The role of wastewater in surveillance and emergence of antibiotic resistant bacteria

Marion Hutinel

Department of Infectious Diseases,
Institute of Biomedicine at Sahlgrenska Academy
University of Gothenburg

UNIVERSITY OF GOTHENBURG

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marion.hutinel@gu.se

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“Nous sommes solidaires, emportés par la même planète, équipage d’un même navire”

“We stand together, carried along by the same planet, crew of a single ship”

Antoine de Saint-Exupery
Terre des Hommes
Wind, Sand and Stars

ABSTRACT

As antibiotic resistance spreads among bacterial pathogens, it reduces treatment options and increases treatment failures of infectious diseases. Strategies employed to reduce this spread or adapt to its consequences need to be based on reliable surveillance data which is lacking in many countries, often due to limited resources. Wastewater contains pooled excreted bacteria, including common pathogens such as *Escherichia coli*, from the population connected to the sewers. Hence, analysis of wastewater has the potential to be used as a resource-efficient surveillance system for antibiotic resistance. In the sewers, human-associated bacteria are also mixed with environmental bacteria and exposed to many substances known to induce horizontal gene transfer (HGT), a major driver for the acquisition of antibiotic resistance in bacteria. The studies presented in this thesis aimed to develop and assess several ways in which the analysis of wastewater samples could be used to provide clinically relevant antibiotic resistance data and evaluate the effects of wastewater on HGT.

The resistance rates of *E. coli* from wastewaters were determined. Additionally, the abundance of different carbapenemase-producing Enterobacterales (CPE) and antibiotic resistance genes (ARGs) were quantified in wastewater. Resistance rates in wastewater *E. coli* were strongly correlated with resistance rates in clinical isolates and the detection of CPE in wastewater was coherent with the detection of similar CPE in the contributing population. The concentrations of some carbapenemase genes (namely $bla_{\text{OXA-48}}$, bla_{NDM} and bla_{KPC}) were in accordance with the occurrence of CPE carrying those genes in wastewater. Further, a rise of *bla*OXA-48 in wastewater preceded detection of corresponding CPE in patients, indicating that monitoring of ARGs in wastewater could serve as an early warning system. Hence, it is noteworthy that many ARGs of emerging concern (*cfr*, *optrA*, *mcr-1*, *mcr-3*, *mcr-4*, *mcr-5*, *sul4* and *gar*), which have almost never been detected in Swedish clinical samples, were detected regularly in wastewater.

A HGT assay, where a recipient strain was mixed with a complex donor bacterial community, was used to measure the rate of acquisition of ARGs in the presence of wastewater. Municipal wastewater had no detectable effect on HGT but exposure to hospital wastewater could promote antibiotic resistance.

Overall, this thesis provides evidence supporting the use of antibiotic resistance data from wastewater analyses as a valuable complement to traditional clinical surveillance. Additionally, the thesis highlights a possible role of hospital wastewater in the emergence of antibiotic resistant bacteria.

SAMMANFATTNING PÅ SVENSKA

Spridning av antibiotikaresistens bland patogena bakterier ökar, vilket leder till färre behandlingsalternativ vid infektionssjukdomar och ökar risken för behandlingssvikt. Strategier för att minska denna spridning eller anpassa sig till dess konsekvenser måste baseras på tillförlitlig övervakningsdata, vilket saknas i många länder, ofta på grund av begränsade resurser. Avloppsvatten innehåller utsöndrade bakterier, inklusive vanliga patogener så som *Escherichia coli*, från befolkningen som är ansluten till avloppssystemet. Analys av avloppsvatten har därför potential att användas som ett resurseffektivt övervakningssystem för antibiotikaresistens. I avlopp blandas också human-associerade bakterier med miljöbakterier och exponeras där för många ämnen som har beskrivits kunna inducera horisontell genöverföring, en viktig drivkraft för förvärvandet av antibiotikaresistens bland bakterier. Studierna som presenteras i denna avhandling syftade till att utveckla och bedöma flera sätt på vilka avloppsanalyser kan användas för att tillhandahålla kliniskt relevanta antibiotikaresistensdata samt utvärdera effekterna av avloppsvatten på horisontell genöverföring.

Andelen *E. coli* från avloppsvatten resistenta mot olika antibiotika bestämdes. Dessutom, kvantifierades mängden av olika karbapenemasproducerande Enterobacterales (KPE) och antibiotikaresistensgener (ARG) i avloppsvatten. Andelarna resistenta *E. coli* i avloppsvatten var starkt korrelerade med andelarna resistenta *E. coli* bland kliniska isolat och liknande typer av KPE detekterades i sjukhusavlopp och patienter. I avloppsvatten stämde koncentrationerna av några karbapenemasgener (*bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$ och *bla*$_{\text{KPC}}$) väl överens med förekomsten av KPE som bar på dessa gener. Dessutom, en ökning av *bla*$_{\text{OXA-48}}$ i avloppsvatten observerades före det att motsvarande KPE detekterades hos patienter, vilket indikerar att övervakning av ARG i avloppsvatten kan fungera som ett tidigt varningssystem. Det är därför värt att notera att flera ARG, som nästan aldrig har upptäckts i svenska kliniska prover (*cfr*, *optrA*, *mcr-1*, *mcr-3*, *mcr-4*, *mcr-5*, *sul4* och *gar*), upptäcktes regelbundet i avloppsvatten.

Ett system för att studera horisontell genöverföring, där en mottagarbakterie blandades med ett komplext givarbakteriesamhälle, användes för att mäta förvärvandet av ARG i närvaro av avloppsvatten. Kommunalt avloppsvatten hade ingen påvisbar effekt på horisontell genöverföring men exponering för sjukhusavlopp kunde främja antibiotikaresistens.

Sammantaget ger denna avhandling stöd för användandet av antibiotikaresistensdata från avloppsvattenanalyser som ett värdefullt komplement till traditionell klinisk övervakning. Dessutom belyser avhandlingen en möjlig roll för sjukhusavloppsvatten i uppkomsten av antibiotikaresistenta bakterier.

PUBLICATION LIST

The thesis is based on the following articles:

I. Marion Hutinel, Patricia Maria Catharina Huijbers, Jerker Fick, Christina Åhrén, Dan Göran Joakim Larsson, and Carl-Fredrik Flach
Population-level surveillance of antibiotic resistance in *Escherichia coli* through sewage analysis
Eurosurveillance, 2019, 24(37), 1800497

II. Carl-Fredrik Flach, Marion Hutinel, Mohammad Razavi, Christina Åhrén, and Dan Göran Joakim Larsson
Monitoring of hospital sewage shows both promise and limitations as an early-warning system for carbapenemase-producing Enterobacterales in a low-prevalence setting
Manuscript

III. Marion Hutinel, Dan Göran Joakim Larsson, and Carl-Fredrik Flach
Antibiotic resistance genes of emerging concern in Swedish municipal and hospital wastewaters
Manuscript

IV. Marion Hutinel, Jerker Fick, Dan Göran Joakim Larsson, and Carl-Fredrik Flach
Investigating the effects of municipal and hospital wastewaters on horizontal gene transfer
Environmental Pollution, 2021, 276, 116733

Additional articles not included in this thesis:

Nadine Kraupner, Stefan Ebmeyer, Marion Hutinel, Jerker Fick, Carl-Fredrik Flach, and Dan Göran Joakim Larsson.
Selective concentrations for trimethoprim resistance in aquatic environments
Environment International, 2020, 144, 106083

Nadine Kraupner, Marion Hutinel, Kilian Schumacher, Declan Alan Gray, Maja Genheden, Jerker Fick, Carl-Fredrik Flach, and Dan Göran Joakim Larsson
Evidence for selection of multi-resistant *E. coli* by hospital effluent
Environment International, 2021, 150, 106436

CONTENT

ABBREVIATIONS

ARB	Antibiotic resistant bacterium / Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
CPE	Carbapenemases-producing Enterobacterales
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum beta-lactamase
HGT	Horizontal gene transfer
LB	Lysogeny broth
MALDI-TOF	Matrix-assisted laser desorption/ionization- time of flight
MGE	Mobile genetic element
MH	Mueller Hinton
MIC	Minimum inhibiting concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
VRE	Vancomycin-resistant enterococci
WBE	Wastewater-based epidemiology
WWTP	Wastewater treatment plant

1. BACKGROUND

1.1. Antibiotics and antibiotic resistance

1.1.1. Antibiotics

Antibiotics have revolutionized medicine by providing efficient treatments against bacterial infections. From the beginning of their mass production during the Second World War to modern days, they have saved countless lives and completely changed our perception of bacterial infections from a ubiquitous deadly threat to (for the most of us) an occasional inconvenience. Beyond their use for the treatment of bacterial infections, antibiotics are needed as prophylaxis for the care of patients with many other pathologies. They are for example essential to allow safe surgical procedures, protect immuno-compromised persons (e.g. patients under cancer chemotherapy, receivers of organ transplants, patients with acquired immunodeficiency syndrome (AIDS)) or patients in intensive care (da Costa et al., 2020; Ying Wang et al., 2021; Multani et al., 2020; Minozzi et al., 2021). Through all these usages, antibiotics have become a cornerstone of modern medicine. Further, well spread accounts of their success and their relatively low price and easy access have contributed to their frequent overuse and misuse (Giacomini et al., 2021). As it has become known that such excesses have a direct effect on rendering antibiotics inefficient, numerous actors are now involved worldwide to understand, monitor and mitigate the development of antibiotic resistance, and ensure our ability to treat bacterial infections in the future.

1.1.2. Acquisition of antibiotic resistance

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."
(Fleming, 1945)

As illustrated by Alexander Fleming's warning in his speech when receiving the Nobel Prize for the discovery of penicillin, the first cases of bacteria developing resistance toward antibiotics were observed almost as early as the discovery of antibiotics themselves. Over the following years, bacteria have evolved resistance against all the antibiotics developed and the proportion of bacteria resistant toward each antibiotic class has increased. High resistance rates have led to the relinquishing of previously widely used antibiotics or the need to combine them with other drugs to counter the resistance mechanism (e.g. amoxicillin and clavulanic acid) (Neu and Fu, 1978). The acquisition of resistance toward multiple antibiotics by bacterial strains can render the treatment options extremely scarce or even inexistent in some cases. Bacterial infections caused by resistant bacteria are associated with increased complications and mortality even when there are still effective treatment options available (Peralta et al., 2007). Additionally to the

cost in human lives, antibiotic resistance represents a substantial financial cost for health care systems and/or affected individuals caused by increased hospitalizations, multiplication of medical tests, use of more expensive treatments and extra side effects from the treatments (Lautenbach et al., 2001; Cosgrove and Carmeli, 2003; Roberts et al., 2009; Shamsrizi et al., 2020). Infections with antibiotic resistant bacteria (ARB), which are forecasted to increase in prevalence, are therefore expected to cause considerable public health and economic issues in the coming years.

