Dissemination Of Antibiotic Resistance Via Wastewater And Surface Water

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Propositions

accompanying the dissertation

"Dissemination Of Antibiotic Resistance Via Wastewater And Surface Water"

by

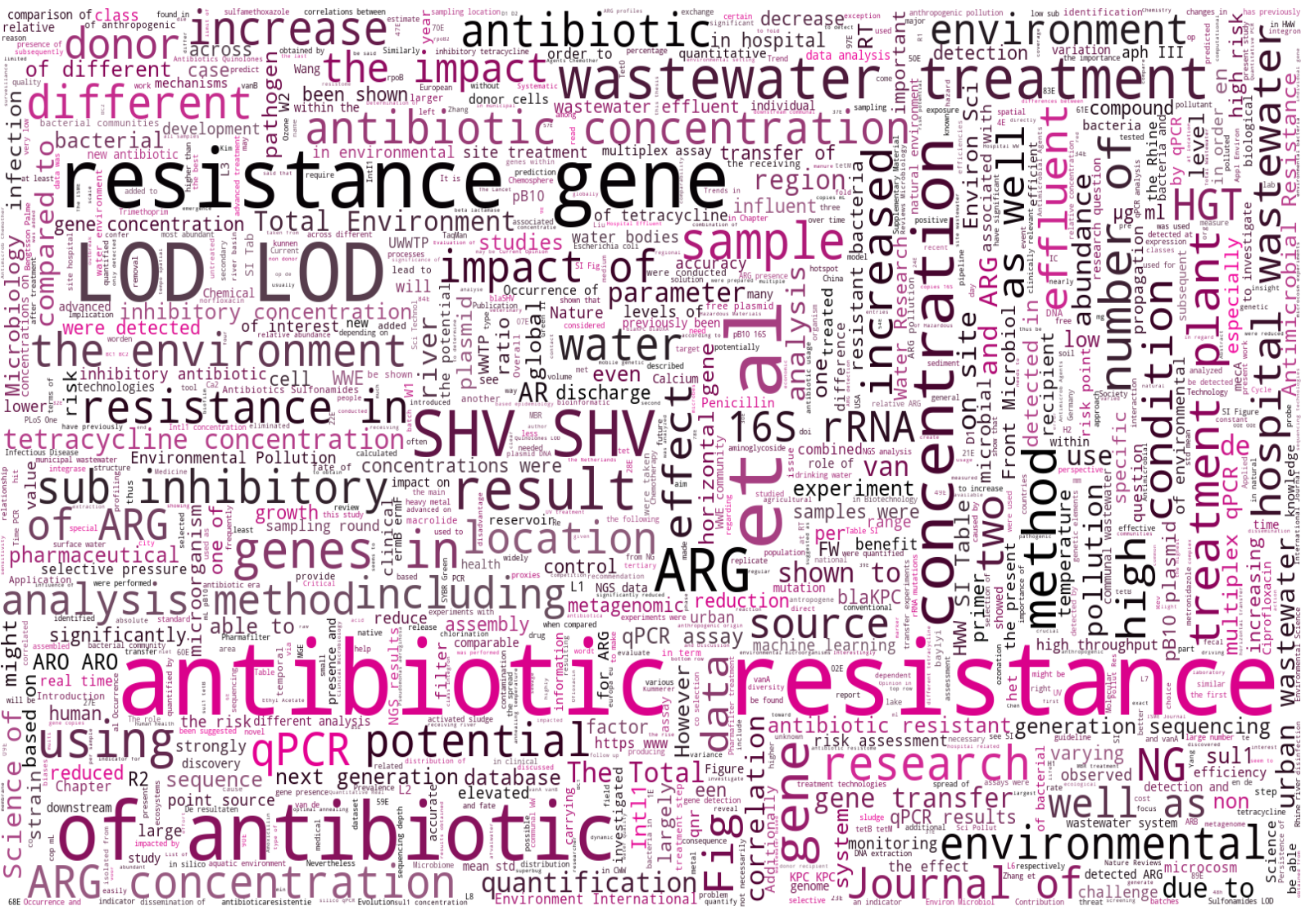
Gabriela Karina PAULUS

- Tackling antibiotic resistance of anthropogenic origin at the source is most efficient [this thesis, Chapter 2]
- Continuous advocacy for qPCR as the "gold standard" in ARG research could well be an example of the sunk-cost fallacy at work [this thesis, Chapter 5]
- Ignoring the point of intersection between high antibiotic and AR – concentration anthropogenic environments and aquatic environments increases risk for HGT and ARG propagation [this thesis, Chapter 4]
- More standards and conventions, as well as better proxies for antibiotic resistance of anthropogenic origin are sorely needed in the field of environmental research of antibiotic resistance [this thesis, Chapter 3]
- Continuous above-average efficiency is achievable only with extensive periods of rest in between
- To increase women ratios in STEM we need to stop propagating the stereotype of what a "typical" scientist looks or acts like and how she is supposed to express herself
- Machine learning is the future of all scientific disciplines. It will substantially help the efforts against antibiotic resistance
- Regularly hitting the gym improves academic performance
- While big data could help bring solutions to many issues today, big data centers are ravaging water conservation efforts and might lead to disastrous outcomes if unregulated, especially in arid areas
- Health care and education are not privileges, they are human rights

These propositions are regarded as opposable and defendable, and have been approved as such by promotors Prof. dr. G. J. Medema and Prof. dr. T. Beredonk.

"Dissemination Of Antibiotic Resistance Via Wastewater And Surface Water"

Gabriela Karina Paulus



"Dissemination Of Antibiotic Resistance Via Wastewater And Surface Water"

Dissertation

for the purpose of obtaining the degree of doctor
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by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J. van der Hagen,
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by

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Dr. L. M. Hornstra has contributed greatly to the preparation of this dissertation.

An electronic version of this dissertation is available at

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"Aprender a dudar

es aprender a pensar"

Octavio Paz

Summary

Antibiotic resistance is one of the biggest threats society is facing around the globe and has been on the rise worldwide. While antibiotic resistances play crucial roles in shaping and coordinating microbial communities in natural environments, they can lead to disastrous results when acquired by pathogens in clinical environments. Effective antibiotics not only enable the functioning and interactions necessary for our highly globalized world, but also drive advances in healthcare and are the deciding factor facilitating life-saving medical intervention such as open-heart surgery, organ transplants and chemotherapy. Increasing resistance