement resistance mechanisms. A total of 160 ARG subtypes were registered from the WWTP effluent metagenome, with *bacA*, *sul1* and *aadA* (6.20%, 5.38% and 1.17%, respectively) being the most prevalent (Paper III). In comparison, all these parameters exceed the ones recorded from Västerås WWTP (Sweden), an example of a large scale WWTP in Baltic Sea region, where the total proportions of ARGs in the microbial communities were 18.5±0.4%, and the *bacA*, *sul1* and *aadA* gene proportions were 3.97±0.01%, 0.31±0.10%, and 0.54±0.04%, respectively (Nõlvak *et al*., 2018).

The quantified ARG abundances and their relative abundances in the prokaryotic community of Nõo WWTP effluent were in the range of 10^3–10^6 copies/mL and 0.0008–2.04%, respectively, where the *sul2* was the least and *sul1* the most abundant ARG (Paper III). A similar range of individual ARG abundances has been reported in effluents of several large-scale WWTPs in Sweden and Finland (Börjesson *et al.,* 2010; Laht *et al.,* 2014; Nõlvak *et al.,* 2018), while the reported ARG abundances in effluents of larger WWTPs than Nõo in Estonia (e.g., Tallinn, Tartu) tend to exceed this ARG abundance level (Laht *et al.,* 2014).

In the effluent of the HSSF CW treating municipal wastewater, all the targeted ARGs were detectable with tetracycline resistance encoding *tetA* and sulfonamide resistance encoding *sul1* being most abundant, which is explained by tetracycline and sulfonamide being among the most commonly used antibiotics in human and veterinary medicine in Estonia (Estonian State Agency of Medicines). The quantified ARG abundances remained in the range of 10^5–10^8 copies/L and relative abundances in the range of 0.0001–1.10% of the microbial community of CW effluent (Paper I, Table A.1, Table A.2). The abundances of bacterial 16S rRNA and most targeted ARGs were generally lower in the final CW effluent compared to WWTP effluent. Notably, the HSSF CW proved much more efficient compared to WWTP in reducing sulfonamide resistance encoding *sul1* concentrations (on average 1.6×10^7 copies/L and 3.2×10^9 copies/L, respectively) and relative abundances in microbial community (on average 0.042% and 2.04%, respectively) of wastewater treatment facility effluents. This finding also suggests CWs as a feasible WWTP effluent polishing options in the matter of improved ARG removal.

5.2. Antibiotic resistance in receiving waterbodies

5.2.1. Nõo stream and Elva river

The strong effect of WWTP effluent on receiving stream water and sediment antibiotic resistome was evident in close vicinity downstream of WWTP. Although the proportion of antibiotic resistome in the stream water samples preceding (upstream) and following (downstream) the WWTP discharge point (23.9% and 24.3%, respectively) remained virtually unchanged, shifts in resistome structure towards an increase in resistance types prevalent in WWTP effluent, as well as a higher number of detected ARG subtypes and generally increased ARG abundances were recorded 0.3 km downstream (Paper III). This complements findings that WWTP effluents introduce great shifts in microbial community structure in receiving stream or river water closely downstream of WWTP effluent discharge points (Price *et al*., 2018; Mansfeldt *et al.,* 2020) that are apparently also mirrored in antibiotic resistome profile. The upstream sample was dominated by an inactivation mechanism utilizing β-lactam resistance-type determinants (especially *blaTEM* subtypes). In contrast, the downstream sample was dominated by multiresistance (especially of the RND-efflux type) determinants and had a higher proportion of sulfonamide resistance and a lower proportion of β-lactam resistance determinants than upstream.

Based on the ARGs quantification data, the abundances of almost all targeted ARGs (except for *blaTEM1*) were higher (in the range of 10^2–10^4 copies/mL) in the close vicinity of WWTP outflow compared to the rest of the stream (the range of 10–10^3 copies/mL). Osińska *et al* (2020) has also recorded higher (10^1–10^5 copies/mL) ARG concentrations in the river water downstream of the WWTP effluent compared to upstream (10–10^4 copies/mL). Also, elevated proportions of sulfonamide and aminoglycoside resistance types (specifically, *sul1*, *sul2* and *aadA*), as well as β-lactam-resistance-encoding *blaOXA* genes, forming one behavioral cluster (Paper III) and previously reported as characteristics of heavily impacted waterbodies (Corno *et al*., 2019), were recorded in the water and sediment 0.3 km downstream of the WWTP outflow. Upstream of the WWTP, the abundances of these particular ARGs were low in the sediments and non-detectable in water. A substantial decrease in the proportion of β-lactam resistance was noted in the stream water following WWTP discharge point, specifically of *blaTEM*, which has previously been shown to be characteristic of freshwater bodies (Corno *et al*., 2019). The quantitative data suggested that this effect could arise from the excess input of microbes (including ARG-carriers) introduced by WWTP effluent rather than from an actual change in the abundance of *blaTEM* genes. Conversely, in sediments, the proportion of β-lactam resistance increased downstream of the WWTP discharge point, mainly due to *blaOXA* and *blaLRA* gene families, which have been documented from river sediments as minor β-lactam resistance determinants (Jiang *et al*., 2018).

In general, the ARG abundances in receiving stream water decreased along the distance gradient further downstream, and the WWTP effluent-associated

sul1–sul2–aadA–blaOXA2 cluster became virtually undetectable from the 2.7 km location onward (Paper III). In the sediments, the changes in the structure of the resistome along a distance gradient indicated recovery from the impact of WWTP effluent as the proportions of sulfonamide, aminoglycoside, and β-lactam resistance, as well as the of *sul1–sul2–aadA–blaOXA2* cluster, gradually decreased, and in the river sediments 3.7 km downstream of WWTP, the resistome was remarkably similar to the resistome of the stream sediments upstream of the WWTP. This confirms previous suggestions of at least partial recovery of impacted communities controlled by distance from the effluent source (Price *et al*., 2018).

Our results also suggest that archaea form a substantial portion (~10%) of WWTP effluent and might contribute to the spread of antibiotic resistance determinants in receiving waterbodies, as archaeal abundances showed positive relationships with *acrB*, *blaCTX-M*, *mexF*, *tetW*, *sul1* and *sul2* abundances. The partial correlations approach suggested that especially the sulfonamide-resistance-encoding *sul2* gene might be prominently related to the archaeal community. This result coincides with previous findings of positive correlations between genus *Methanothrix* abundance and the *sul1* and *sul2* genes (Yang *et al*., 2020), suggesting that the role of archaea in conferring and possibly mediating antibiotic resistance in waterbodies merits more focused in-depth research in the future.

5.2.2. Baltic Sea

5.2.2.1. The microbial community of the Baltic Sea

The bacterioplankton of the Baltic Sea was dominated by the Actinobacteria, Protobacteria and Bacteroidetes phyla (Paper II), which are conventionally considered to be characteristics of freshwater ecosystems (Neuenschwander, *et al*., 2018; Zwart *et al*., 2002; O'Sullivan *et al*., 2005), but also seem to thrive in brackish environmental conditions. The PCoA plot based on the Bray–Curtis distance matrix showed that the main difference between bacterioplankton samples is related to the sampling time, although variation in community structure among sampling locations is also large.