Bacteria can acquire resistance via mutations in their genomes or acquisition of genetic material from other bacteria. The latter pathway, called horizontal gene transfer (HGT), encompasses several mechanisms (**Figure 1**). Bacterial transformation is the acquisition of free DNA from the environment around the bacteria. In some cases, bacteriophages, which are viruses infecting bacteria, transport genetic material from one bacterium to another. Yet, the HGT mechanism likely to play the biggest role in the spread of antibiotic resistance genes (ARGs) is conjugation, by which a piece of circular independently-replicating DNA called a plasmid is injected by one bacterium into another (Norman et al., 2009).

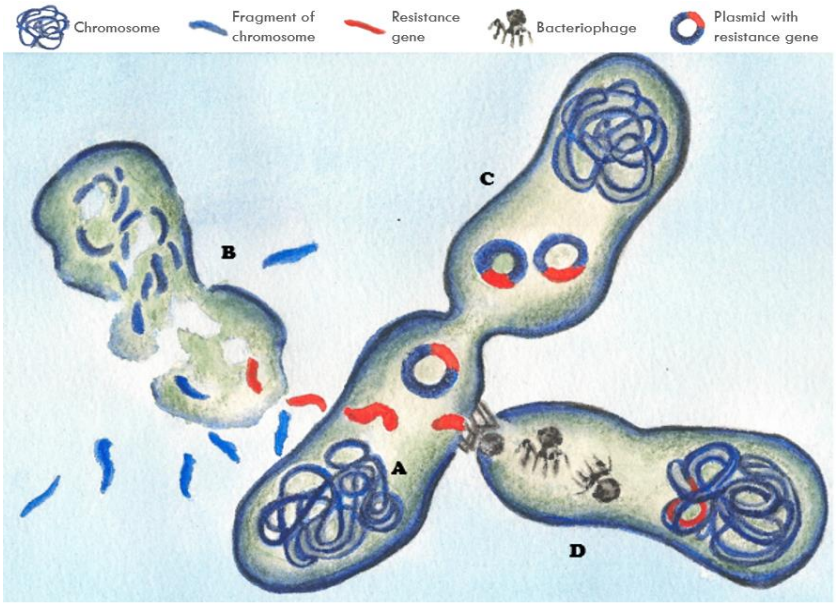

Figure 1: Main mechanisms of horizontal gene transfer between bacteria. A recipient bacterium **(A)** can acquire antibiotic resistance genes (in red) through transformation **(B)**, conjugation **(C)** or transduction by bacteriophages **(D)**. Illustration by Odile Pfennig.

Acquisition of resistance toward multiple antibiotics by one bacterial strain can be a progressive journey with successive acquisition of new mutations and/or HGT events over generations, but sometimes the evolution toward multi-resistance can take a critical leap forward by the acquisition of resistance toward several

antibiotics at once. Indeed, some resistance mechanisms provide cross-resistance toward several classes of antibiotics. This can be the case, for example, of a mutation leading to the overexpression of an efflux pump that can eject several different antibiotics out of the bacterial cell (Nikaido, 1998; Webber and Piddock, 2003). In other cases several ARGs can be encoded on the same mobile genetic element (MGE) and are therefore co-acquired. It is indeed not uncommon for MGEs to accumulate ARGs and/or virulence factors providing thereby their bacterial host with an array of genes advantageous in infection situations (Cepas and Soto, 2020).

1.1.3. Selection of antibiotic resistance

The acquisition of antibiotic resistance, although its frequency can be influenced by some external conditions, is largely random (Hughes and Andersson, 2017; Toprak et al., 2011). The main driving force behind the increase in the proportion of resistant bacteria is the subsequent selection, exerted mainly by antibiotics themselves. When a bacterial community including antibiotic resistant strains is exposed to antibiotics inhibiting the susceptible strains, the resistant strains will become dominant over time (**Figure 2**). Indeed, even if the resistant strains were initially very rare, they will likely be able to multiply thanks to the space and nutritional resources left unused by the affected susceptible bacteria. Such selection is strongest when bacterial communities are exposed to antibiotic concentrations above the minimum inhibiting concentration (MIC) of the susceptible strains, as is the desired case when an individual undergoes antibiotic treatment. However, it is enough that the environmental conditions provide a growth advantage to the resistant strains compared to the susceptible ones for the resistant strains to over time represent a larger part of the bacterial community (as the resistant strains multiply faster than the susceptible stains) (Gullberg et al., 2014; Sandegren, 2014). Therefore, selection can also happen at concentrations below the ones needed to completely inhibit susceptible bacteria and such concentrations expected to select for resistance can be found in diverse environments (see 1.2 below). There are also cases when a specific resistance mechanism can be co-selected for by several different substances. When a resistance mechanism provides cross-resistance toward several substances, each of those substances can select for resistance to the other ones. When several genes are linked together on a genetic element, the entire genetic element can be selected for by any of the conditions in which it provides a growth or survival advantage to its host bacterial strain. This can promote resistance toward an antibiotic through selection by other antibiotics or completely unrelated substances such as metals or disinfectants (Pouwels et al., 2019; Baker-Austin et al., 2006; Pal et al., 2017; Wales and Davies, 2015; Akimitsu et al., 1999; Kampf, 2018).

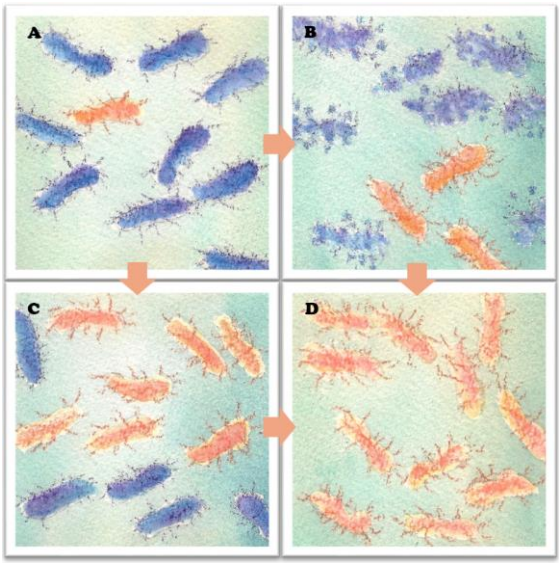

Figure 2: Selection of antibiotic resistant bacteria. The bacterial community starts with a majority of susceptible bacteria (blue) and a few resistant bacteria (orange) **(A)**. When the bacterial community is under selective pressure, either susceptible bacteria are killed **(B)** or resistant bacteria have a growth advantage **(C)**. Over time, this leads to resistant bacteria representing the majority of the community or even its entirety **(D)**. Illustration by Odile Pfennig.

1.1.4. Prescription practices

The decisions to use antibiotics and which ones, are complicated choices influenced by many factors and participants. Although antibiotics can be purchased over the counter or even on the black market in some cases, in many countries, including in Sweden where our studies were carried out, antibiotic dispensation requires a prescription by a physician or a veterinarian. Since inappropriate use of antibiotics creates unnecessary opportunities for selection of ARB which could make the treatment of future bacterial infections more problematic, the prescriber faces an important responsibility both for ensuring the health of the current patient (human or animal) but also for preserving the effectiveness of antibiotics for future treatments. Ideally, antibiotics should only be used to treat infections from bacteria and among bacterial infections, only the ones that will benefit from a treatment. In some cases, where the occurrence of a bacterial infection is likely, antibiotics can also be used preventively (Bernabeu-Mira et al., 2020; Chan et al., 2020; da Costa et al., 2020; DeNegre et al., 2020; Jury et al., 2021; Lee, 2020; Lodi et al., 2021; Minozzi et al., 2021; Rankine-Mullings and Owusu-Ofori, 2021). Additionally, prescribers should choose the most targeted treatment possible, which will be efficient against the bacteria responsible for the infection while resulting in the minimum amount of side effects and limiting the selection of antibiotic resistant bacteria (WHO, 2019; Dellit et al., 2007). Knowing the susceptibility profile of the pathogen is an important tool to fulfill those objectives. However, in practice, the antibiotic treatment often has to

be started before the results of antibiotic susceptibility testing (AST) can be obtained, when such tests are performed at all. This leads to most antibiotic prescriptions being empiric, i.e. done without AST information. In those cases, antibiotic choices are informed by other factors. The prescriptions are based on local, national or international recommendations. They are also guided by the practitioner's professional experience, resistance information available (e.g. surveillance data, scientific literature) and sometimes even personal or local prescription habits.

Antibiotics are classified to prioritize their use with regard to their risk-benefit ratio for the patient and their potential for selection of resistances (Society for Healthcare Epidemiology of America et al., 2012; WHO, 2019). First line antibiotics usually represent the first recommended treatment choices. Second line antibiotics are to be used in more serious infections, in case of allergy to certain antibiotics or when the pathogen is resistant to the first line antibiotics. Finally, last resort antibiotics are to be reserved for critical cases. Although, some last resort antibiotics may cause serious adverse side effects, they are considered of crucial importance for humans since they are the last treatment options for some highly multi-resistant bacteria. A typical illustration of that is colistin. Because of its nephrotoxicity and neurotoxicity, its use has been avoided in human medicine for decades and instead it was mainly employed in animals. However, the emergence of highly multi-resistant Gram negative bacteria, in particular carbapenemases-producing Enterobacterales (CPE), has made colistin an essential last resort antibiotic since some of those strains can be resistant toward all other available treatments (Li et al., 2006; El-Sayed Ahmed et al., 2020; Hughes et al., 2020). Following the discovery of mobile colistin resistance (*mcr*) genes that can be spread between bacterial strains via HGT, colistin use in animals has been drastically reduced to slow their spread (Liu et al., 2016; Xavier et al., 2016; Yin et al., 2017; Carattoli et al., 2017; Borowiak et al., 2017). In cases of multi-resistant Gram positive bacteria, linezolid can be used as a last resort, but similarly to colistin, several mobile genes providing resistance toward that antibiotic have been discovered recently (Contreras et al., 2019; Turner et al., 2019; Bender et al., 2018; Vester, 2018). The antibiotic resistance situation has to be constantly monitored to adapt to such changes.

1.1.5. Clinical surveillance of antibiotic resistance

Clinical surveillance of antibiotic resistance is based on the microbiological diagnostics and in particular the collection of the results of AST performed on the bacteria isolated from individual patients. It has the advantage of providing, in the first place, information that can be used to adapt the treatment of the specific patient. Secondly, the AST data can be reported to local, national or international agencies which compile them and provide reports (Swedres-Svarm, 2019; ECDC, 2020; WHO, 2020). Additionally, some countries require the systematic reporting of specifically problematic resistant pathogens, sometimes accompanied by further characterization of the resistance mechanism. In Sweden, *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBL) or

carbapenemases (CPE) suspected to be encoded on plasmids, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant Streptococcus pneumoniae and vancomycin-resistant enterococci (VRE), are subjected to such mandatory reporting (Swedres-Svarm, 2019). The surveillance data thereby gathered is essential for guiding empirical treatments, inform antibiotic resistance stewardship and policies, and follow the effect of such actions (Cornaglia et al., 2004; Dellit et al., 2007; Pulcini et al., 2019; Giacomini et al., 2021).