antibiotics is threatening the medical status quo, as well as social and economic stability (***Chapter 1***). Water environments, especially anthropogenically impacted environments such as wastewater treatment plants, are suspected to be - not only - reservoirs for antibiotic resistance genes but also hotspots for horizontal gene transfer. Knowledge about the impact of anthropogenically impacted aqueous environments is needed in order to be able to uncover the processes, parameters and mechanisms underlying and facilitating the transfer of antibiotic resistance genes in order to be able to implement practical, useful and efficient measures in order to reduce the spread of antibiotic resistance and to reduce anthropogenic impact of antibiotic and antibiotic resistance gene pollution in the environment (***Chapter 1***).

Equally important for accurate risk assessment, is the question how persistent anthropogenically sourced antibiotic resistance gene pollution is in the environment. The answer to this question is not solely important from an environmental and ecological point of view. Persistence of antibiotics and resistance genes in natural environment also increase risk for transfer and re-introduction to anthropogenic environments. The results of ***Chapter 3*** show that anthropogenically introduced antibiotic resistance does not necessarily persist in the aqueous phase of large rivers. They further show that regional environmental and anthropogenic factors can strongly affect antibiotic concentrations and variety locally. High-risk point sources, such as wastewater effluents from the pharmaceutical industry can have an especially pronounced impact. Results also show, that better proxies are needed in order to estimate overall antibiotic resistance gene pollution from anthropogenic sources.

To further investigate the importance of potential high-risk point sources, the lasting impact of highly polluted hospital wastewater on the subsequent communal wastewater system was investigated in ***Chapter 2***. To this end two hospitals and the receiving communal wastewater systems, including influents and effluents from the receiving wastewater treatment plants were monitored. Advanced on-site treatment of hospital wastewater was conducted at only one of these two locations. The results confirm that hospital wastewaters are more hazardous than regular communal wastewater in terms of antibiotic as well as antibiotic resistance genes variety and concentration, with hospital wastewaters harboring 0.4 to 1.8-fold higher relative antibiotic resistance genes concentration. They further show, that untreated wastewater from high-risk point sources have a significant and lasting impact on the entire receiving wastewater system including introduction of hospital-associated antibiotic resistance genes and antibiotics. This impact extends to the communal wastewater treatment plant effluent, which directly affects the receiving natural water body. On-site treatment of high-risk point sources could thus be shown to have a significant positive impact in term of risk mitigation and effective hazard control. Nine out of thirteen detected antibiotic resistance genes could not be detected anymore after advanced, on-site treatment.