The 16S rDNA sequencing data of the microbial communities of the Baltic Sea water was reanalyzed in this thesis (on the consideration that during the seven years since the original publishing of Paper II, the reference databases have been improved significantly) to see if the updates in the databases affect the original assessment of community structure. However, the effect of enhanced databases on taxonomic assignment proved to be minute, as the initial analysis had covered 97.3±1.0% of the microbial community from the new version of the taxonomic assignment.

In the current thesis, the microbial community of the Baltic Sea water was also assessed in more detail on genus level. The clustering of the seawater samples based on the proportions of top 50 bacterial genera in bacterial communities (Figure 5; Table A.3) indicates large variability between communities of different locations and times. This result supports the original conclusions from PCoA analysis (Paper II) as well as the results obtained from ARG quantification data.

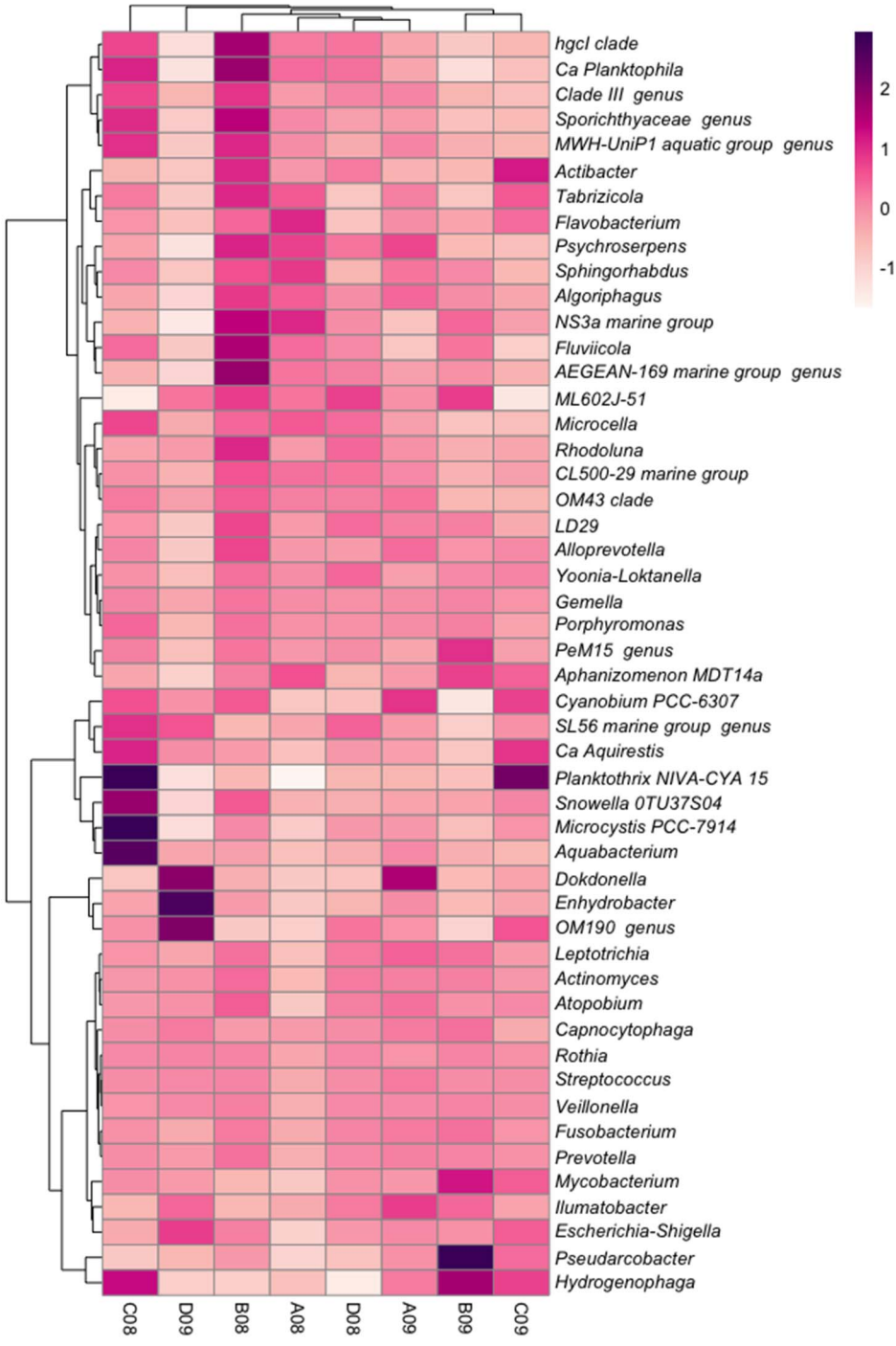

Figure 5. Heatmap showing the clustering of the Baltic Sea water samples according to the proportions (%) of the top 50 bacterial genera (clr-transformed). A – Tallinn Bay, B – Gulf of Finland, C – Narva Bay, D – Gulf of Riga. 08 and 09 in sample labels denote years 2008 and 2009, respectively.

On genus level, the microbial community of the brackish Baltic Sea water proved to be an intriguing mix of typical freshwater bacterioplankton members such as *Ca. Planktophila* and *Rhodoluna* (Neuenschwander, *et al*., 2018, Pitt *et al*., 2019), mostly wastewater associated ARG-carriers like *Hydrogenophaga* (Gan *et al.,* 2017, Fang *et al.,* 2021), human gastrointestinal disease associated ARG-carriers like *Pseudarcobacter* (Pérez-Cataluña *et al.,* 2018), natural ARG-carriers like *Mycobacterium* (Johansen *et al*., 2020) and especially in Narva Bay several bloom generating (Huisman *et al.,* 2018) cyanobacterial genera (i.e. *Planktothrix, Microcystis, Snowella*). The mixed composition of the seawater microbial community mirrors the unique conditions (i.e. brackish water, limited water exchange, large catchment area, intense anthropogenic pressure) of the Baltic Sea and suggest the necessity of further in depth research to its microbial community and antibiotic resistome evolvement and interactions as conclusions drawn based on seawater communities in other regions are probably not applicable in the Baltic Sea area due to its aforementioned unique nature.

5.2.2.2. The antibiotic resistome of the Baltic Sea

All seven targeted ARGs, except for *ampC* at Tallinn Bay and Gulf of Riga in 2008, were detected and quantified from the bacterioplankton of all the Baltic Sea sampling sites in 2008 and 2009 (Paper II). The quantified ARG abundances and their relative abundances were in the range of 10^2–10^4 copies/L and 0.001–0.047%, respectively, which coincides with the results of Muziasari *et al.* (2016) who found that the relative abundances of ARGs in the Baltic Sea were in the range of 0.001–0.048%. The relative abundance of targeted ARGs carrying bacteria within bacterioplankton mainly remained around 0.01% for each tested ARG except for *tetA* carrying bacteria, which showed up to two orders of magnitude higher proportion. The abundances and relative abundances of each targeted ARG differed in the samples obtained from different regions of the Baltic Sea, and there were also remarkable differences for individual ARGs between two study years at one sampling point. A strong association between ARG abundance data and bacterioplankton phylogenetic composition was also found (Paper II).