To fulfill all its functions, an antibiotic resistance surveillance system should provide the resistance rates of pathogens and be able to alert in case of emergence of new resistance threats or unexpected increases in types of resistance that used to be rarely encountered. Hence, to be accurate and relevant, it needs to be based on a large amount of samples from the population of interest and regularly updated to reflect changes in the antibiotic resistance situation. This requires financial means and infrastructures that are not available in many countries. Even in countries where surveillance from an international perspective is extensive, most of the uncomplicated infections diagnosed in primary care are treated empirically and are therefore not included in surveillance systems (Gupta et al., 2011; Kornfält Isberg et al., 2019).

In an effort to develop surveillance of antibiotic resistance in more countries, the World Health Organization is implementing the Global Antimicrobial Resistance Surveillance System (GLASS) (WHO, 2020). The program has managed to enroll 91 countries or territories (out of 196) but only 66 have reported antibiotic resistance data. Even among the countries reporting, information can be very limited and highly biased toward severe or complicated cases (with as few as 19 isolates reported for a country). Further, the program suffers from the lack of standardization of the sampling strategies, AST methods and antibiotics tested. This illustrates that despite the willingness of some countries to develop their antibiotic surveillance system, for many areas of the world, surveillance data is limited, if existing at all.

1.2. Role of the environment in antibiotic resistance

The environment has long been known as a transmission pathway for bacterial pathogens including antibiotic resistant ones (Cabral, 2010; Coleman et al., 2012; Graham et al., 2014). It is for example well established that contaminated drinking water can transmit cholera, typhoid or bacterial dysentery. More recently the environment has also been suggested to play important roles for the evolution of ARB (Finley et al., 2013; Wellington et al., 2013; Ebmeyer et al., 2021). Indeed, most antibiotics are derived from natural compounds which means that bacteria have been exposed to them in the environment for a considerable amount of time (Moloney, 2016; Herrmann et al., 2016). Consequently, bacteria have developed antibiotic resistance mechanisms in the environment long before antibiotics were used by humans, and environmental bacterial communities are likely to be an important reservoir of antibiotic resistance determinants (Hall and Barlow, 2004; D'Costa et al., 2011; Berglund et al., 2017, 2020).

Over the last century, anthropogenic activities have led to the release of antibiotics into the environment, exposing the bacterial communities to those compounds on a completely different scale compared to what could occur naturally before (Larsson, 2014a). Man-made antibiotics can reach the environment in many different ways. The highest environmental antibiotic concentrations have been observed in areas contaminated by industrial discharges from antibiotic production sites (Bielen et al., 2017; González-Plaza et al., 2019; Larsson, 2014b; Thai et al., 2018). However, the most common sources of antibiotics to the environment are humans and animals undergoing antibiotic treatment. Indeed, the treatment often results in the excretion of active antibiotic residues via urine, feces or sweat. Manure from farm animals contaminated with antibiotic residues is often applied on agricultural fields while human excreta either go directly to the environment or enter the sewers to undergo treatment (see 1.3.1. below) (Christian et al., 2003; Heuer et al., 2011; Massé et al., 2014; Verlicchi and Zambello, 2015; UNICEF and WHO, 2020). The sludge resulting from the treatment might also be applied on soils and treated wastewater is discharged into water bodies. Finally, except where good programs to take back unused medicines are in place and adhered to, many discarded pharmaceuticals end up in landfills (Chen et al., 2017; Song et al., 2016). Those main pathways and others lead to increased concentrations of antibiotics in the environment which could cause selection of ARB and increase HGT of mobile ARGs. Additionally to antibiotics, ARB and ARGs are also released in the environment by some of those same pathways (i.e. excretion by humans or animals) contributing even more to the prevalence of antibiotic resistance in the environment and the likelihood of (re-)transmission of ARB to humans and/or ARGs to pathogens (Heuer et al., 2011; Wolters et al., 2019).

1.3. Sewers, sewage and wastewater

1.3.1. The sewer system

Sewer systems collect wastewater and in particular the urine and feces from the connected population, also called sewage. Therefore they contain a large amount of bacteria originating from human microbiota and especially from the gut flora. Numerous substances also go down the drain and are therefore mixed together in the sewers. Notably, the consumed antibiotic and/or metabolites thereof (some of them with an antibiotic activity) excreted by individuals under antibiotic treatment. To the basic household wastewater, disposals from hospitals and/or industries can be added depending on the design of the sewer networks and local regulations. Hospitalized patients are more likely to be treated with antibiotics than the general population and hospitals are known to be hosting more resistant strains, hence those can be expected to be found in higher concentrations in hospital wastewater (Nicolle and WHO, 2001; Cooke et al., 2010; Galvin et al., 2010; Le Corre et al., 2012; Ko et al., 2013; Lax and Gilbert, 2015; Lax et al., 2017; Versporten et al., 2018; Paulshus et al., 2019; Hassoun-Kheir et al., 2020). The sewers are also likely to collect rain water, intentionally or not. Indeed, even when there is a separate system for the collection of rainwater in cities, sewer lines are

rarely watertight enough to keep water from pouring through the ground bringing with it an additional set of substances and environmental bacteria (Hurley et al., 2007; Kamei-Ishikawa et al., 2016; Oliveira et al., 2020). In the sewers all those various elements are mixed together by the flow bringing them to a discharge location or a wastewater treatment plant (WWTP).

1.3.2. Wastewater based epidemiology (WBE)

Since, sewage can be looked upon as pooled urine and feces from a large number of individuals, it is in many aspects a reflection of the population that contributes to it. Accordingly, wastewater analysis has emerged as an attractive means for population-level surveillance (Gracia-Lor et al., 2017; Thomas and Reid, 2011) of, for example, pharmaceutical (Yuan et al., 2016) and illicit drug consumption (Ort et al., 2014; van Nuijs et al., 2011) and viral pathogens (Bisseux et al., 2018; Hellmér et al., 2014) including more recently the SARS-CoV-2 virus in the context of the global COVID-19 pandemic (Daughton, 2020; Farkas et al., 2020; Randazzo et al., 2020; Venugopal et al., 2020). Additionally, several studies have shown that the detection of *Salmonella* in wastewater is a relevant indicator of salmonellosis in the population (Vincent et al., 2007; Yan et al., 2018; Diemert and Yan, 2019). In a similar manner, WBE could potentially be used for the surveillance of antibiotic resistances in bacteria from human populations (Huijbers et al., 2019). Several, potentially complementary approaches could be employed. Bacteria of clinical interest could be isolated from wastewater and their resistance profile determined. This would provide resistance information based on many bacterial isolates with the need to involve only relatively few people and resources. This could therefore potentially be the way to quickly gather antibiotic resistance data for places where it has been lacking. Additionally, in places with a well-developed clinical antibiotic surveillance system, analyses of resistance in wastewater bacteria could bring supplementary information potentially less biased toward complicated clinical cases. Bacteria could also be isolated from wastewater based on their ability to resist an antibiotic. Alternatively, ARGs could directly be quantified in wastewater. Such culture-independent approach should be even less laborious than the previously mentioned culture-based approaches. The two latter methods, combining a technique with a low detection limit with samples originating from a large number of individuals, could allow screening of ARB or ARGs on a scale impossible for classical clinical surveillance systems. They should therefore be well suited for the detection of rare resistance determinants.

Many studies have analyzed ARB or ARG in wastewater but very few have compared resistance rates between wastewater bacteria and bacteria from the corresponding population. In most of the cases when it has been done, the wastewater samples were gathered after going through treatment, which might have modified the resistance rates in the wastewater bacterial community (Reinthaler et al., 2013; Yang et al., 2009). In 2015, Kwak and colleagues noted some coherence between the resistance rates observed in *Escherichia coli* isolated from untreated wastewater in Stockholm and the trends observed during previous years at the national level (Kwak et al., 2015). Despite the appeal, the

relevance of the use of wastewater analyses to predict the clinical antibiotic resistance situation remains largely un-evaluated and information critical for proper implementation of the methodology as well as for the interpretation of the results is lacking. There are several factors that could affect the relationship between what is found in wastewater and samples from humans:

- **Bacteria excreted by people to the sewers are not necessarily the same ones as the ones causing infections.**
  One of the main goals of antibiotic resistance surveillance systems is to provide the information needed to optimally use antibiotics in cases of bacterial infections. Therefore, the most important information is resistance rates in the bacterial strains causing infections. Yet, humans mainly excrete their fecal bacteria to the sewers. It has been shown that in several types of bacterial infections, the infecting bacterial strain is likely to originate from the patient's own microbiota (Yamamoto et al., 1997; Moreno et al., 2008; Cogen et al., 2008; Sekirov and Finlay, 2009). However, the bacteria excreted by the patient in the sewers are not limited to the ones causing infections, and previous studies have indicated that bacteria isolated from the gut flora were less likely to be resistant than the ones causing infections (Clermont et al., 2017; Dang et al., 2013; Nielsen et al., 2014). Additionally, some pathogens are not present in the gut microbiota or would not survive in the wastewater environment. It is worth mentioning that even if the bacteria isolated from wastewater would not correspond exactly to the ones causing infections in patients, characterization of the former could still be informative of the situation in the latter if resistances in the two bacterial populations were correlated.

- **Modifications of the bacterial community by the wastewater environment.**
  The conditions in the sewers are extremely different to the ones in the environments bacteria originated from. It is therefore likely that the composition of the wastewater bacterial community is shaped, at least in part, by the physical and chemical conditions encountered in the sewers. As a matter of fact, a shift toward bacteria more tolerant of aerobic conditions in wastewater compared to fecal sample has been observed (Bengtsson-Palme et al., 2016). Sampling the wastewater early in the sewer system might reduce that influence. In any case, wastewater that has been exposed to a treatment would not be ideal for WBE purposes since the treatment will strongly affect the bacterial composition and possibly the antibiotic resistance characteristics of bacteria (Bengtsson-Palme et al., 2016; Quintela-Baluja et al., 2019; Verburg et al., 2021).

- **The presence of bacteria of non-human origin in wastewater.**
  It is not guaranteed that bacteria and ARGs found in wastewater samples originate from humans. Even if similar bacterial strains have sporadically been identified in wastewater and clinical samples, it is often difficult to find the same strains in both types of samples since only a small

proportion of the vast diversity of wastewater bacteria is analyzed and only a small portion of the population's microbiota is analyzed (Kühn et al., 2003; Mahon et al., 2017; Drieux et al., 2016; Jørgensen et al., 2017; Iversen et al., 2004). This still raises the question of the representativeness of the wastewater bacterial community with regard to the human microbiota. Although a large proportion of the bacteria in wastewater can be expected to originate from humans, bacteria in the wastewater can also originate from other sources (i.e. animals and the environment). Additionally, it is possible that the wastewater contains bacteria originating from the sewers themselves in the sense that some bacteria attached to the surfaces in biofilms might have evolved there into strains found in no other environment. The proportion of bacteria from non-human origin in wastewater is largely unknown. Therefore, it would be difficult to be sure of the origin of ARB isolated from wastewater. This issue is even more problematic when quantifying ARGs directly as the bacterial host species would likely be unknown and unavailable for further investigations.