A lot of research has been done to investigate the effect of different antibiotic concentrations on antibiotic resistance. A large part of this research is, however, focused on inhibitory concentrations and isolated bacteria (often pathogens). However, wastewater largely contains quite low antibiotic concentrations in the sub-inhibitory range and bacterial interactions and competition can play a significant role in the propagation of antibiotic resistance genes. ***Chapter 4*** aims to examine the effect of various tetracycline concentrations, including sub-inhibitory concentrations, on horizontal gene transfer and resistance and growth of complex microbial communities. The results reveal that low sub-inhibitory tetracycline concentrations can be a significantly stronger driver of antibiotic resistance gene propagation than higher concentrations. Under some conditions, subinhibitory tetracycline conentrations of 0.0015 µg/ml increased resistance-carrying plasmid concentrations up to 2-fold more than tetracycline concentrations of >10 µg/ml. The results in this chapter further show, that the effect of different antibiotic concentrations on horizontal gene transfer are strongly matrix-dependent. The possession of antibiotic resistance genes may not be sufficiently advantageous for enhanced bacterial growth, even under moderately selective conditions, when resistant bacteria are embedded in a complex bacterial community. A non-native donor bacterium could thus be shown to have no selective advantage over native wastewater community bacteria under inhibitory tetracycline concentrations. An advantage could only be documented under 10-fold elevated inhibitory tetracycline concentrations. The significance of matrix effects should not be underestimated and a lot more research will be necessary in this area. Environments that serve as contact zones for high-resistance, anthropogenically polluted wastewater effluent and low antibiotic concentration natural environments might be of especial interest for risk assessment, as high-resistance bacteria in combination with sub-inhibitory antibiotic concentration may notably increase the risk for horizontal gene transfer.

One peculiarity of environmental biological research is that the largest part of environmental microorganisms cannot be cultured in laboratory environments. This increases the dependency on molecular methods for research. Real-time (quantitive) polymerase chain reaction has long since been the method of choice to obtain insight into the inner workings of non-cultivable environmental microorganisms. With the rise of next generation sequencing as well as steadily decreasing costs hereof, an increasing number of research is being conducted with this newer method. Despite the vastly different molecular mechanisms behind these two technologies, results are frequently compared across the board. ***Chapter 5*** concerns itself with the more basic (but deeply relevant) question of method comparability. Further, the status quo of qPCR as the "gold standard" for environmental antibiotic resistance research is challenged. The results in this chapter show, not only that NGS has vast advantageous in antibiotic resistance gene detection (presence of the gene), but is also nearly equally accurate for relative antibiotic resistance gene quantification. 50% of all WGS quantification results were within the range of -50% to + 100% of qPCR results, while 86% was quantified with <1-log difference from qPCR results. Machine-learning models were shown to be especially beneficial to antibiotic resistance gene detection, when compared to qPCR or non-machine-learning bioinformatic pipelines for NGS data analysis. Genes present at very low concentrations were the exception as their concentration was often vastly overestimated by NGS methods. A combination of initial NGS screening of samples with subsequent targeted, informed choice of genes of interest tailored to the research question (based on NGS results), can be recommended as the most advantageous plan of action. Additionally, qPCR results could be analyzed in context and potential selection bias on results and conclusions thus be reduced.

Chapter 6 offers a concluding perspective. This chapter includes a reconciling discussion, taking up the "red threat" of this thesis and unifying the different chapters. It further points out recommendations and implications of the combined thesis results and gives a brief

outlook, highlighting the importance of machine learning and wastewater-based epidemiology for the future of antibiotic resistance research.