The recorded sulfonamide resistance encoding *sul1* gene, suggested as a marker for anthropogenic influence (Czekalski *et al*., 2015), abundances and relative abundances in the community were very variable at different sampling locations and times (abundance of 3.69×10^2–5.85×10^4 copies/L and relative abundance of 0.0001–0.0230%), supporting the notion of showcasing human influence, especially in Narva Bay sample of 2009, over a stable natural background. Nevertheless, the *sul1* relative abundances in the community recorded in this study were lower than previously found in European freshwater environments (Czekalski *et al*., 2012; Berglund *et al.,* 2015), probably owing to the greater dilution factor of the allochthonous microbial community in seawater compared with freshwater lakes and rivers.

The expansion of ARG quantification targets for this thesis revealed that *blaCTX-M*, which is an ESBL encoding gene, was by far the most abundant

individual ARG in seawater universally in all sampling locations (abundance of 1.2–8.4×10^6 copies/L and relative abundance of 0.4–0.8%), surpassing the abundance of other targets by at least two orders of magnitude (Table A.4) and relative abundance of most ARGs at least 4-fold (Table A.5). The prevalence of *blaCTX-M* gene could be an indicator of fecal contamination, as it is carried by *Enterobacteriaceae* such as *E. coli*. Still, this gene has been shown to be incorporated in the resistome of gut microbiota of seagulls (Alves *et al.*, 2014). The possible recording of anthropogenic influence event in Narva Bay in 2009, suggested by high *sul1* abundance recorded in the original study (Paper II) was further supported by singular detection of *aadA* gene with high abundance in the same sample (Table A.4) and placement of this sample separately from the rest of samples along the first axis in PCA plot (Figure 6A). Coinertia analysis results indicated that the correlation between the relative abundance of ARGs and bacterial community structure (Figure 6B) is weak (correlation estimate RV=0.54) and statistically not significant (p=0.11).

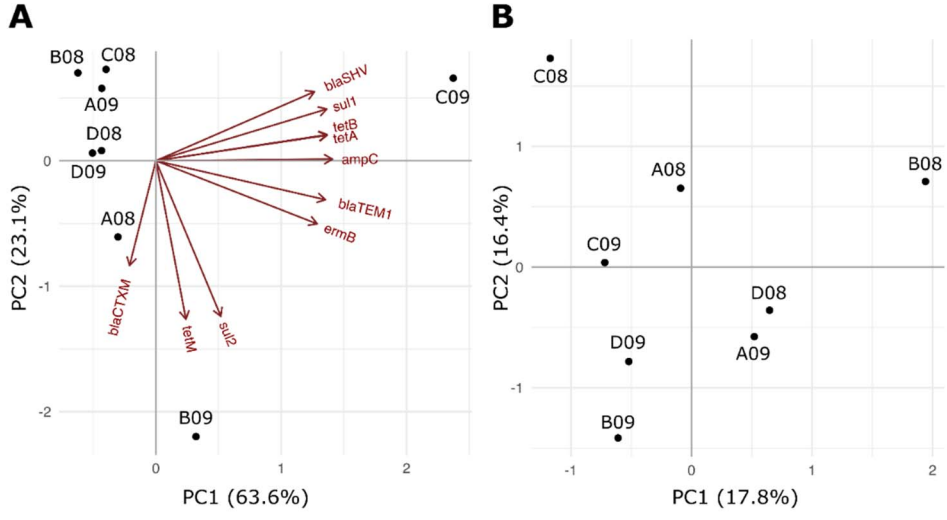

Figure 6. Ordination of the water samples according to the principal component analysis (PCA) based on the correlation matrix of antibiotic resistance gene relative abundances (**A**) and proportions of bacterial genera in the microbial community (**B**) in the Baltic Sea water. Sample labels: A – Tallinn Bay, B – Gulf of Finland, C – Narva Bay, D – Gulf of Riga; 08 and 09 denote years 2008 and 2009, respectively.

5.3. Comparison of the quantified section of antibiotic resistome in targeted water environments

The PCA analysis was applied in this thesis to visualize the differences in the structure of the antibiotic resistome in the different sections of the ARG propagation pathway from the wastewater treatment facilities (WWTP, CW) effluent through the primary receiving waterbody (stream) to the final receiving waterbody (the Baltic Sea). The PCA analysis revealed a difference in the structure of ARG communities between all the tested media (Figure 7). This result complements the findings of Li *et al.,* 2015, who reported distinctive grouping of environments, including different water environments, based on ARG proportions in metagenomic data. The first two axes of the PCA accounted for 75.6% of the total variance in case of ARG abundance data (Figure 7A, B) and 51.1% of the total variance in the case of ARG relative abundance data (Figure 7C, D). The CW and WWTP effluent samples distinguish from one another by ARG absolute and relative abundance values, indicating that the ARGs released to the environment are very different between these two wastewater purification methods. The stream water sample in close vicinity to the WWTP outflow (single outlier point of stream water samples in Figure 7) is strongly affected by the WWTP outflow. ARG carrying microbial community of this sample is more similar to WWTP effluent than the rest of the stream water samples. Still, other stream and river samples have a distinctly different resistome. Moving along the distance gradient, the Baltic Sea has its own distinct resistome characterized by the lowest amount of studied ARGs.