It is essential to consider the factors above when designing and/or interpreting results of WBE studies with the aim to predict the antibiotic resistance situation in humans. Most importantly, it is fundamental to proceed to a rigorous comparisons between the data obtained by WBE and by classical antibiotic resistance surveillance to verify if they are linked by a stable relationship before WBE can potentially be employed to predict antibiotic resistance in infectious pathologies or in the microbiota of human populations.

1.3.3. Wastewater as an arena for the acquisition and spread of antibiotic resistance

Apart from being a potential tool for the surveillance of antibiotic resistance in the contributing population, wastewaters have also been pointed out as environmental hotspots for the evolution and dissemination of antibiotic resistance (Rizzo et al., 2013). Wastewater might exert an influence on antibiotic resistance though the following three main mechanisms:

Selection of ARB

The many substances present in wastewater have the potential to affect the bacterial community present in the sewers. Indeed, the antibiotics, disinfectants and metals which are present in that environment might select for ARB. For example, tetracycline, ciprofloxacin and trimethoprim in untreated wastewater can be found at concentrations higher than the ones that have been shown individually to be able to select for resistant bacteria within complex bacterial communities (1 µg/L, 1 µg/L and 100 µg/L, respectively) (Lundström et al., 2016; Kraupner et al., 2018, 2020; Karthikeyan and Meyer, 2006; Lindberg et al., 2014, 2005; Bengtsson-Palme et al., 2016; Östman et al., 2017; Kasprzyk-Hordern et al., 2009; Faleye et al., 2019). Several studies have investigated the influence of the wastewater treatment process on the proportions of ARB and ARGs with varying results

(Bengtsson-Palme et al., 2016; Hrenovic et al., 2017) but a study carried out at the Rya WWTP (from which the municipal wastewater samples were obtained for the studies presented in this thesis) concluded that there was no evidence of selection for antibiotic resistance in *E. coli* during the treatment (Flach et al., 2018).

Induction of HGT

The mixing of bacteria of different origins could provide the physical circumstances necessary for the spread of ARGs between bacteria by HGT. Further, HGT can also be induced by several substances found in wastewater; e.g. pharmaceuticals (Wang et al., 2019, 2020; 2021) in particular antibiotics (Whittle et al., 2002; Beaber et al., 2004; Jutkina et al., 2016; Scornec et al., 2017; Jutkina et al., 2018; Liu et al., 2019), metals (Zhang et al., 2018, 2019), biocides (Zhang et al., 2017; Jutkina et al., 2018), food preservatives (Cen et al., 2020) and non-nutritive sweeteners (Yu et al., 2021). Even though the effect concentrations of the substances tested individually are above the ones that are usually found in wastewater, some are within the range of reported concentrations. Besides, the complex mixture found in wastewater could lead to additive or synergistic effects increasing the rate of HGT (Gullberg et al., 2014). Not only could such inductive conditions increase the proportion of bacteria carrying an ARG (particularly if combined with selection) but the wastewater setting (as a meeting point of bacteria of different origins) could be the scene of particularly critical transfer events that would introduce an ARG in a pathogenic strain not harboring it before. It is therefore essential to evaluate the potential of wastewater to induce HGT.

Dissemination of ARB, ARGs and antibiotics to the environment

WWTPs play an essential role in reducing the amount of bacteria that are discharged into the environment via wastewater. However, a portion of ARB, ARGs, antibiotics and other substances that could influence antibiotic resistance are still emitted from the WWTPs and can thus reach water bodies and agricultural land. In those environments, they might influence the composition of the local microbial communities and ARB might be (re-)transmitted to humans, either directly or through the consumption of water and possibly food products (Huijbers et al., 2015; Coleman et al., 2012; Leonard et al., 2018; Søraas et al., 2013; Laurens et al., 2018; Murray et al., 2019).

2. AIMS

The first three studies presented can be grouped under the overall aim to develop and assess several ways in which the analysis of wastewater samples could be used to provide clinically relevant antibiotic resistance data. The fourth study aimed to evaluate the role of wastewater in the emergence of ARB. The specific objectives of each paper were as follows:

Paper I Characterize the relationship between resistance rates generated from wastewater and clinical isolates collected both at a hospital and in a broader municipal setting.

A second objective was to evaluate a method developed for performing resistance profiling of a large number of bacterial isolates.

Paper II Investigate the relationship between CPE in hospital wastewater and observations of CPE in samples from the corresponding hospitalized population.

A second objective was to evaluate if direct quantification of carbapenemase genes in wastewater could provide information as reliable as the more resource-demanding culture-based approach.

Paper III Monitor through wastewater analysis the prevalence of a set of mobile ARGs of emerging concern (*optrA*, *cfr*, *mcr*, *sul4* and *gar*) which have rarely or never been detected in clinical isolates in Sweden.

A second objective was to evaluate the likelihood of those ARGs being carried by bacterial pathogens or gut bacteria by comparing their abundance with the variations in abundance of the bacterial taxa present in the wastewater samples.

Paper IV Evaluate the effects of the complex abiotic mixture present in hospital and municipal wastewaters on the transfer of mobile antibiotic resistance genes between bacteria through horizontal gene transfer.

A second objective was to characterize the MGEs captured from the wastewater bacterial community.

3. METHODOLIGICAL CONSIDERATIONS

3.1. Wastewater sampling

WBE is based on the principal that the wastewater samples contain urine and feces from many individuals of the targeted population. Hence, it is essential to have a sampling strategy that ensures this is the case. The sampling point should get input from many contributors from the targeted group and limited contribution from other sources. Additionally, the composition of the wastewater changes during the day depending on the temporality of the various inputs (Teerlink et al., 2012; Coutu et al., 2013). Therefore, pooling of several sub-samples is likely to give a more general representation of the overall chemical and microbiological composition of wastewater. Moreover, the composition of wastewater might be affected by storage. Hence it is important that samples are processed quickly and stored under conditions that will maintain the studied characteristics as close to their original state as possible.

All wastewater samples used in the presented studies were constituted of sub-samples taken over 24h-periods and maintained at 4°C during and after collection. Several types of wastewater samples were collected in Gothenburg (Sweden). Untreated wastewater was gathered from the main site of the Sahlgrenska University Hospital and from the inlet of the Rya municipal WWTP (Gryaab AB). Additionally, treated wastewater was collected from the outlet of the WWTP.

With 1950 beds, the Sahlgrenska University Hospital is the largest hospital in Sweden and one of the largest hospitals in Europe. The wastewater sampling point is situated within the perimeter of the hospital, receives contributions from most of the wards of the site and is equipped with an automatic sampler taking sub-samples every ninth minutes.

The Rya WWTP is one of the largest WWTP in Scandinavia serving close to 800 000 persons in 2020. It treats mainly household wastewater with only 11% of its biochemical oxygen demand originating from other sources (Davidsson, 2020). At the WWTP, samples were constituted of sub-samples taken flow-proportionally every 1000 m^3.

3.2. Antibiotic resistance in wastewater bacteria

3.2.1. Culture-based methods

Clinical bacteriological diagnostics rely mainly on culture-based methods to identify bacterial pathogens and reveal their antibiotic resistance profile. Therefore, the use of this type of methodologies to analyze antibiotic resistance in wastewater allows a relatively direct comparison of the results with data from individual patients and clinical surveillance.

Bacterial enumeration and isolation

Cultivation of a small volume of wastewater on agar plates allows the estimation of bacterial concentrations by counting colony-forming units (CFUs) and individual strains can be isolated and stored for further analyses. A wide variety of culture media can be used for making agar plates. Rich media like Lysogeny Broth (LB) or Mueller Hinton (MH) incubated at 37°C allow the rapid growth of many bacteria including pathogens within a day. The less nutritive R2A medium incubated at room temperature allows detection of a wider range of bacteria especially ones accustomed to poorer nutritional conditions as can be found in water samples, but this can take up to a week. Chromogenic media such as CHROMagar MH Orientation or CHROMagar ECC (CHROMagar, France), the latter being specialized for coliforms, allow the distinction between several bacterial species by coloring the bacterial colonies (**Figure 3**). Antibiotics can be added to the agar plates to only allow the growth of bacteria resistant to those antibiotics and some commercial plates can even be selective for a specific resistance mechanism; e.g. ChromID OXA-48 (Biomerieux, France), a chromogenic media selecting for *bla*$_{\text{OXA-48}}$ carrying CPE.

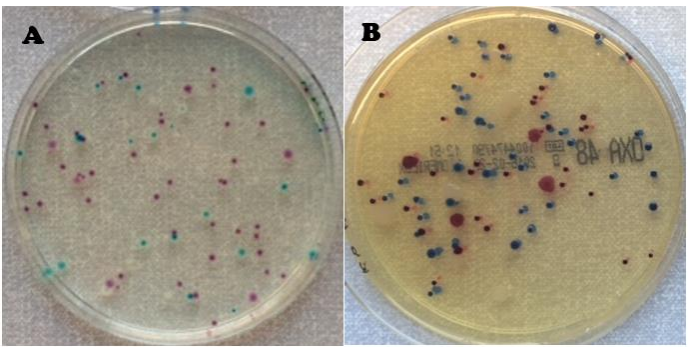

Figure 3: Chromogenic plates. A: CHROMagar ECC (CHROMagar, France) chromogenic plate inhibiting the growth of non-coliform bacteria. *E. coli* colonies are colored in blue and other coliforms in pink. **B:** ChromID OXA-48 (Biomerieux, France) chromogenic and selective agar plate for the screening of *bla*$_{\text{OXA-48}}$ harboring CPE. *E. coli* colonies are colored in pink/red and other coliforms colonies are colored in blue/green.

Species identification by MALDI-TOF

Even if some bacterial species can be identified via their growth characteristics, colony morphology and/or their coloration on chromogenic media, this process can be prone to errors. The use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry can reveal or confirm the species of a bacterial isolate (Croxatto et al., 2012). The bacteria mixed with a matrix are bombarded with a laser, which desorbs and ionizes their proteins. The ionized peptides then travel through vacuum to a detector at a speed dependent of their mass. Recording of the time necessary for the peptides to reach the detector

provides a spectrum that can in many cases be assigned to a bacterial species by comparing it to existing spectral databases.

In **Paper I**, MALDI-TOF confirmed that 99.7% of the 1256 presumed *E. coli* isolated from CHROMagar ECC plates were correctly identified. In **Paper II**, all the presumed *E. coli* isolated from ChromID OXA-48 or ECC-meropenem plates were confirmed by MALDI-TOF (92 and 130 isolates, respectively). Additionally, all the other bacteria isolated from those plates were confirmed to be Enterobacterales and their species or at least genera could be revealed (149 and 61 isolates, respectively).

Antibiotic resistance profiling

The disk diffusion method is the most common and standardized type of AST in clinical laboratories. Small disks containing specific amounts of antibiotics are placed on agar plates covered by an inoculum of the bacterial isolate to be tested (**Figure 4**). After a defined incubation, the diameter of the zone around the disk in which the growth of the isolate has been inhibited is measured. Since the disks create reproducible gradients of antibiotic concentrations by diffusing their antibiotics into the agar around them, the resistance ability of the isolate can be deduced from the size of the inhibition zone.