Samenvatting

Antibioticaresistentie is een van de grootste bedreigingen waarmee de samenleving vandaag de dag wereldwijd wordt geconfronteerd en is wereldwijd in opkomst. Hoewel antibioticaresistentie een cruciale rol speelt bij de vorming en coördinatie van microbiële gemeenschappen in een natuurlijke omgeving, kan het leiden tot desastreuze resultaten wanneer het door ziekteverwekkers in een klinische omgeving wordt verworven. Werkende antibiotica maken niet alleen het functioneren en de interacties mogelijk die nodig zijn voor onze sterk geglobaliseerde wereld, maar stimuleren ook de vooruitgang in de gezondheidszorg en zijn de doorslaggevende factor voor levensreddende medische interventies zoals openhartoperaties, orgaantransplantaties en chemotherapie. De toenemende resistentie-antibiotica bedreigt de medische status quo en de sociale en economische stabiliteit (***hoofdstuk 1***). Watermilieus, met name antropogene omgevingen zoals afvalwaterzuiveringsinstallaties, worden verdacht van - niet alleen - reservoirs voor antibioticaresistentiegenen, maar ook van hotspots voor horizontale genoverdracht. Kennis over de impact van antropogene beïnvloede wateromgevingen is nodig om de processen, parameters en mechanismen die ten grondslag liggen aan en de overdracht van antibioticaresistentiegenen te kunnen blootleggen, zodat praktische, nuttige en efficiënte maatregelen kunnen worden genomen om de verspreiding van antibioticaresistentie te beperken en de antropogene impact van antibiotica- en antibioticaresistentiegenenvervuiling in het milieu te verminderen (***hoofdstuk 1***).

Even belangrijk voor een nauwkeurige risicobeoordeling is de vraag hoe hardnekkig de antropogene genverontreiniging door antibioticaresistentie in het milieu is. Het antwoord op deze vraag is niet alleen van belang vanuit milieu- en ecologisch oogpunt. Persistentie van antibiotica en resistentiegenen in het natuurlijke milieu verhogen ook het risico op overdracht en herintroductie naar een antropogene omgeving. De resultaten van ***hoofdstuk 3*** laten zien dat antropogene geïntroduceerde antibioticaresistentie niet noodzakelijkerwijs in de waterige fase van grote rivieren blijft bestaan. Ze tonen verder aan dat regionale milieu- en antropogene factoren lokaal een sterke invloed kunnen hebben op de antibioticaconcentraties en -variatie. Vooral risicovolle puntbronnen, zoals afvalwater van de farmaceutische industrie, kunnen een grote invloed hebben. De resultaten tonen ook aan dat er betere proxies nodig zijn om de totale antibioticaresistentiegenenvervuiling door antropogene bronnen in te schatten.

Om het belang van mogelijke risicovolle puntbronnen verder te onderzoeken, werd in ***hoofdstuk 2*** de blijvende impact van sterk vervuild ziekenhuisafvalwater op het daaropvolgende gemeentelijke afvalwatersysteem onderzocht. Daartoe werden twee ziekenhuizen en de ontvangende gemeentelijke afvalwatersystemen, inclusief de influenten en effluenten van de ontvangende afvalwaterzuiveringsinstallaties, onderzocht. Op slechts één van deze twee locaties werd een geavanceerde behandeling van het ziekenhuisafvalwater ter plaatse uitgevoerd. De resultaten bevestigen dat ziekenhuisafvalwater gevaarlijker is dan gewoon gemeentelijk afvalwater, zowel wat betreft de verscheidenheid aan antibiotica als de antibioticaresistentiegenen en de concentratie. Ze tonen verder aan dat onbehandeld afvalwater van hoogrisicobronnen een significante en blijvende impact heeft op het hele ontvangende afvalwatersysteem, inclusief de introductie van ziekenhuisgerelateerde antibioticaresistentiegenen en antibiotica. Deze impact strekt zich uit tot het effluent van de gemeenschappelijke afvalwaterzuiveringsinstallatie, dat een directe impact heeft op het ontvangende natuurlijke waterlichaam. De behandeling ter plaatse van bronnen met een hoog risico zou dus een significant positief effect kunnen hebben op het gebied van risicobeperking en effectieve risicobeheersing.