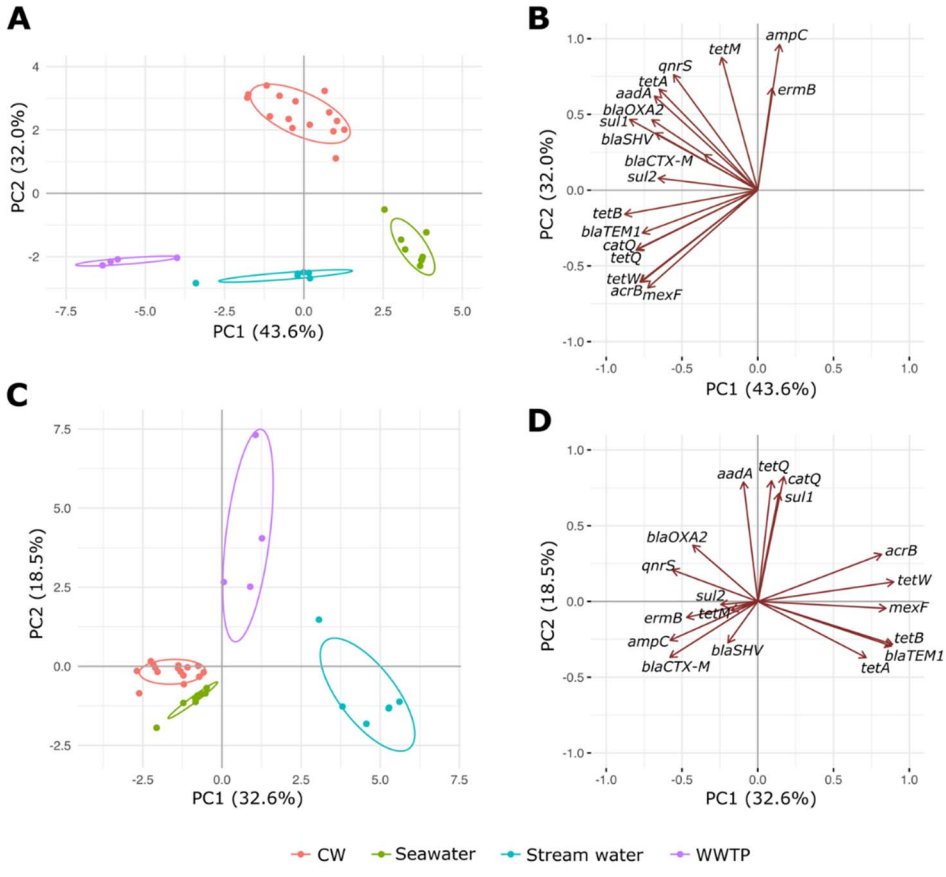

Figure 7. Ordination of the water samples according to the principal component analysis (PCA) based on the correlation matrix of antibiotic resistance gene (ARGs) abundances (**A** and **B**) and relative abundances (**C** and **D**) from different media – constructed wetland (CW), seawater, stream water, and wastewater treatment plant (WWTP) effluent. Plots A and C show the ordination of water samples, and plots B and D show ARGs' correlation with the first two PCA axes.

5.4. Comparison of old and improved gene quantification methodology

The quantification of gene copy numbers by qPCR can be affected by many factors, such as primer coverage, inhibition, low amplification efficiency (Yang *et al.,* 2013) as well as differences in the amplification efficiency of standard dilutions and environmental samples (Nõlvak *et al.,* 2012a). In the current thesis, the improved gene quantification methodology compared to the original research (Paper I, II) consisted of newer 16S rRNA primers with higher coverage as well as improved gene copy number calculation methodology, which takes into account the amplification efficiency of each standard curve dilution and targeted samples of environmental origin (Nõlvak *et al.,* 2016).

The previously quantified bacterial 16S rRNA gene abundance in CW effluent samples varied from 2.58×10^{9} to 5.24×10^{10} copies/L (Paper I), whereas with newer primers of higher coverage and improved calculation methodology, the 16S rRNA gene abundance range was 1.73×10^{9} to 1.27×10^{11} copies/L (Table A.1). The differences in ARG abundances and relative abundances of CW effluent and seawater samples between the original (old) and improved (new) target gene abundance calculation methodology are shown in Figure 8. The abundances of ARGs quantified in the CW effluents in the original paper (Paper I) were in the range of 2.94×10^{3} to 3.52×10^{7} copies/L and the recalculated ARGs abundances ranged from 8.96×10^{3} to 1.23×10^{8} copies/L (Table A.1). While most of the determined ARGs abundances in CW effluents were only slightly underestimated, the abundance of *ampC* abundance was underestimated by almost two orders of magnitude in the original study (Figure 8A). In the case of ARGs relative abundance in CW effluents, the harnessed improved quantification methodology indicated that the minute relative abundances of *tetB* and *tetM* remained virtually the same. At the same time, *ampC*, *qnrS* and *tetA* genes proved to be underestimated and *sul1* and *ermB* gene relative abundances were slightly overestimated in the original paper (Figure 8C).

In the case of the Baltic Sea seawater, the previously quantified bacterial 16S rRNA gene abundance was in the range of 5.55×10^{7} to 6.32×10^{8} copies/L (Paper II), while the improved quantification methodology yielded the abundance range of 2.53×10^{8} to 1.2×10^{9} copies/L (Table A.4). The abundances of ARGs quantified in the original paper were in the range of 25 to 6.82×10^{4} copies/L, while the recalculation gave the range of 3 to 3.02×10^{5} copies/L (Table A.4). Revisiting determined ARG abundances in the Baltic Sea water with improved calculation methodology indicated that the abundances of *ermB, sul1, tetB* and *tetA* were somewhat underestimated and the abundance of *blaSHV* overestimated in the original research (Figure 8B). The relative abundances of *blaSHV* and *tetM* genes proved to be slightly overestimated. In contrast, *ermB* and *tetB* genes were slightly underestimated, and *tetA* and *sul1* genes were substantially underestimated in the original paper (Figure 8D).

Although the total abundance of bacterial 16S rRNA gene was underestimated in the original papers I and II, and the improved estimations of ARG abundances also varied compared to original research, these specifications did not affect the main conclusions made in the papers.

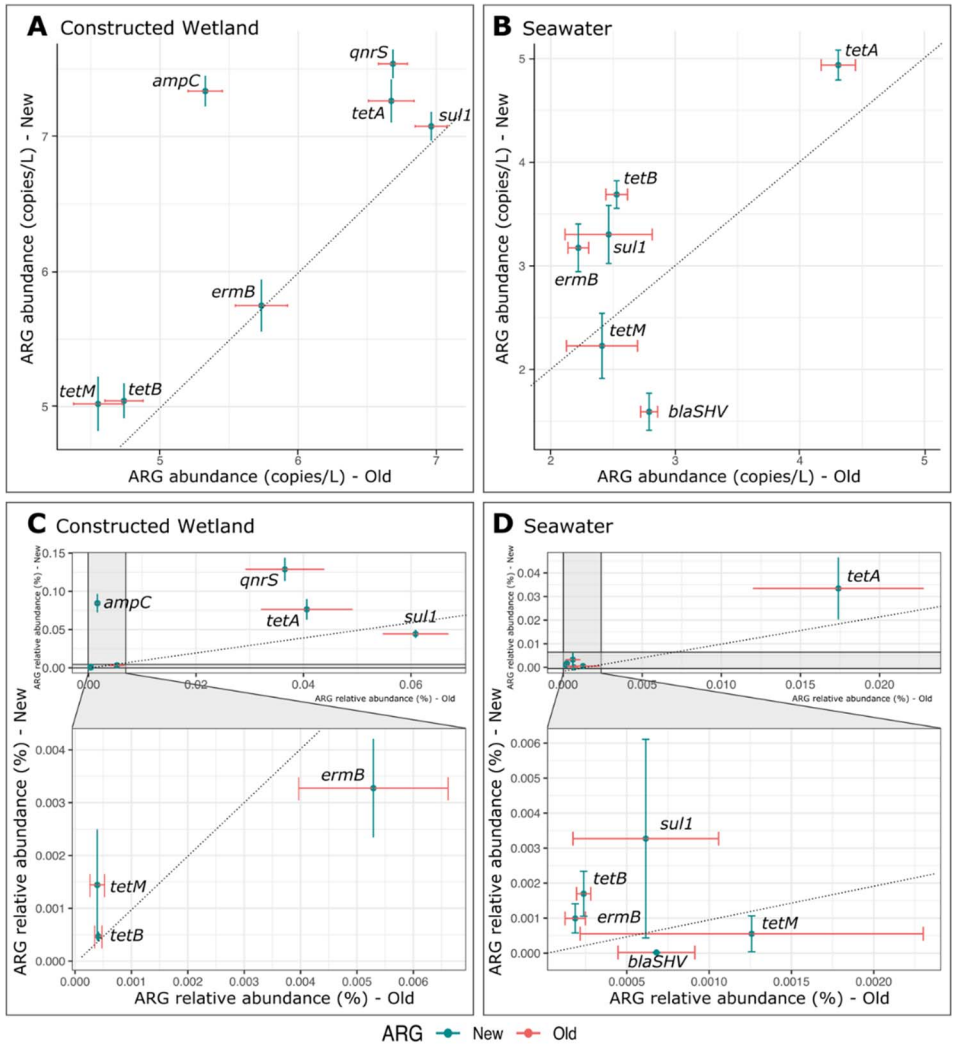

Figure 8. The comparison of the results of original (Paper I, II; old) and improved (new) antibiotic resistance gene (ARGs) abundance and relative abundance quantification calculation methodology in constructed wetland effluent (**A**, **C**) and seawater (**B**, **D**) samples, visualized as scatterplots with bidirectional error bars. The axes of ARG abundance graphs (A; B) are in logarithmic scale with only the values of exponents of ten depicted.