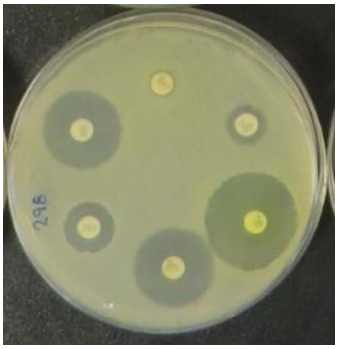

Figure 4: Antibiotic susceptibility testing by disk diffusion. The size of the inhibition zones can be measured around the antibiotic disks and compared to breakpoint values.

The disk diffusion method requires the processing of each bacterial isolate individually, one after another. To test many isolates in a short amount of time and in a less expensive way, we employed an alternative AST methodology. The bacterial isolates were inoculated in liquid media containing antibiotics at breakpoint concentrations in 96-well-plates (**Figure 5**). The ability of the isolates to grow in the presence of antibiotics could be assessed visually after incubation (see detailed description of the methodology in **Paper I**).

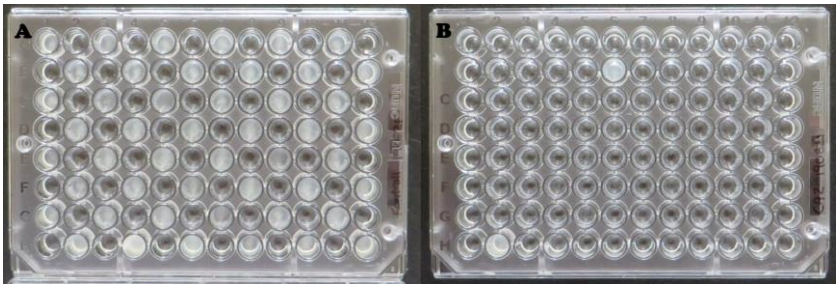

Figure 5: Antibiotic susceptibility testing in 96-well-plates with liquid culture media. A: Control plate without antibiotics where isolates grew in all 48 inoculated wells. One well out of two is left un-inoculated to verify non-contamination of neighboring wells. **B:** Plate inoculated identically as the control plate but containing an antibiotic to which only two isolates are resistant.

The two abovementioned AST methods were compared in **Paper I** by determining the resistance of 155 bacterial isolates to eight antibiotics with both methods. The two methods gave very similar results. The only exception might be tests with the antibiotic cefadroxil, which had the tendency to identify more isolates as resistant when done in the 96-well-plates with liquid medium compared to disk diffusion. This led to a positive predictive value of approximately 60% for resistance to cefadroxil with our methodology. However, cefadroxil is in both cases (in clinical laboratories and with our methodology) used to detect potential ESBL-producing isolates, which are then tested with other more clinically relevant cephalosporins (i.e. cefotaxime and ceftazidime). It is therefore easy to bypass this divergence by directly comparing resistance rates for the more clinically relevant cephalosporins if necessary.

The production of carbapenemases can be tested with the Carba NP reaction (Dortet et al., 2014). Suspected CPE are lysed and placed in a medium containing imipenem. If the bacterial strain hydrolyzes the antibiotic, the medium changes color from red to yellow or orange confirming the production of carbapenemases by the strain. In **Paper II**, this method could show carbapenemase production by a large proportion of the *E. coli* isolated from ChromID OXA-48 or ECC-meropenem plates (87% and 79 %, respectively). This was also the case of most *Klebsiella pneumoniae* (100% and 86%, respectively). *Raoultella spp.*, isolated from ChromID OXA-48 plates were producing carbapenemases (5 isolates) but not the ones isolated from ECC-meropenem plates (7 isolates). The other bacterial species isolated from those plates rarely produced carbapenemases.

Sub-species classification

Biochemical fingerprinting by rapid screening PhenePlates for *E. coli* (PhPlate Microplate Techniques AB, Sweden) was employed to assess the diversity of the *E. coli* isolated from wastewater samples in **Paper I**. The method allows to distinguish different *E. coli* based on their ability to metabolize 11 substrates. The specific culture medium contains bromothymol blue which changes color from blue to yellow as the substrate is consumed and pH changes (**Figure 6**). An isolate

can be assigned a score for each substrate that together constitute its biochemical fingerprint, which can then be compared to the biochemical fingerprints of the other isolates.

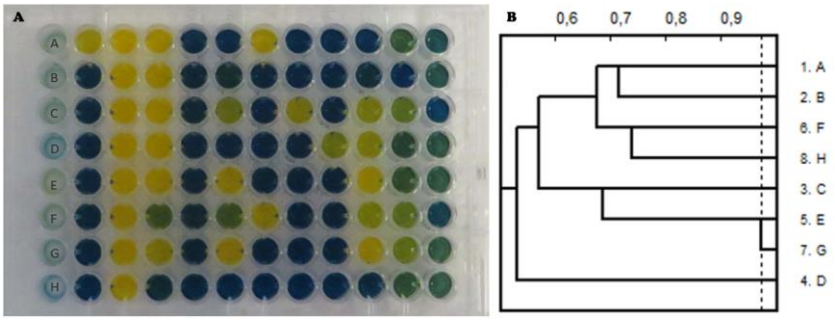

Figure 6: Biochemical fingerprinting with PhenePlate. A: Reaction of the *E. coli* isolates A to H (one in each row) with the different substrates (in each column). **B:** Dendrogram illustrating the relatedness of the *E. coli* isolates A to H between each other. Isolates E and G are considered to have the same biochemical fingerprint since they are more than 97.5% similar (dashed line).

Genotypic characterization of isolated bacteria

Polymerase Chain Reaction (PCR) is a common laboratory procedure allowing to detect a gene by amplifying it. Small fragments of single-stranded DNA called primers are designed to be complementary to each strand of the targeted gene. If the targeted gene is present in the PCR reaction with the primers, the fragment between the two primers is copied by a DNA polymerase. After several amplification cycles the fragment is present in so many copies (i.e. amplicons) that it can be visualized by adding a dye. PCR is useful for identifying the ARG responsible for an observed antibiotic resistance phenotype. The detection of an ARG can also, in many cases, be predictive of the resistances phenotype but the relationship can be more complex and subjected to the influence of other genes or factors modifying the expression of the ARG. In **Paper II**, PCR was used to identify which carbapenemase genes were present in the isolated CPE. In **Paper IV,** PCR was used to characterize the type of plasmid acquired by HGT by targeting replicon genes characteristic of different plasmid incompatibility groups. The PCR-based replicon typing kits (PBRT 2.0, Diatheva, Italy), consisting of 8 PCR mixes containing primers targeting 30 plasmid replicons, were used to characterize plasmids in a few isolates at first. Once it was clear which plasmid types were likely to be found in the isolates and that the isolates usually harbored only one plasmid, PCR with primers targeting the common plasmid types were employed to characterize the plasmids of many isolates.

Another, more complete way to genetically characterize bacteria is to sequence them. Whole genome sequencing can be performed with different methodologies. Next generation sequencing, in particular Illumina sequencing, allows the production of a lot of sequencing data with few errors but only in the form of small fragments (i.e. reads) of 150 to 300 base pairs. The short reads thereby produced have to be assembled together to form a more legible image of the original genetic

content of the bacterial cells. The assembly process is not without challenges and either because of lack of sequencing reads covering some areas of the genome or the presence of regions with repeated sequences, it is often not possible to reconstitute the complete genetic sequences from short reads. Other sequencing methods produce longer reads but also more frequent sequencing errors. This is the case of single-molecule real-time sequencing by PacBio (Pacific Bioscience, USA) which produces reads of 10 kilo bases on average. The recently developed nanopore sequencing (Oxford Nanopore Technology, United Kingdom) also allows the generation of long reads and the possibility to purchase a sequencer for a limited initial investment (allowing to perform sequencing on site opposite to other sequencing technologies which usually require the involvement of specialized sequencing facilities) which make it an attractive option. Long reads can be assembled to reveal the genetic content of the chromosome and plasmids of a bacterial strain as in **Paper IV**. Alternatively, hybrid assemblies using both short and long read can permit resolution of the genomic structure with long reads and correction of sequencing errors with the short reads as was done in **Paper II**.

3.2.2. Genetic analyses on the whole wastewater bacterial community

It is also possible to proceed to a genetic analysis on the whole content of wastewater without preceding isolation of bacterial strains. DNA extracted directly from wastewater contains the pooled genomes of the wastewater bacterial community called metagenome. The wastewater metagenomes can be revealed by sequencing but methods allowing to target only the genes of interest in a metagenome often have a better sensitivity at lower costs. This is why in **Paper II** and **Paper III** quantitative PCR (qPCR) was used to measure the concentration of ARGs in wastewater. This method, also called real-time PCR, is based on the same mechanism of DNA amplification as the PCR described above but allows to determine the initial quantity of the gene by following the amount of amplicons generated during the assay.

The qPCR method has the advantage of having a low detection limit allowing the detection of rare genes in complex DNA samples. It can allow the absolute quantification of a gene in the DNA added to the PCR reaction, but this requires the comparison with the results of another sample for which the gene concentration is already known. Further, in the case of the quantification of ARG it is more informative to know the amount of the ARG relative to the amount of bacteria rather than relative to the total DNA since the samples can contain DNA from other organisms and in varying proportions. An approximation of that is the quantification relative to a ubiquitous bacterial gene. The most commonly used gene for that purpose is the gene coding for the ribosomal RNA 16S, which is part of the small subunit of the ribosome in prokaryotes. Since ribosomes are essential to the functioning of the cell, each bacterial cell contains at least one gene coding for 16S rRNA. The 16S rRNA gene has some conserved regions, which can be targeted by PCR primers. It also has hypervariable regions that differ between bacterial taxa and therefore can be used to identify them. Hence, in addition to its

use for the normalization of qPCR results, 16 rRNA genes can be sequenced to unravel the taxonomic composition of a bacterial community in a sample as was done in **Paper III** (Lane et al., 1985; Tringe and Hugenholtz, 2008; Yang et al., 2016).

Metagenomic methods can allow the rapid and large scale characterization of complex samples. They also bypass culture steps and therefore take into account the bacterial community as a whole including bacteria that cannot be cultivated in laboratories. However, the DNA of all the different bacteria is fragmented and mixed together in the metagenome therefore it is often not possible to link a mobile gene to its host with the methods mentioned above. Further, the bacteria studied in metagenomic analyses are lysed and therefore not available for subsequent characterization.

3.3. Collection of available data for comparison with wastewater data

For **Paper I,** which included measurements of resistance rates in *E. coli* from wastewater samples, the results of AST performed on *E. coli* isolated from patients were collected. At the hospital, resistance data about *E. coli* isolated from blood and urine clinical samples from inpatients hospitalized in the wards and emergency department connected to the wastewater sampling point during the study period was collected. Additionally, for comparison with *E. coli* from municipal wastewater, resistance data about the *E. coli* isolated from urine samples taken in the primary care centers located in the municipalities connected to the WWTP was collected. Patients can be sampled multiple times especially when they suffer from an infection that is difficult to treat. Therefore, to avoid overrepresentation of the strains of some patients and potential bias toward more resistant bacteria, only the first sample from each patient was included for the calculation of resistance rates in the clinical settings.