Er is veel onderzoek gedaan naar het effect van verschillende antibioticaconcentraties op de antibioticaresistentie. Een groot deel van dit onderzoek is echter gericht op remmende concentraties en geïsoleerde bacteriën (vaak ziekteverwekkers). Afvalwater bevat echter grotendeels vrij lage antibioticaconcentraties in het subremmende bereik en bacteriële interacties en concurrentie kunnen een belangrijke rol spelen bij de verspreiding van antibioticaresistentiegenen. In ***hoofdstuk 4*** wordt het effect van verschillende tetracyclineconcentraties, waaronder subremmende concentraties, op de horizontale genoverdracht en de resistentie en groei van complexe microbiële gemeenschappen onderzocht. De resultaten laten zien dat lage subremmende tetracyclineconcentraties een significant sterkere motor kunnen zijn voor de vermeerdering van antibioticaresistentiegenen dan hogere concentraties. De resultaten in dit hoofdstuk tonen verder aan, dat het effect van verschillende antibioticaconcentraties op horizontale genoverdracht sterk matrix-afhankelijk is. Het bezit van antibioticaresistentiegenen is mogelijk niet voldoende voordelig om de groei van bacteriën te bevorderen, zelfs onder matig selectieve omstandigheden, wanneer resistente bacteriën ingebed zijn in een complexe bacteriële gemeenschap. Een niet-inheemse donorbacterie zou dus aantoonbaar geen selectief voordeel hebben ten opzichte van inheemse bacteriën uit de afvalwatergemeenschap onder remmende tetracyclineconcentraties. Een voordeel kon alleen worden gedocumenteerd onder 10-voudige verhoogde remmende tetracyclineconcentraties. Het belang van matrixeffecten mag niet worden onderschat en er zal veel meer onderzoek nodig zijn op dit gebied. Omgevingen die dienen als contactzones voor hoge resistentie, antropogeen verontreinigd afvalwater en lage antibioticaconcentratie natuurlijke omgevingen, kunnen van bijzonder belang zijn voor de risicobeoordeling, aangezien bacteriën met hoge resistentie in combinatie met subremmende antibioticaconcentratie met name het risico op horizontale genoverdracht kunnen verhogen.

Een bijzonderheid van milieubiologisch onderzoek is dat het grootste deel van de micro-organismen in het milieu niet in een laboratoriumomgeving kan worden gekweekt. Dit vergroot de afhankelijkheid van moleculaire methoden voor onderzoek. Realtime (kwantitatieve) polymerasekettingreactie is allang de methode bij uitstek om inzicht te krijgen in de innerlijke werking van niet-kweekbare micro-organismen in het milieu. Met de opkomst van next generation sequencing en de gestaag afnemende kosten hiervan, wordt steeds meer onderzoek gedaan met deze nieuwere methode. Ondanks de sterk verschillende moleculaire mechanismen achter deze twee technologieën worden de resultaten vaak over de hele linie met elkaar vergeleken. ***Hoofdstuk 5*** gaat over de meer fundamentele (maar zeer relevante) kwestie van de vergelijkbaarheid van de methode. Verder wordt de status quo van qPCR als "gouden standaard" voor onderzoek naar antibioticaresistentie in het milieu op de proef gesteld. De resultaten in dit hoofdstuk laten zien dat NGS niet alleen een groot voordeel heeft bij de detectie van antibioticaresistentiegenen (aanwezigheid van het gen), maar ook bijna even nauwkeurig is voor de relatieve kwantificering van antibioticaresistentiegenen. Machine-learning model bleek vooral gunstig te zijn voor antibioticaresistentie gen detectie, in vergelijking met qPCR of niet-machine-learning bioinformaticapijplijnen voor NGS data analyse. Genen die bij zeer lage concentraties aanwezig waren, waren de uitzondering, omdat hun concentratie vaak enorm werd overschat door NGS-methoden. Een combinatie van de eerste NGS screening van monsters met de daaropvolgende gerichte, geïnformeerde keuze van interessante genen, afgestemd op de onderzoeksvraag (op basis van NGS resultaten), kan worden aanbevolen als het meest accurate plan van aanpak. Bovendien kunnen de qPCR-resultaten in de context worden geanalyseerd en kunnen potentiële selectievooroordelen op de resultaten en conclusies zo worden gereduceerd.

Hoofdstuk 6 biedt een sluitend perspectief. Dit hoofdstuk bevat een samenvattende discussie, de verschillende hoofdstukken worden samengevoegd, en wijst op de aanbeveling

en implicatie van de resultaten van het proefschrift en geeft een kort toekomstperspectief, waarbij het belang van machinaal leren en op afvalwater gebaseerde epidemiologie voor de toekomst van het onderzoek naar antibioticaresistentie wordt benadrukt.

TABLE OF CONTENTS

1. Introduction

1.1. Motivation for this research or "The post-antibiotic era – a dystopian future?"

Antibiotic resistance is on the rise globally. Bacterial microorganisms have proven to be formidable adversaries. Up to date, they have been able to render every single one of our antimicrobial weapons useless. Steadily increasing hygienic standards have been able to keep humanity a step ahead in the majority of cases. However, even today people are dying from infections caused by resistant microorganisms. The most recent viral pandemic has shown the global community its limits; it has demonstrated the fragility of societal structures and the pitfalls of a globalized world in the face of nature's scourges unleashed.