6. CONCLUSIONS

The effluents of WWTPs and other types of wastewater treatment facilities, such as CWs, are major contributors of nutrients as well as microbes, including ARG-carrying bacteria and archaea, as well as pathogens, to receiving waterbodies' ecosystems. In both stream water and sediments, the WWTP effluent's impact on the microbial community abundance and structure, its antibiotic resistome composition and abundance, and the pathogenic community structure are highest at a close vicinity location (0.3 km) downstream of the WWTP discharge point. The further downstream, gradual recovery of impacted communities along the distance gradient from WWTP was recorded, culminating in the mostly comparable state of river water and sediment parameters 3.7 km downstream of WWTP to the stream water and sediments upstream of the WWTP discharge point. Our results also suggest that archaea form a substantial proportion of the microbial community of WWTP effluent and receiving stream and possibly contribute to the spread of antibiotic resistance determinants. The role of archaea as well as the state and abundance of pathogenic communities in WWTP effluent-receiving waterbodies merits further in-depth research with combined community analysis and quantitative methods approach.

The abundance of 16S rRNA and ARGs, and the proportions of ARGs in the microbial community, were generally reduced during the wastewater treatment process in the CW. ARG concentrations in system effluent were comparable to those observed for conventional wastewater treatment facilities except for sulfonamide resistance encoding *sul1* concentration, which was reduced more efficiently by HSSF MCs than in traditional wastewater treatment systems.

The most numerous ARG in the bacterioplankton of the Baltic Sea was *tetA*, which also showed the highest relative abundance compared with the 16S rRNA genes of the whole bacterial community. The dominant phyla in the bacterioplankton of the Baltic Sea were Actinobacteria, Protobacteria, and Bacteroidetes, and the structure of the bacterial community varied over time and in space. The results from the multivariate analysis revealed that each of the analyzed water environments (WWTP, CW, stream water and seawater) has its own distinct ARG resistome.

ARGs are ubiquitous in natural environments, hence, it is becoming increasingly clear that the environment plays an important role in the dissemination of ARGs. Elevated levels of ARGs in aquatic environments are shown to be in correlation with a proximity to anthropogenic activities. The origin of this increase is likely to be routine discharge of antibiotics and ARGs, for example, via WWTP effluents or run-off from livestock facilities and agriculture. HGT events are likely to be common in different compartments of the aquatic environment and integrons, in particular, are well suited for mediating environmental dissemination of ARGs.

Although our results are indicating that the effect of WWTP on the receiving waterbodies is decreasing gradually downstream, given the specifics of the Baltic

Sea and the high pollution load due to WWTP outflows in the coastal region, the Baltic Sea is vulnerable to ARG contamination. On top of the fact that the Baltic Sea is used for fishing, fish farming and the beaches are used for recreational purposes, there is a high probability of transmitting ARGs from the environment to humans. Considering these factors, there is a need for comprehensive large-scale studies about the diversity and abundance of ARGs in the different compartments of the Baltic Sea. Such studies would allow relating spatial and temporal variation in ARG carrying microbes in the Baltic Sea microbiome to environmental conditions and anthropogenic activities and assess the potential health-related risks and need for improved wastewater treatment technologies.

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SUMMARY IN ESTONIAN

Antibiootikumiresistentsus omavahel seotud tehislikus ja looduslikus veekeskkonnas

Antibiootikumide kasutuselevõtuga 20. sajandi alguses muutusid seni surmavad infektsioonid ravitavaks, kuid kuna kõigi antibiootikumide vastu kujuneb mikroorganismidel ühel hetkel välja resistentsus, on tänapäeval bakteriaalsed infektsioonid muutunud taas ohtlikuks. Antibiootikumiresistentsus ei ole ainult meditsiinisektori probleem, vaid resistentsuse tekke ja leviku taga on ka antibiootikumide kasutamine põllumajanduses ja loomakasvatuses ning selle levik keskkonnas. On näidatud, et kliiniliselt olulised antibiootikumiresistentsusgeenid (ARG) pärinevad sageli hoopis looduslikust keskkonnast. Kuna keskkonnabakteritelt võivad ARG-id levida omakorda inimpatogeenidele, on antibiootikumiresistentsuse vastu võitlemiseks vajalik uurida ARG-ide päritolu ning levikut keskkonnas.

Reoveepuhastusjaamade heitvesi on üks peamisi teid, kuidas ARG-id tehiskeskkonnast looduslikku keskkonda pääsevad, kuna reoveepuhastuse käigus ei eemaldata veest kõiki resistentseid baktereid ega ARGe. Kuna reoveepuhastusjaamades on baktereid väga palju ja tihedalt koos ning ARG-id asuvad enamasti mobiilsetel geneetilistel elementidel, saab seal toimuda horisontaalne geeniülekanne erinevast keskkonnast pärit bakterite vahel ning seega mõnel juhul hoopiski ARG-ide kontsentreerumine. Kõrvuti enimlevinud aktiivmudaprotsessil põhinevate reoveepuhastusjaamadega kasutatakse ka alternatiivseid reoveepuhastussüsteeme, näiteks tehismärgalasid. Reoveepuhastusjaamade heitvesi juhitakse enamasti looduslikesse veekogudesse, näiteks ojadesse või jõgedesse, kus ARG-ide hulk suureneb reoveepuhasti väljavoolust allavoolu jäävatel aladel. Antibiootikumijäägid, resistentsed bakterid ja ARG-id võivad kanduda põhjavette, jõgedesse ja lõpuks ka merre. Sealt võivad nad omakorda tagasi inimestele kanduda kas otsese kokkupuute kaudu, näiteks veekogus ujudes või saastunud vett juues, või kaudseid teid pidi, näiteks süües mereande. Hetkel on siiski veel vähe teada ARG-ide leviku-, ülekande- ja akumuleerumismehhanismide kohta inimtegevusest mõjutatud looduslikus veekeskkonnas.