For **Paper II**, all cases of CPE detected in patient samples at the hospital during the study period were reported. Those included CPE detected due to clinical infections but consisted mainly of CPE identified through routine fecal screening as part of the infection control program at the hospital. Indeed, patients with specific risk factors for CPE carriage such as patients hospitalized in a foreign country during the previous year or with a recent refugee status are routinely screened upon admission to the hospital to reduce the risk of transmission of those resistant bacteria. Additionally, patients particularly vulnerable to infections are also tested systematically (e.g. patients admitted in the hematology or transplantation units).

For **Paper III**, the ARGs studied have been detected so rarely in the Swedish clinical setting that there was little data available. This is probably due to a low prevalence of those genes in bacteria causing infections but also to the fact that resistance to colistin and linezolid are infrequently tested (e.g. only for isolates with other types of resistance or specific clinical conditions). Further, when resistance

to those antibiotics is encountered, the underlying genetic mechanism is rarely investigated. In 2018, a total of six *mcr* carrying isolates had ever been found in Swedish patients (Swedres-Svarm, 2018). A few cases of linezolid resistance in enterococci and *Staphylococcus aureus* were reported nationally in the last years but it is not specified if those cases are due to mutations in the bacteria or to the acquisition of mobile resistance genes such as *optrA* or *cfr* (Swedres-Svarm, 2019). The first *optrA* carrying VRE in Stockholm was only reported this year (Fang et al., 2021). Finally, the last two ARGs investigated (*sul4* and *gar*) have been discovered very recently and their involvement in the resistance phenotype of pathogens has rarely been investigated (Razavi et al., 2017; Böhm et al., 2020).

3.4. Effects of wastewater on bacteria

The effect of raw hospital and municipal wastewaters on HGT was studied in **Paper IV**. In order to reduce variations in the concentrations of possible chemical inducers, the municipal wastewater was only collected on days when the flow was low indicating a limited contribution of rainwater. This in turns signifies that the concentrations of chemicals of anthropogenic origin should be at the higher end of their possible ranges in the municipal wastewater samples tested.

The abiotic fraction of wastewater was separated from the bacteria by filtration through 0.45 µm pore-sized filters. The filter-sterilized wastewater samples were then freeze-dried and stored at -20°C. This allowed the replacement of the water content of the wastewater by an equal volume of culture medium (LB agar). The plates prepared in such manner should then contain the chemicals of the wastewater at original concentrations. To verify their sterility, plates prepared with filtered wastewater were incubated overnight at 37°C.

HGT between a complex natural donor bacterial community and a recipient *E. coli* was studied. The recipient strain was resistant to rifampicin and kanamycin, and produced the green fluorescent protein allowing to isolate and identify it reliably even when mixed with other bacteria. The bacterial community was prepared from treated wastewater in order to provide a diverse source of MGEs from various donor species. The donors and the recipient strains were mixed together and placed on filters which were then placed on top of the agar plates prepared with the freeze dried wastewater or on LB plates prepared in parallel but unsupplemented as control. The plates were placed at 30°C for 3h before the mating was stopped by suspending the bacteria in 4°C phosphate buffer saline. Such mating conditions were chosen according to the results of a study by Jutkina and colleagues, which concluded they were the most optimal to allow sufficient HGT while limiting the risk of selection (Jutkina et al., 2016). HGT was assessed by the number of recipients that had acquired resistance to sulfamethoxazole (called transconjugants) since sulfamethoxazole resistance genes are common and can be found on various plasmids, often associated with other ARGs (Poirel et al., 2018). At the end of the assay, the total amount of recipient was quantified additionally to the number of transconjugants as a way to correct for potential variations introduced by differing amounts of recipients. This quantification of the

recipients at the end of the assay also allowed to point out detrimental effects off the tested substances on the growth and survival of the bacteria.

The chemical composition of the wastewater sample was characterized by liquid chromatography-mass spectrometry. The concentration of a set of antibiotics chosen according to the local prescription practices was measured as well as other pharmaceuticals and biocides that had been described as inducing HGT were measured. This method was also used to quantify antibiotics in the wastewater samples of **Paper I**.

4. SUMMARY OF THE RESULTS AND DISCUSSION

4.1. Wastewater based-surveillance of antibiotic resistance

4.1.1. Estimation of resistance rates in pathogens

The primary purpose of antibiotic resistance surveillance is to provide antibiotic resistance rates in pathogens causing infections to guide empirical therapy. In **Paper I**, we investigated if such information could be deduced from analysis of *E. coli* isolated from wastewater. *E. coli* can be responsible for a wide variety of infections, from relatively mild urinary tract infections to life threatening blood infections. The strains causing those extra-intestinal infections can also be residents of the human gut microbiota and therefore be excreted in feces (Yamamoto et al., 1997; Moreno et al., 2008; Kaper et al., 2004). In the first study, we isolated *E. coli* from wastewater samples and compared the resistance rates among them to the resistance rates in *E. coli* isolated from clinical samples (urine or blood from patients). The comparison was done during a one-year period and on two different scales in the Gothenburg area. On the one hand, the comparison was carried out at the hospital. Wastewater was sampled at the hospital and resistance rates of wastewater *E. coli* were related to resistance rates in *E. coli* from patients hospitalized in the wards connected to the wastewater sampling point. Since this approach targets a limited population which is extensively sampled and tested for antibiotic resistant bacteria, it could be expected that this setting would allow a closer comparison between clinical and wastewater resistance rates. On the other hand, the comparison was carried out for a much broader population. Wastewater was sampled at the entry of the municipal WWTP and resistance rates of those wastewater *E. coli* were related to resistance rates in *E. coli* from primary care patients sampled in the municipalities connected to the treatment plant.

Resistance rates in wastewater and clinical *E. coli* isolates were found to be strongly correlated in both studied settings (hospital or municipal) and regardless of the type of clinical samples (urine or blood) (r^2= 0.82 to 0.95). The *E. coli* load was similar in both types of wastewaters, but *E. coli* from hospital wastewater were more resistant than *E. coli* from municipal wastewater. The relationship between clinical and wastewater resistance rates were also different for the two studied settings. Resistance rates in hospital wastewater *E. coli* were very similar to those in hospital clinical isolates while resistance rates in municipal wastewater *E. coli* were approximately half of those measured in primary care *E. coli*. Further, the resistant rates measured in hospital wastewater *E. coli* were more variable between the different sampling occasions than the ones measured in municipal wastewater *E. coli* and those variations didn't seem to correspond to variations observed in the clinical setting. The most staggering example of that was a large increase in ciprofloxacin resistance among hospital wastewater *E. coli* sampled on the 22nd June 2016 while resistance toward ciprofloxacin was not increased among *E. coli* isolated from patients around that date (**Figure 7**).

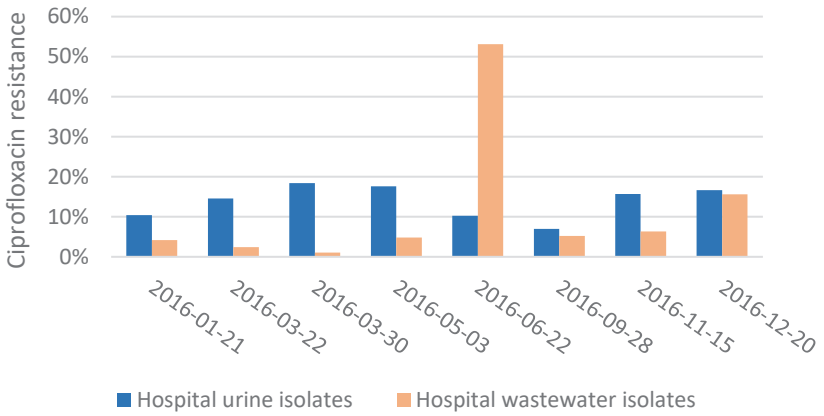

Figure 7: Ciprofloxacin resistance rates among *E. coli* isolated from patients' urine samples or wastewater samples at the hospital. Dates (in YYYY-MM-DD format) correspond to the wastewater sampling date. For each date, the resistance rate in urine isolates was calculated for *E. coli* isolated from patients' samples between one week before and one week after the wastewater sampling date.

The biochemical typing of the *E. coli* isolated from hospital wastewater on occasions with extreme resistance rates showed that identical bacterial isolates represented a large proportion of the bacteria isolated from those samples. The resistance profile of those over-represented clones heavily influenced the observed resistance rates in those samples. We hypothesized that sampling the wastewater as close to the source as was done for the hospital wastewater was probably not allowing enough mixing of the fecal material coming from different individuals. This led to a higher probability of isolating bacterial strains coming from the same individual several times rather than a diverse set of strains more likely to be informative of the whole targeted population. A similar reduction in the diversity of bacteria isolated from hospital wastewater samples has been observed in other studies, therefore there is a risk that this problem would affect all studies based on this type of samples or maybe even more generally on samples collected early in the sewers (Colque Navarro et al., 2014; Kwak et al., 2015; Paulshus et al., 2019). In our study, this bias was likely reduced by sub-sampling and random picking of bacterial colonies, but the most important component to reduce the influence of that phenomenon was probably pooling data from several sampling occasions (as can be seen on **Figure 7**). This observation shows the importance of a good sampling strategy when it comes to predicting resistance rates on a population-level.

Finding higher resistance rates in *E. coli* from hospital wastewater compared to municipal wastewater could be expected based on the assumption that there should be higher resistance rates in the microbiota of the hospital population compared to the wider municipal population. In concordance with that, resistance

rates were higher in *E. coli* from hospitalized patients than primary care patients. The closer resistance rates between hospital wastewater *E. coli* and hospital clinical *E. coli* compared to municipal wastewater *E. coli* and primary care *E. coli*, could have been explained by the rare sampling of uncomplicated cases of urinary tract infections in primary care likely to lead to an overestimation of resistance rates in that setting (see section 1.1.5). However, the observation that resistance rates in hospital wastewater *E. coli* were so close to clinical resistance rates was surprising, considering that resistance rates in fecal bacteria are expected to be lower than in the bacteria causing infections (Clermont et al., 2017; Dang et al., 2013; Nielsen et al., 2014). At the time, we suspected that the chemical and in particular the antibiotic content of the hospital wastewater might select for more resistant *E. coli* but the measured antibiotic concentrations in wastewater samples did not support that hypothesis. However, the results of two of our studies carried out afterwards have shown that the content of hospital wastewater is capable of causing selection of resistant *E. coli* (see **Paper IV** and (Kraupner et al., 2021)). In **Paper IV**, we showed that a strain was more able to survive or grow in the presence of hospital wastewater for 3h at 30°C when it had acquired plasmids conferring resistance toward multiple antibiotics compared to that same bacterial strain without a plasmid or with a plasmid conferring resistance only toward sulfamethoxazole. In the study by Kraupner et al., we demonstrated the selective effect of hospital wastewater in several laboratory conditions including ones close to what can be expected to be found in the sewers, i.e. at 20°C, under agitation without any addition of nutrients. Under those conditions, hospital wastewater had a bactericidal effect on susceptible bacterial strains that was measurable within five hours. Even through it is not certain that such selection would have had time to occur in the sewers before the sampling of hospital wastewater, there is also a possibility that it would occur during the 24h while sampling is proceeding and therefore there is a risk that it could have influenced the resistance rates measured in hospital wastewater.