Fortunately, COVID-19 is but *one* virus and the collaborative efforts for a vaccine started strong and fast. In the cases of bacterial microbes, we are even more fortunate, as most bacteria are still susceptible to at least a portion of antibiotics in our arsenal. Scientists are intensely researching antibiotic resistance to avoid a future in which humanity does not have any safeguard against bacteria left. Such a future would be appallingly worse than the latest pandemic crisis and the probability to be able to design effective, safe vaccines for the myriad of bacterial pathogens would be slim to none.

The general public has recently had a peek into what a post-antibiotic era could look like. Similar to the current pandemic, a large majority of the world's population would be at risk in case of infection and the potential for a collapse of the health care systems would once again become tangible, due to missing effective treatments. Society and the global economy could suffer the consequences of constant on-alert health policies. Critical and selective medical procedures could be compromised due to the increased risk for infection (e.g. open-heart surgery) or the danger to compromise patients' immune systems (e.g. chemotherapy treatment) without the safety blankets of effective treatment against infections.

The work in this thesis was conducted in order to generate knowledge about antibiotic resistance, its spread and the importance of its reduction, and thus contribute to the global fight against antibiotic resistance.

1.2. Background

1.2.1 A SHORT HISTORY OF ANTIBIOTIC DISCOVERY

Penicillin, the first commercially produced antibiotic, was first discovered by Alexander Fleming in 1928 [1]. After a lagging period during which Penicillin was largely ignored, US American officials brought into life a large-scale collaboration to overcome corporate reluctance to spend resources investigating this little-known compound. The aim of this collaboration was to provide large-scale production of Penicillin for military efforts, based on successful early human trials and the development of methods to mass-produce Penicillin at

Oxford. During World War II, Penicillin was seen as a tool that would play "a critical role in the recovery of manpower" [2].

After the war, pharmaceutical companies rapidly developed an interest in antimicrobial research and advanced antibiotic discovery based on a method introduced by Selman Waksman that systematically screens soil antibiotic-producing soil microbes. From the 1940s to the 1960s a "golden era" for antibiotic research emerged, during which a large number of antibiotic classes with different modes of action were discovered [3]. The discovery of chloramphenicol (1949), was quickly followed by tetracyclines (1950), macrolides/lincosamides/streptogramins (1952), glycopeptides (1956), rifamycins (1957), nitroimidazoles (1959), quinolones (1962) and trimethoprim (1968) [4]. Antibiotic compounds were further designed by (semi-) synthesis in order to increase efficacy and the scale of production or to reduce side-effects and toxicity for otherwise efficient antibiotics [5]. In the last few decades advanced sequencing technologies have additionally enabled new opportunities for antibiotic discovery by taking advantage of the enormous wealth of microbial genomes [5].

Nevertheless, discovery of new antibiotics has drastically decreased since the 1970s and has come to a near halt in the last three decades [3–8]. This pronounced decline is clearly apparent in the low number of new antibiotics that have made it to market for clinical use in the last decades, for example: only one antibiotic from a newly discovered antibiotic class has been marketed for clinical use within the last 50 years [7,9]. Recently, a research team from The Hong Kong Polytechnic University has discovered a new antibiotic class with promising pharmacokinetic properties [10], however investigations into clinical potential and safety are in the early stage at best.

1.2.2 The emergence of antibiotic resistance

Parallel to the new abundance of clinical antibiotics in the 20th century, came the increasing resistance of bacterial pathogens [11,12]. However, before clinical resistance was natural resistance. As commercial antibiotics largely originated from (soil) microorganisms, it will not come as a surprise that natural antibiotic resistance is ubiquitous in nature. Apart from "antibiotic warfare" and the use of antibiotics for selective advantages against competitors, environmental bacterial communities use antibiotics for a large array of tasks, including signaling, metabolic diversification and community forming [13–22]. Resistance can occur due to different reasons [23]: (1) some bacteria are intrinsically resistant to certain antibiotic, e.g. gram-negative bacteria are resistant to a number of antibiotics that attach the cell wall owing to their outer cell wall which prevents certain compounds from passing through it (2) spontaneous mutation can lead to resistance (3) horizontal gene transfer of mobile genetic elements can propagate resistance from resistant to susceptible microorganisms.