Eelkirjeldatust tulenevalt oli siinse töö eesmärgiks kirjeldada reoveepuhastusjaama heitveest pärinevate ARG-ide levikut vastuvõtvates veekogudes. Peamised uurimismeetodid, mida antud töös kasutati olid amplikonipõhine ja kogu DNA sekveneerimine ning kvantitatiivne polümeraasi ahelreaktsioon (qPCR). Töö käigus saadud tulemused on järgmised:

- Uuritud tehismärgalas reoveepuhastusprotsessi käigus 16S rRNA geeni ja ARG-ide üldarvukus ning nende osakaal mikroobikoosluses vähenesid üldiselt. Uuritud ARG-id Nõo WWTP väljavoolus jäid vahemikku 0.0008–2.04% kogu mikroobikooslusest, sarnaselt teistele WWTP-dele Läänemere piirkonnas. ARG-ide kontsentratsioonid CW süsteemi heitvees olid võrreldavad konventsionaalsete reoveepuhastitega, välja arvatud sulfoonamiidi resistentsust kodeeriva *sul1* geeni puhul, mille eemaldamine tehismärgalas oli efektiivsem.

- Reoveepuhastusjaamade ja tehismärgalade heitveed on olulised vastuvõtvate veekogude toitainete, aga ka mikroobide (sealhulgas ARG-e kandvad bakterid ja arhed ning patogeenid) allikad. Reoveepuhastusjaama väljavoolul oli kõige suurem mõju vahetus läheduses (0,3 km) olevale oja vee ja sette mikroobikooslusele, nii selle arvukusele ja struktuurile kui ka antibiootikumiresistoomile ning patogeenide sisaldusele. Kaugemale allavoolu jäävate alade setete mikroobikooslus taastus järk-järgult ning juba 3,7 kilomeetrit eemal oli jõesette bakterikooslus võrreldav reoveepuhastist ülesvoolu jääva alaga. ARG-ide arvukus ojavees vähenes reoveepuhastusjaamast kaugemale allavoolu liikudes. Saadud tulemused viitavad ka sellele, et arhed moodustavad arvestatava osa reoveepuhasti heitvee ja vastuvõtvate veekogude mikroobikooslusest ning võivad aitadata kaasa ARG-ide levikule.
- Läänemere bakteriplanktoni antibiootikumiresistoomi võib teiste tegurite kõrval mõjutada üle 3100 Läänemere valgalas asuva reoveepuhastusjaama väljavoolu. Uuritud ARG-idest kõige arvukam oli tetratsükliini resistentsust kodeeriv *tetA* geen, mis oli ka suurima osakaaluga ARG kogu Läänemere bakterikoosluses. Läänemere bakteriplanktoni domineerivateks hõimkondadeks olid *Actinobacteria, Protobacteria* ja *Bacteroidetes* ning bakterikoosluse struktuur varieerus ajas ja ruumis.
- Mitmemõõtmelise analüüsi tulemused näitasid, et igal analüüsitud veekeskkonnal (reoveepuhastusjaama väljavool, tehismärgalapuhasti väljavool, ojavesi ja merevesi) on oma selgelt erinev ARG resistoom.
- Kuigi töö tulemused näitavad, et Nõo reoveepuhasti mõju vastuvõtvatele veekogudele väheneb järk-järgult allavoolu, siis tulenevalt Läänemere eripäradest ja suurest saastekoormusest rannikualadel, on Läänemeri väga tundlik ARG-ide reostuse suhtes. Läänemeres on ka mitmeid kalakasvatusi, ning randu kasutatakse sageli meelelahutuslikel eesmärkidel, mistõttu on suur oht ARG-ide ülekandumiseks looduslikust keskkonnast inimestele.

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APPENDIX

Table A.1. The abundances of bacterial and archaeal 16S rRNA genes (B16S and A16S, respectively) and antibiotic resistance genes in the microbial community of the effluent of horizontal subsurface flow filters of Nõo constructed wetland. Gene copy numbers are presented as gene copy numbers per liter of water. NA – not applicable, ND – not detected.

Sample	B16S	A16S	*aadA*	*bla CTX-M*	*bla OXA2*	*bla TEM1*	*sul1*	*sul2*	*tetA*	*tetB*	*tetM*	*ermB*	*ampC*	*qnrS*
26_I	3.33×10^{10}	2.21×10^{9}	3.28×10^{7}	ND	NA	NA	1.30×10^{7}	NA	9.86×10^{6}	1.92×10^{5}	1.26×10^{5}	2.94×10^{8}	1.36×10^{7}	3.78×10^{7}
26_II	2.77×10^{10}	2.33×10^{9}	2.53×10^{7}	1.82×10^{8}	1.55×10^{6}	3.50×10^{5}	1.68×10^{7}	NA	1.24×10^{7}	2.60×10^{5}	2.17×10^{5}	NA	3.37×10^{7}	4.88×10^{7}
26_III	2.04×10^{10}	2.72×10^{9}	2.47×10^{7}	1.71×10^{8}	8.38×10^{5}	NA	1.32×10^{7}	NA	6.95×10^{6}	1.48×10^{5}	3.36×10^{4}	NA	1.97×10^{7}	3.83×10^{7}
45_I	6.02×10^{10}	2.81×10^{9}	5.97×10^{7}	3.70×10^{8}	2.49×10^{6}	9.91×10^{5}	2.23×10^{7}	NA	6.26×10^{7}	3.39×10^{5}	7.80×10^{4}	1.50×10^{8}	4.25×10^{7}	6.45×10^{7}
45_II	4.72×10^{10}	5.23×10^{9}	8.80×10^{7}	NA	NA	NA	2.48×10^{7}	NA	7.40×10^{7}	1.90×10^{5}	7.55×10^{6}	3.78×10^{8}	6.57×10^{7}	7.19×10^{7}
45_III	3.22×10^{10}	2.70×10^{9}	3.20×10^{7}	2.01×10^{8}	1.05×10^{6}	7.47×10^{5}	1.50×10^{7}	2.08×10^{7}	2.22×10^{7}	7.62×10^{4}	1.32×10^{5}	NA	3.14×10^{7}	4.35×10^{7}
64_I	8.36×10^{10}	3.20×10^{9}	1.35×10^{8}	6.40×10^{8}	5.66×10^{6}	1.33×10^{6}	3.63×10^{7}	1.49×10^{7}	1.12×10^{8}	3.24×10^{5}	6.80×10^{5}	NA	9.75×10^{7}	1.05×10^{8}
64_II	3.34×10^{10}	1.85×10^{9}	4.93×10^{7}	9.12×10^{7}	1.27×10^{6}	7.45×10^{5}	2.53×10^{7}	4.73×10^{6}	6.60×10^{7}	2.92×10^{5}	1.02×10^{5}	7.44×10^{7}	5.94×10^{7}	7.33×10^{7}
64_III	1.27×10^{11}	2.45×10^{9}	1.49×10^{8}	3.12×10^{8}	4.36×10^{6}	1.82×10^{6}	2.93×10^{7}	1.16×10^{7}	1.23×10^{8}	5.57×10^{5}	8.32×10^{5}	NA	4.75×10^{7}	8.51×10^{7}
94_I	4.29×10^{10}	2.11×10^{9}	2.60×10^{7}	1.62×10^{8}	1.46×10^{6}	2.69×10^{5}	1.18×10^{7}	NA	2.24×10^{7}	8.22×10^{4}	1.36×10^{5}	3.42×10^{8}	1.25×10^{7}	3.43×10^{7}
94_II	3.00×10^{10}	4.07×10^{8}	1.81×10^{7}	NA	NA	NA	7.31×10^{6}	NA	1.46×10^{7}	6.16×10^{4}	1.65×10^{4}	1.24×10^{8}	1.18×10^{7}	2.12×10^{7}
94_III	2.41×10^{10}	8.11×10^{8}	1.98×10^{7}	9.09×10^{7}	ND	NA	1.34×10^{7}	NA	1.97×10^{7}	4.30×10^{4}	4.48×10^{4}	4.53×10^{8}	1.05×10^{7}	3.88×10^{7}
150_I	3.25×10^{10}	3.16×10^{8}	8.16×10^{6}	6.65×10^{7}	2.70×10^{5}	8.83×10^{5}	4.56×10^{6}	4.18×10^{6}	5.01×10^{6}	3.86×10^{4}	2.44×10^{4}	3.00×10^{8}	1.39×10^{7}	1.32×10^{7}
150_II	1.37×10^{10}	2.65×10^{8}	3.62×10^{6}	9.19×10^{7}	2.27×10^{5}	NA	2.98×10^{6}	NA	3.74×10^{6}	1.45×10^{4}	8.96×10^{4}	7.68×10^{7}	1.00×10^{7}	8.63×10^{6}
150_III	1.73×10^{9}	5.17×10^{7}	NA	1.95×10^{7}	3.38×10^{4}	2.29×10^{4}	1.38×10^{6}	3.83×10^{5}	9.65×10^{5}	1.90×10^{4}	2.23×10^{4}	5.92×10^{5}	2.45×10^{6}	4.01×10^{6}