Overall, we showed in **Paper I** that the resistance rates in *E. coli* isolated from wastewater have a strong relationship with the resistance rates in *E. coli* isolated from clinical samples, which is an important first step toward the development of a surveillance system for clinical antibiotic resistance through WBE. Yet the relationship observed was dependent of the sampling location and therefore will require further characterization before clinical resistance rates could be deduced from the analysis of wastewater sampled in new locations.

4.1.2. Monitoring of rare resistance traits

To follow the spread of rare resistance traits can be challenging for classical clinical surveillance systems. Indeed, even if the investigated ARB is present in the gut microbiota of a portion of the population, it could require the sampling of many individuals before the ARB is detected and even more to detect it enough to measure the extent of its dissemination. Additionally, if the ARB is rarely or never involved in infections, it would not be investigated by routine clinical surveillance but could only be detected through large scale fecal screening programs. Yet

many of such rare ARB are likely to be present in wastewater and could be monitored there. Since the ARB should be rare among the wastewater bacteria also, their monitoring would require the use of methods with low detection limits such as the use of selective culture media or qPCR targeting the genes responsible for the resistance phenotype.

Paper II focused on the detection of CPE in wastewater. CPE are rarely detected in patients in Sweden and are often imported from foreign countries. As a matter of fact, carriage of CPE was only detected in eight patients residing at the investigated hospital site over the two years period of the study. In this second study, CPE were isolated from hospital wastewater and their occurrence was compared to the detection of CPE in patients' samples during the same period. Further, a set of five carbapenemase genes were quantified in the wastewater samples to evaluate if such methodology would provide reliable estimations of the abundance of CPE in wastewater and potentially also in the hospital patients.

The *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{NDM}}$ genes were the most common carbapenemase genes found in both patient and wastewater CPE. The direct quantification of those two genes was also consistent with the detection of CPE in wastewater. During the study period, the *bla*$_{\text{KPC}}$ gene was never detected in samples from patients. Coherently, it was not detected in wastewater, neither by qPCR nor in any of the CPE isolated. On the contrary, the quantification of two other carbapenemase genes was not a good indicator of the presence of CPE carrying them. The concentration of *bla*$_{\text{VIM}}$ varied a lot while the one of *bla*$_{\text{IMP}}$ was consistently high. However, none of the CPE isolated from the wastewater samples carried those genes and *bla*$_{\text{VIM}}$ was detected only once in a CPE from a patient during the study period, suggesting that those genes are likely to be harbored by non-Enterobacterales in wastewater. Because of this, quantification of *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ in wastewater is likely to be of limited pertinence for estimating CPE abundances.

The *bla*$_{\text{OXA-48-like}}$ genes were detected in almost all samples but in higher concentration from the end of 2015. The detection of *bla*$_{\text{OXA-48-like}}$ carrying CPE from wastewater increased within the same period, with a peak from January to May 2016. Those months also corresponded with the detection of CPE carrying *bla*$_{\text{OXA-48-like}}$ genes in several patients. Sequencing revealed that the *bla*$_{\text{OXA-48-like}}$ genes were carried by different strains in wastewater compared to patients' samples. Nonetheless, the *bla*$_{\text{OXA-48-like}}$ carbapenemase genes were located on resembling MGEs. The *bla*$_{\text{OXA-48}}$ gene were in both settings carried by IncL plasmids closely resembling pE71T (KC335143), as many plasmids carrying the *bla*$_{\text{OXA-48}}$ gene characterized in the clinical setting worldwide. However, the plasmids found in bacteria isolated from a patient had, among other small differences, a deletion of a segment that was present in the plasmids from wastewater isolates. The *bla*$_{\text{OXA-244}}$ gene was found to be chromosomally encoded within a composite transposon in another clinical isolate, a composite transposon with only one nucleotide differing was found in a wastewater isolate. Even though, the peaks of detection of *bla*$_{\text{OXA-48-like}}$ genes and *bla*$_{\text{OXA-48-like}}$ carrying CPE in wastewater were in fairly good concordance with the clinical observations, the baseline detection of *bla*$_{\text{OXA-48-like}}$ might reflect something else than human carriage of CPE. Indeed, the peaks

observed in wastewater were of a 1000-fold magnitude. It is highly unlikely that a 1000 times more people would carry CPE in the hospital during an outbreak without more cases being detected. A possible explanation could be that the baseline levels detected would be due to *bla*$_{\text{OXA-48-like}}$ carrying CPE coming from other sources such as biofilms in the sewers or hospital sinks rather than directly from human feces. This hypothesis would also be supported by the repeated isolation from wastewater of *bla*$_{\text{OXA-48-like}}$ carrying CPE strains of sequence types not found in the clinical samples (i.e. *E. coli* of sequence type 2450 and *K. pneumoniae* of sequence type 147). Yet, the fact that strains from wastewater and patients were carrying resembling *bla*$_{\text{OXA-48-like}}$ harboring MGEs, could indicate that the wastewater strains would have acquired their *bla*$_{\text{OXA-48-like}}$ genes from gut bacteria via HGT in the past.

The *bla*$_{\text{NDM}}$ genes were detected on only one occasion in wastewater (in April 2017). CPE carrying *bla*$_{\text{NDM}}$ were also only isolated from that same wastewater sample. Additionally, both an *E. coli* and a *K. pneumoniae* carrying *bla*$_{\text{NDM}}$ were isolated from a patient close to the wastewater sampling date. Sequencing showed that the patient's strains were identical to CPE strains carrying *bla*$_{\text{NDM}}$ isolated from wastewater. Moreover, the plasmids harboring *bla*$_{\text{NDM}}$ had very few differences between the two bacterial species (only 6 nucleotides differed between *E. coli* and *K. pneumoniae* plasmids) and were also identical between wastewater and patient's isolates of each respective species. Such observations indicate that the *bla*$_{\text{NDM}}$ genes detected in wastewater predominantly originate from the connected population and therefore are a direct indication of *bla*$_{\text{NDM}}$-CPE carriage in this case. Still, the *bla*$_{\text{NDM}}$ genes were not detected in any other wastewater sample while it was detected in isolates from three other patients during the study period. It is likely that CPE from those patients were also present in wastewater at some time point but were missed by our sampling campaign. Indeed, the wastewater samples were always collected more than two weeks apart from those detections in patients. More regular sampling (or ideally even continuous sampling) could then resolve this issue.

Paper III, used a qPCR methodology similar to what had been tested in **Paper II** to quantify, in wastewater, ARGs which are even more rarely detected than carbapenemase genes in the Swedish clinical setting. The set of ARGs included mobile resistance genes providing resistance toward the last resort antibiotics colistin (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*) and linezolid (*optrA* and *cfr(A)*), as well as two ARGs recently discovered though targeted metagenomic methods, *sul4* and *gar*, providing resistance toward sulphonamides and aminoglycosides, respectively. Since most of those ARGs have never been detected in clinical samples, their prevalence in Sweden is largely unknown. To carry out a large scale screening of those ARGs of emerging concern and potentially follow their spread, we quantified them in raw wastewater samples from the hospital and at the inlet to the WWTP as well as in treated wastewater from the outlet of the WWTP collected over a five-year period.

All the targeted ARGs were detected in wastewater samples except *mcr-2*. The genes providing resistance to linezolid were only detected in hospital wastewater

whereas the other ones were detected in all types of wastewater samples. The *cfr(A)* gene was detected in all hospital samples while *optrA* was exclusively detected during 2017. The *mcr-1* gene was detected in approximately half of all wastewater samples while *mcr-3*, *mcr-4* and *mcr-5* were almost always detected and were most abundant in municipal wastewater. The *sul4* and *gar* genes were detected in all samples and were at their highest abundance in the last hospital samples collected.

While it is possible that differences in ARG abundances between hospital and municipal wastewater (as observed for *cfr(A)* , *optrA*, *mcr-3*, *mcr-4* and *mcr-5*) could be a reflection of differences in the prevalence of those genes in the two contributing populations, a characterization of the bacterial composition in the wastewater samples indicated another possible explanation. The sequencing of 16S rRNA in the wastewater samples showed marked differences in the composition of the bacterial communities, not only between raw and treated wastewater, but also between hospital and municipal wastewater. The latter is likely a consequence of the fact that hospital and municipal wastewater represent different stages of a shift of the bacterial community due to the conditions encountered in the sewers (Bengtsson-Palme et al., 2016; Buelow et al., 2018; Quintela-Baluja et al., 2019; Verburg et al., 2021). Indeed, being sampled at the end of the sewer system, the municipal wastewater bacterial community had more time to be modified by the wastewater environment compared to hospital wastewater bacterial community that resembled more a fecal bacterial community. Such shift in bacterial composition could influence the abundance of some ARGs in wastewater since the differentially abundant taxa include known potential hosts of the genes in question. It is consequently important to consider that differences in ARG abundances between wastewater sampled in different locations might be influenced by different levels of modification of the bacterial community and hence might not reflect real differences in carriage of the ARG in the corresponding populations even if the ARGs are harbored by gut bacteria.

The detection of *optrA* during only a limited period in the hospital wastewater samples could indicate that the gene was part of a silent outbreak of linezolid resistant bacteria during that year. Such an outbreak could have remained undetected locally since resistance to linezolid is not tested systematically. Further, the ARG could have been carried by bacterial strains distinct from the ones responsible for infections. Nevertheless, it is worth pointing out that a small increase in linezolid resistance among enterococci isolates was reported at the national level during the year when *optrA* was detected in wastewater (Swedres-Svarm, 2019). In a similar manner, the increased prevalence of *sul4* and *gar* at the end of the sampling campaign could denote an acceleration of the spread of those genes, although further sampling would be needed to verify if this would be a lasting tendency or only a temporary surge.

The detection of the ARGs in wastewater is notable in itself since those ARGs of emerging concern had either never been identified in samples from Swedish patients or at most a handful of times. Yet, it is difficult to evaluate how close those genes are to causing problems in the local clinical setting without further

information on which bacteria are hosting those genes. It would indeed be very different if the ARGs are carried by pathogenic bacteria, human commensal bacteria or bacteria not associated with humans. Unfortunately, the direct quantification of the ARGs does not reveal that information. To get an indication of the potential carriers of the ARGs in the wastewater samples, the correlations between the variations in the ARGs concentrations and the variations in the concentration of the bacterial taxa present in wastewater were analyzed with extra attention to already known potential hosts for each ARG. Although such analysis cannot be entirely conclusive, it highlighted some likely hosts of the ARGs in the samples. In particular, several *Aeromonadaceae* operational taxonomic units correlated strongly with *mcr-3* and *mcr-5* which could indicate that this family is likely to be hosting those *mcr* genes in the wastewaters. A few percent of the population carry *Aeromonas spp*. in their guts (Millership et al., 1983; Svenungsson et al., 2000; Igbinosa et al., 2012). Hence it is possible that this genera would be a host of *mcr-3/5* genes in the human population, but human carriage of those *mcr* genes could also be associated with other bacterial taxa as suggested by a recent metagenomic survey (Andrade et al., 2021). *Aeromonadaceae* were more abundant in municipal than hospital wastewaters therefore their increase could account for a portion of the increase in *mcr-3/5* genes in municipal wastewater. In addition to the shift in the bacterial community mentioned above, the increase in this particular bacterial taxa could be due to the incorporation of bacteria originating from sewer biofilms into the wastewater as it flows through the sewers. Indeed *Aeromonas spp.* thrive in the sewer environments and are avid biofilm producers (McLellan and Roguet, 2019).