Natural resistance had, however, not been a problem before the introduction of commercial antibiotics. The larger problem was the appearance of clinical resistance. Shortly after the large-scale introduction of antibiotics, euphoria ensued and the general public as well as some experts believed that a total triumph over infectious diseases was in grasping distance [4]. Mortality from infectious diseases dropped drastically[5,8,24], a 20-fold decrease of death rates from infectious disease could be recorded from 1900 to 1980 [24]. And while a large part of that decrease is a result of better hygienic standard and vaccinations [25], calculations from the U.S. government estimate that the use of antibiotic might have added as much as 10 years to life expectancy in the United States [26].

The euphoria regarding a victory over pathogens was premature. As early as 1945, Alexander Fleming had warned against the dangers of antibiotic resistance. During his Nobel Prize lecture he stated that: "[…]*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. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant*.[…]" [27]. Looking back at the development of antibiotic resistance over the last few decades, Fleming's words have a prophetic sound to them. In fact, the first cases of antibiotic resistance were published in 1940, even before the commercial introduction of penicillin [28]. Penicillin resistance threatened to throw society back to the pre-antibiotic era, but new antibiotics and antibiotic classes were discovered and disaster postponed [29].

At the same time antibiotic overuse, misuse, inappropriate prescriptions, non-essential agricultural use as well as inadequate disposal and insufficient guidelines intensified issues regarding antibiotic resistance [29]. The race between science and bacteria started, and resistances to most newly introduced antibiotics developed in clinically relevant strains years or sometimes mere months after their introduction [29]. One example of the speed and ease with which resistances can form is P.*aeruginosa*. The first ceftazidime resistant strain was discovered in 1991, only 5 years after its introduction into the markets. The reason was the mutation of the β-lactamase resistance gene bla_{OXA10}, two mutations on this gene were enough to result in bla_{OXA11} and confer resistance to ceftazidime [30]. Society has been in this race against microorganisms for decades and in the last few years, the sharply decreased number of new antibiotics has tilted the board in favor of the microorganisms.

1.2.3 Current situation and societal impact

Antibiotic resistance has been on the rise. It has reached a level at which it is a peril to human life, public health, society and the economy [31]. Multidrug resistance is a widespread issue and the cause of a large number of nosocomial infections [32–36].In the United States more than 35.000 people die from infections with antibiotic resistant organisms per year [37], in the European Union that number is approximately 33.000 [38]. This translates into nearly one death every 15 minutes in each of the two regions. Additionally, current estimated expenses caused by antibiotic resistance range as high as 1.5 Billion Euros per year, in Europe alone [39]. Globally, some estimations predict that, by 2050, as many as 10 million people will die from antibiotic resistance annually and global GDP could be lowered by up to 3.5% from direct and indirect causes associated with antibiotic resistance [40].

The damage to human well-being and risk to society cannot easily be monetarily captured but can be said to be one of the most devastating effects of antibiotic resistance. Antibiotic resistance pathogens are further not solely a threat in and of themselves, they have also been shown to be able aggravate patients health as secondary infections, which could be especially perilous during situations such as the current COVID-19 pandemic [41]. Apart from being therapeutics against infections, antibiotics have long since become the backbone of modern medicine. They are extensively used for prophylactic purposes and essential medical interventions, such as chemotherapy, invasive surgery or neonatal care (to just name a few), would in many cases become unacceptably more dangerous without efficient antibiotics [29,42].

Globalization and increased global mobility are rapidly increasing the spread and advance of antibiotic resistance, across regions and ignoring national borders [43–46]. However, the

medical, societal and economic damage is not and will not be equally distributed. Poor countries are disproportionately more at risk [47]. In India, 58.000 newborns died from infections with antibiotic resistant bacteria, passed on by their mothers, within one year in 2013 [48]. Of the estimated 10 million annual deaths from antimicrobial resistance by 2050, nearly 90% are calculated to occur in Africa and Asia [49]. Among the many reasons to be found for this misbalance are often underdeveloped national antibiotic stewardship programs [50] and frequent antibiotic misuse and abuse [51]. Many of these countries further have a scarcity of clean water or access to it. Introducing water and sanitation infrastructure was shown to decrease the cases of water-borne diarrhea and associated antibiotic use and have a decrease potential of up to 60% [40]. More generally, a report by the world bank states that "putting resources into AMR containment now is one of the highest-yield investments countries can make", with low and middle income countries obtaining the largest benefit from such investments [47].