Table A.2. The relative abundances (%) of antibiotic resistance genes in the microbial community of the effluent of the horizontal subsurface flow filters of Nõo constructed wetland. NA – not applicable.

Sample	*aadA*	*blaCTXm*	*blaOXA2*	*blaTEM1*	*sul1*	*sul2*	*tetA*	*tetB*	*tetM*	*ermB*	*ampC*	*qnrS*
26_I	0.0925	NA	NA	NA	0.0367	NA	0.0278	0.0005	0.0004	0.8276	0.0383	0.1065
26_II	0.0841	0.6063	0.0052	0.0012	0.0561	NA	0.0412	0.0009	0.0007	NA	0.1124	0.1627
26_III	0.1071	0.7408	0.0036	0.0000	0.0571	NA	0.0301	0.0006	0.0001	NA	0.0853	0.1657
45_I	0.0948	0.5872	0.0040	0.0016	0.0353	NA	0.0994	0.0005	0.0001	0.2376	0.0675	0.1024
45_II	0.1679	NA	NA	NA	0.0473	NA	0.1411	0.0004	0.0144	0.7201	0.1254	0.1372
45_III	0.0917	0.5749	0.0030	0.0021	0.0430	0.0597	0.0635	0.0002	0.0004	NA	0.0899	0.1247
64_I	0.1551	0.7377	0.0065	0.0015	0.0419	0.0172	0.1290	0.0004	0.0008	NA	0.1123	0.1214
64_II	0.1397	0.2584	0.0036	0.0021	0.0716	0.0134	0.1872	0.0008	0.0003	0.2109	0.1685	0.2077
64_III	0.1149	0.2411	0.0034	0.0014	0.0226	0.0089	0.0949	0.0004	0.0006	NA	0.0367	0.0657
94_I	0.0578	0.3604	0.0032	0.0006	0.0263	NA	0.0497	0.0002	0.0003	0.7604	0.0278	0.0762
94_II	0.0596	NA	NA	NA	0.0240	NA	0.0479	0.0002	0.0001	0.4067	0.0386	0.0697
94_III	0.0795	0.3643	NA	NA	0.0536	NA	0.0790	0.0002	0.0002	0.0018	0.0419	0.1555
150_I	0.0248	0.2026	0.0008	0.0027	0.0139	0.0127	0.0152	0.0001	0.0001	0.9145	0.0423	0.0403
150_II	0.0258	0.6559	0.0016	NA	0.0212	NA	0.0267	0.0001	0.0001	0.5486	0.0714	0.0616
150_III	NA	1.0972	0.0019	0.0013	0.0776	0.0215	0.0542	0.0011	0.0013	0.0332	0.1373	0.2250

Table A.3. The proportion (%) of top 50 bacterial genera in the bacterial community of the Baltic Sea. A – Tallinn Bay, B – Gulf of Finland, C – Narva Bay, D – Gulf of Riga. 08 and 09 in sample labels denote years 2008 and 2009, respectively.

Genus	A08	B08	C08	D08	A09	B09	C09	D09
CL500-29 marine group	33.59	44.29	24.08	32.70	26.87	16.28	20.40	16.12
Mycobacterium	2.85	3.88	6.52	6.31	5.85	21.65	10.42	5.52
hgcI clade	6.33	28.42	10.63	6.75	3.87	2.36	3.01	1.63
Streptococcus	1.61	2.47	2.25	2.27	2.72	2.29	2.27	2.39
Veillonella	1.35	2.40	1.90	2.18	2.17	2.27	2.02	2.15
Clade III genus	1.26	3.60	2.97	1.63	1.64	0.90	0.79	0.88
Rothia	0.84	1.25	1.17	1.20	1.03	1.23	1.06	1.23
Cyanobium PCC-6307	0.38	1.40	1.56	0.41	2.05	0.21	1.77	0.81
SL56 marine group genus	0.60	0.48	2.05	1.28	0.68	0.33	0.80	1.43
Fusobacterium	0.64	1.14	0.91	1.05	1.11	1.25	0.85	0.66
Prevotella	0.62	1.22	0.96	0.98	1.08	1.01	0.91	0.78
Gemella	0.95	1.20	1.02	0.89	0.93	1.02	0.85	0.67
Actinomyces	0.47	1.17	0.74	1.03	0.97	0.95	0.74	0.80
Enhydrobacter	0.14	0.27	0.25	0.19	0.32	0.18	0.24	4.42
NS3a marine group	1.37	1.97	0.31	0.51	0.24	0.73	0.40	0.12
Microcella	1.01	0.91	1.24	0.85	0.49	0.29	0.32	0.43
OM43 clade	0.70	0.94	0.73	0.67	0.74	0.35	0.36	0.48
PeM15 genus	0.49	0.71	0.65	0.55	0.40	1.44	0.44	0.28
Psychroserpens	1.00	1.36	0.35	0.59	0.95	0.26	0.25	0.13
LD29	0.45	1.09	0.49	0.75	0.62	0.62	0.37	0.24
Planktothrix NIVA-CYA 15	0.03	0.09	2.76	0.10	0.10	0.08	1.39	0.04
Porphyromonas	0.49	0.68	0.71	0.47	0.51	0.57	0.38	0.29
Sphingorhabdus	1.03	0.81	0.47	0.26	0.55	0.45	0.26	0.20
Ca Aquirestis	0.19	0.31	1.10	0.32	0.30	0.17	0.91	0.37