The detection of *mcr-1*, *mcr-3*, *mcr-4*, *mcr-5*, *sul4* and *gar* in the treated wastewater discharged into the river could also raise concerns. Indeed, contamination of the environment by those ARGs could constitute a risk for (re-)transmission of bacteria carrying those genes to humans, but once again this risk would be dependent on the identity of the bacterial hosts of those ARGs.

Overall, **Paper II** showed that the detection of CPE in wastewater was coherent with the detection of such bacterial strains in patients contributing to the wastewater. It also indicated that the quantification of some ARGs in wastewater samples could be representative of the situation in the connected population provided that those ARGs are mainly carried by bacterial strains of clinical interest in wastewater. Further, the detection of an increase in $bla_{\text{OXA-48-like}}$ genes in wastewater ahead of the detection of CPE carrying those genes in several patients suggests that monitoring of some ARGs in wastewater could serve as an early warning system. Considering those findings, the detection in wastewater of ARGs rarely found in clinical samples in **Paper III** could indicate their silent dissemination in the microbiota of the local population. Such finding highlights the importance of monitoring closely the spread of those ARGs and to comply with antibiotic usage guidelines as to not create unnecessary selection accelerating their propagation.

4.2. Effects of wastewater on bacteria

Paper IV investigated the potential of the abiotic content of hospital and municipal wastewater to induce HGT. An *E. coli* recipient strain was mixed with a complex bacterial community and exposed to filter-sterilized wastewater. The exposure to municipal wastewater did not provoke any changes compared to the control conditions, suggesting that this type of wastewater does not increase HGT in places with relatively low antibiotic consumption like Sweden. In contrast, exposure to hospital wastewater led to an increased proportion of recipients that acquired new resistance genes (i.e. transconjugants). It additionally led to a stark decrease in the number of viable recipient bacteria at the end of the assay. Similarly to what was observed in **Paper I**, the antibiotic concentrations measured in the wastewater samples were below what could be expected to cause such a decrease individually. The concentrations of antibiotics and other pharmaceuticals were also below concentrations known to be able to induce HGT. Still, the two observed effects were caused by the chemical content of hospital wastewater. They could be the result of substances not measured and/or not known to cause such effects, or be due to the mixture of several substances together. Whatever the cause, we determined that transconjugants that had acquired resistance toward many antibiotics were less affected by the suppressing effect of hospital wastewater than the recipient strain or a transconjugant that had acquired resistance toward sulfamethoxazole only. Hospital wastewater even had a clear bactericidal effect on the two latter less resistant strains when they were exposed to it individually. The better ability of multi-resistant strains to grow and/or survive in the presence of hospital wastewater could have influenced the final ratio of transconjugants among recipients. Indeed, there is a possibility that transconjugants would have been selected for by hospital wastewater after their acquisition of a plasmid harboring resistance genes. Such possible selection, although an interesting observation in itself and further investigated in another of our studies (Kraupner et al., 2021), make it challenging to isolate the role of HGT on the witnessed increased proportion of transconjugants. However, if a strong selection of antibiotic resistance had taken place during the assays, it should have increased resistance toward antibiotics among transconjugants exposed to hospital wastewater compared to the ones from the control condition and the ones exposed to municipal wastewater. Yet, no such change in resistance prevalence was observed, indicating limited selection in the conditions of the assay. It is therefore possible that hospital wastewater causes both an increase in HGT and selection of resistant bacteria, and those two mechanisms combined could act together to promote antibiotic resistance in the sewers.

In the study presented in **Paper IV**, we additionally characterized the MGEs acquired by the recipient strain from the treated wastewater bacterial community through a combination of AST, PCR amplification of plasmid replicons and long read sequencing by nanopore technology. The AST of transconjugants showed that resistance toward several other antibiotics was commonly acquired in addition to resistance against sulfamethoxazole. Resistance toward ampicillin, trimethoprim, streptomycin and tetracycline, in particular, were co-acquired by most transconjugants. Characterization of the plasmid types revealed that the

recipient predominantly acquired IncN plasmids (89%) although the plasmids conferred a wide diversity of resistance profiles and therefore likely originated from distinct donor bacteria. The second most common type of plasmid was lowGC-type plasmids (7%), which had almost exclusively been identified in manure samples or soils affected by manure before (Heuer et al., 2009; Kopmann et al., 2013; Jechalke et al., 2013, 2014; Kyselková et al., 2016). The only other type that was repeatedly characterized were IncP1 plasmids (3%), specifically the subgroups ε and β. This contrasts with the plasmids causing sulfamethoxazole resistance in fecal *E. coli* from healthy individuals or *E. coli* causing urinary tract infections among which IncF plasmids were most common (Wu et al., 2010; Poey and Laviña, 2018). Such disparities could indicate that the donor bacteria might have not been comprised of clinical *E. coli* strains but rather *E. coli* stains not involved in infections or other bacterial species. The latter can provide some support to the assumption that wastewater could be an important setting for the transfer of MGEs from environmental bacteria to pathogens.

Paper IV undoubtedly uncovered a strong effect, probably antibiotic-driven, of the content of the hospital wastewater on bacteria. In the study, we did not see any activity of the municipal wastewater. Yet, it is worth noting that the study was carried out in Sweden where antibiotic consumption is relatively low compared to many other countries. Therefore, it is probable that in other countries hospital wastewater would be even more potent and municipal wastewater might exhibit a similar activity.

5. CONCLUSION AND PERSPECTIVES

This thesis contributed to the development and evaluation of methodologies to infer clinically relevant antibiotic resistance data from wastewater analysis. Through that process we showed that wastewater based epidemiology could be a valuable tool for the surveillance of antibiotic resistance and also unraveled some potential biases/caveats that should be considered when deploying such methodologies.

The results suggest that WBE could be used to inform about resistance rates in bacteria causing infections as well as the occurrence of rare resistance threats in the contributing population. However, the studies presented in this thesis were limited to a single city and before implementation further evaluation in several locations with different antibiotic resistance situations is essential. A study by Huijbers and colleagues indicated that the relationship between resistance rates in *E. coli* isolated from municipal wastewater and national clinical data could be relatively stable for a few antibiotics over a set of European countries, which could indicate that approximate clinical resistance rates could be deduced from untreated municipal wastewater gathered from large WWTP in new locations (Huijbers et al., 2020). Karkman and colleagues also recently suggested that it, to some extent, should be possible to predict clinical resistance rates from metagenomic analysis of wastewater (Karkman et al., 2020).

The quantification of ARGs in wastewater by methods such as metagenomic sequencing or qPCR bypasses the tedious bacterial culture process and thereby includes the analysis of resistance genes in non-cultivable bacteria. However, this also means that it encompasses quantification of ARGs from bacteria with no clinical relevance in addition to the potential pathogens present in the samples. Further, the presence of an ARG does not always coincide with the display of a resistance phenotype in bacteria, and antibiotic resistance can also be the result of unknown ARGs or chromosomal mutations that are not necessarily readily identified by culture-independent methods. Those elements make the prediction of resistance rates in clinical isolates from ARG quantification more complicated than from phenotypic characterization of wastewater isolates. A key addition to ARG based studies could be the characterization of the bacterial hosts of the studied genes in wastewater, which in turn could inform on the origin of the bacteria and likelihood of the gene to cause resistance in infection situations. Culture-independent techniques usually cannot provide that information although recently developed methodologies, such as epicPCR or Hi-C, might allow determination of the bacterial hosts of ARGs (Spencer et al., 2016; Stalder et al., 2019). Such host assignments would facilitate the interpretation of the surveillance data and contribute to a better evaluation of the risks associated with the ARG detection. Gene quantification can also initially be combined with culture-based verifications and comparisons with the clinical situation to support the interpretation of the results for specific ARGs in wastewater, as was done in **Paper II**. Based on such studies, it could be determined for each ARG if its quantification is a relevant marker of carriage of antibiotic resistant bacteria in the population

and/or antibiotic resistance in clinical infections, or if the gene is mostly carried by irrelevant bacteria in the studied setting.

The presented studies also highlighted the changes that occur in wastewater within the sewers and how they can influence WBE. Consequently, the choice of the sampling point in the sewers is likely to influence the results of the WBE and ideally its characteristics should be identified before epidemiological data can be deduced from the wastewater samples. It would for example be valuable to evaluate the diversity of the bacteria collected to ensure that they originate from a wide variety of individuals. It might also be necessary to take into account the antibiotic concentrations in the wastewater and verify that those do not influence the resistance markers analyzed. When such influence is suspected, wastewater samples could be diluted as they are collected to reduce the antibiotic concentrations the bacteria are exposed to during sampling and processing of the samples.

Taking into account the aforementioned elements, WBE has great potential to be used for providing population-level surveillance of antibiotic resistance. WBE strategies for antibiotic resistance surveillance would require the involvement of less people and less infrastructure than the classical surveillance systems and therefore should be most valuable where surveillance systems do not exist because of limited resources. Further, surveillance of antibiotic resistance through wastewater analysis could be combined with other WBE initiatives to facilitate its implementation. Measurements of antibiotics or other chemicals to follow consumption, or surveillance of viruses could be done from the same samples. The association with the environmental surveillance of poliovirus using wastewater should be particularly beneficial since it established regular collection of wastewater samples in low-income countries, which are also most in need of surveillance data for antibiotic resistance (WHO and GPEI, 2019; Årdal et al., 2021). Additionally, the methodology employed to determine resistance rates in *E. coli* in **Paper I** could potentially be extended to other pathogens present in feces such as enterococci, *K. pneumoniae*, *Shigella spp*. and *Salmonella spp.*.

With regard to the role of wastewater in the emergence of antibiotic resistance, **Paper IV** unraveled the ability of hospital wastewater to increase the proportion of resistant bacteria through selection and potentially also induction of HGT. The selective potential of wastewater was confirmed and further defined in a later study (Kraupner et al., 2021) but additional investigations would be needed to truly characterize the role of wastewater in HGT without the interference of selection mechanisms, for example by monitoring the expression of genes involved in conjugation. Additionally, it would be important to evaluate if the effects of wastewater observed under laboratory conditions are actually taking place in the real sewer conditions. The potentially short time of exposure of the wastewater bacteria, the complexity of the wastewater bacterial communities, the temperature and mixing conditions in the sewers are likely to influence such phenomenon. Finally, pointing out the specific substances responsible for the observed effects within the complex wastewater mixture would be critical for designing and evaluating potential mitigation strategies.

Together, the studies presented in this thesis provided evidence that wastewater analysis can be used to generate clinically relevant antibiotic resistance surveillance data and pointed to an ability of the content of wastewater (specifically hospital wastewater) to promote the emergence of antibiotic resistance.

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