Genus	A08	B08	C08	D08	A09	B09	C09	D09
Fluviicola	0.39	1.33	0.38	0.29	0.12	0.35	0.11	0.12
Actibacter	0.26	0.82	0.18	0.35	0.19	0.17	0.92	0.13
Sporichthyaceae genus	0.28	1.16	0.71	0.22	0.23	0.14	0.15	0.11
Tabrizicola	0.47	0.78	0.34	0.13	0.33	0.13	0.47	0.12
ML602J-51	0.33	0.58	0.06	0.56	0.25	0.58	0.07	0.33
Snowella 0TU37S04	0.14	0.37	1.37	0.15	0.17	0.17	0.25	0.08
Pseudarcobacter	0.04	0.10	0.05	0.06	0.11	1.99	0.16	0.06
Alloprevotella	0.26	0.59	0.32	0.24	0.42	0.28	0.31	0.13
Microcystis PCC-7914	0.04	0.11	1.97	0.09	0.09	0.06	0.10	0.03
OM190 genus	0.07	0.08	0.17	0.22	0.16	0.07	0.32	1.34
Yoonia-Loktanella	0.30	0.39	0.28	0.43	0.23	0.31	0.32	0.15
Dokdonella	0.07	0.11	0.07	0.08	0.74	0.09	0.12	1.11
Ca Planktophila	0.24	1.02	0.51	0.23	0.13	0.05	0.09	0.05
MWH-UniP1 aquatic group genus	0.23	0.63	0.57	0.16	0.26	0.15	0.14	0.10
Capnocytophaga	0.21	0.21	0.26	0.25	0.30	0.33	0.17	0.31
Ilumatobacter	0.15	0.12	0.12	0.25	0.46	0.31	0.16	0.31
AEGEAN-169 marine group genus	0.20	0.94	0.10	0.18	0.12	0.15	0.10	0.06
Aphanizomenon MDT14a	0.36	0.23	0.15	0.12	0.17	0.41	0.29	0.07
Flavobacterium	0.52	0.27	0.17	0.09	0.19	0.15	0.26	0.10
Aquabacterium	0.05	0.08	1.23	0.06	0.10	0.06	0.06	0.07
Leptotrichia	0.10	0.26	0.17	0.23	0.29	0.25	0.16	0.14
Rhodoluna	0.14	0.48	0.14	0.25	0.16	0.11	0.12	0.15
Algoriphagus	0.26	0.38	0.12	0.16	0.24	0.17	0.12	0.06
Escherichia-Shigella	0.06	0.19	0.12	0.15	0.17	0.16	0.26	0.37
Hydrogenophaga	0.05	0.04	0.38	0.02	0.13	0.57	0.22	0.04
Atopobium	0.07	0.26	0.15	0.19	0.22	0.16	0.17	0.16

Table A.4. The abundances of bacterial and archaeal 16S rRNA genes (B16S and A16S, respectively) and antibiotic resistance genes in the microbial community of the Baltic Sea. Gene copy numbers are presented as gene copy numbers per liter of seawater. A – Tallinn Bay, B – Gulf of Finland, C – Narva Bay, D – Gulf of Riga. 08 and 09 in sample labels denote years 2008 and 2009, respectively. ND – not detected.

Sample	B16S	A16S	*aadA*	*bla CTX-M*	*bla OXA2*	*bla TEM1*	*sul1*	*sul2*	*tetA*	*tetB*	*tetM*	*ermB*	*ampC*	*blaSHV*
A08	4.15×10^{8}	3.52×10^{6}	ND	2.85×10^{6}	ND	4.44×10^{3}	3.87×10^{2}	3.17×10^{3}	2.10×10^{4}	1.73×10^{3}	70	5.92×10^{3}	ND	69
B08	4.73×10^{8}	2.76×10^{6}	ND	1.83×10^{6}	ND	2.79×10^{3}	7.03×10^{2}	2.09×10^{3}	2.54×10^{4}	4.32×10^{3}	1.33×10^{2}	5.09×10^{2}	4.66×10^{4}	31
C08	2.97×10^{8}	1.58×10^{6}	ND	1.24×10^{6}	ND	2.84×10^{3}	1.19×10^{3}	1.33×10^{3}	1.04×10^{5}	1.69×10^{3}	16	2.44×10^{2}	4.46×10^{4}	45
D08	2.76×10^{8}	6.49×10^{5}	ND	2.30×10^{6}	ND	2.72×10^{3}	6.42×10^{2}	1.20×10^{3}	8.30×10^{4}	2.58×10^{3}	27	7.76×10^{2}	ND	47
A09	5.64×10^{8}	1.50×10^{6}	ND	3.01×10^{6}	ND	2.10×10^{3}	8.31×10^{3}	2.43×10^{3}	9.23×10^{4}	1.09×10^{4}	1.01×10^{2}	3.64×10^{3}	2.01×10^{3}	79
B09	2.81×10^{8}	1.58×10^{6}	ND	2.00×10^{6}	ND	4.80×10^{3}	3.69×10^{2}	2.54×10^{3}	1.03×10^{5}	5.35×10^{3}	1.16×10^{4}	5.98×10^{3}	2.82×10^{5}	3
C09	2.53×10^{8}	1.19×10^{6}	2.95×10^{5}	1.27×10^{6}	ND	7.00×10^{3}	5.85×10^{4}	1.59×10^{3}	3.02×10^{5}	1.51×10^{4}	4.55×10^{2}	8.22×10^{3}	8.02×10^{5}	1.74×10^{2}
D09	1.20×10^{9}	2.35×10^{6}	ND	8.38×10^{6}	ND	1.11×10^{4}	7.14×10^{3}	5.79×10^{3}	2.39×10^{5}	1.11×10^{4}	3.13×10^{2}	2.41×10^{2}	4.72×10^{5}	27

Table A.5. The relative abundances (%) of antibiotic resistance genes in the microbial community of the Baltic Sea. A – Tallinn Bay, B – Gulf of Finland, C – Narva Bay, D – Gulf of Riga. 08 and 09 in sample labels denote years 2008 and 2009, respectively. NA – not applicable.

Sample	*aadA*	*blaCTX-M*	*blaOXA2*	*blaTEM1*	*sul1*	*sul2*	*tetA*	*tetB*	*tetM*	*ermB*	*ampC*	*blaSHV*
A08	NA	0.680025	NA	0.001061	0.000092	0.000758	0.005014	0.000413	0.000017	0.001415	NA	0.000016
B08	NA	0.386042	NA	0.000588	0.000148	0.000439	0.005352	0.000910	0.000028	0.000107	0.009805	0.000006
C08	NA	0.415968	NA	0.000951	0.000398	0.000444	0.034712	0.000566	0.000005	0.000082	0.014937	0.000015
D08	NA	0.831946	NA	0.000982	0.000232	0.000433	0.029947	0.000931	0.000010	0.000280	NA	0.000017
A09	NA	0.532645	NA	0.000371	0.001469	0.000430	0.016307	0.001934	0.000018	0.000643	0.000356	0.000014
B09	NA	0.706762	NA	0.001699	0.000131	0.000901	0.036321	0.001894	0.004115	0.002119	0.099979	0.000001
C09	0.115929	0.497960	NA	0.002751	0.022992	0.000623	0.118776	0.005947	0.000179	0.003232	0.315177	0.000068
D09	NA	0.696543	NA	0.000924	0.000593	0.000481	0.019860	0.000925	0.000026	0.000020	0.039243	0.000002

PUBLICATIONS

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Publikatsioonid:

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DISSERTATIONES TECHNOLOGIAE CIRCUMIECTORUM UNIVERSITATIS TARTUENSIS

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