Tackling antibiotic resistance therefore requires a global "One Health"-approach with combined efforts from governments, legislators, medical personnel, researchers, the general public and commercial pharmaceutical companies. Large pharmaceutical companies have, however, steadily been withdrawing from antibiotic research and development, as profitability from new antibiotics does not economically justify development and regulatory costs [52]. The subsequent depletion of the antibiotic pipeline [52–57], has caused the WHO to issue a new warning in January 2020 [58]. As of December 2019 only 41 antibiotics are in clinical development of which none are potentially active against WHO "critical threat pathogens" [59–61].

Unfortunately, even with full collaboration for antibiotic development, creating an infinite stream of new antibiotics seems unsustainable [52]. Awareness of hygiene and antibiotic stewardship are crucial parts to tackle antibiotic resistance. But finally, the most effective weapon against antibiotic resistance organisms will be a better knowledge of the development, propagation and efficient reduction of antibiotic resistance genes. This will create a scenario that enables us to actively reduce transfer and minimize spread and infection.

1.2.4 The role of water and the environment

One major field that has long been overlooked by researchers investigating antibiotic resistance, is the natural environment [42,62–66]. The reasons for this are manifold, including comparatively low antibiotic and pathogen concentrations [67]. Nevertheless, increased urgency due to growing levels of antibiotic resistance have driven academic research, public awareness and political interest in the last years [65,68,69]. Antibiotic resistance genes have become compounds of emerging concern (CECs), especially with regard to the environment [70].

Aquatic environments should be of special interest, as they provide optimal conditions for horizontal gene transfer, ARG propagation and mobilization. Water bodies are substantially and directly impacted by anthropogenic pollution via wastewater effluents and manure runoff; they can be dynamic and widespread and often connect across many different regions and they provide easily accessible dissolved nutrients to microorganisms [66,71–73]. Lakes, rivers and oceans are further often used for recreational and religious purposes, and as domestic or drinking water supply, creating ideal contact and exchange zones for environmental bacteria and pathogens.

ENVIRONMENTAL SOURCES

Antibiotic resistance and antibiotic resistance genes were detected in "pristine" environments without record of previous anthropogenic contamination [74–81], most of which seem to be efflux pumps encoded chromosomally [77,79,81]. It is hypothesized that some environmental microorganisms constitutively produce small amounts of antibiotics in sub-therapeutic concentrations for fighting competitors and thus gaining competitive advantage over them [82–84]. However, recent research suggests that the main reason environmental microorganisms produce antibiotics, is to use them as signal molecules which help to organize and shape the structure of and interactions in natural bacterial communities [13–22,62,85,86]. It was shown that ARG are further naturally present on mobile genetic elements (MGE), including plasmids, transposons and integrons. Relative abundances of MGEs can reach up to 30% [78], which is problematic as these are structures that enable horizontal gene transfer between microorganisms [87].

Challenges regarding natural antibiotic resistance arise from distinct issues, including substantial gaps in knowledge. Antibiotic resistance genes on MGEs harbor the risk to be transferred to (potential) human pathogens. The environmental resistome is frequently referred to as a "reservoir" of antibiotic resistance genes [88–92] and harbors an tremendously large number of antibiotic resistance genes, especially compared to clinical pathogens [93]. Fitness cost of non-essential antibiotic resistance genes can be overcome, even in absence of antibiotics, so that resistances can persist over time [83] Indigenous bacterial communities regularly exchange genetic material (including antibiotic resistance genes) by horizontal gene transfer via conjugation, transformation and transduction [94–97,66,98]. While transfer of antibiotic resistance genes between environmental and clinical microorganisms has been investigated, studies directly showing transfer are missing [98–100]. This knowledge gap is exacerbated by the unpredictability of risk associated with different environmental antibiotic resistance genes. While rifampicin resistance is abundant in environmental strains, clinical resistances are all but nonexistent [101]. Genes that do not confer resistance in natural habitats, may confer resistance in clinical strains [102] or may be significantly up-regulated in clinical or pathogenic strains [103].

ANTHROPOGENIC SOURCES

With increasing use of antibiotics, anthropogenic sources of antibiotics and ARGs have been expanding. There are three major sources and pathways of anthropogenic antibiotic and ARG pollution into the environment: (urban) WWTP effluents, pharmaceutical manufacturing plants and farming (which includes agriculture, animal husbandry and aquacultures) [104]. Out of these three, (urban) WWTP effluents is the largest contributor in volume and has been shown to have significant impact on levels of antibiotic resistant genes and bacteria [105–112]. Hospital WW could be shown to contribute the most to antibiotic resistance in urban WW systems due to high numbers and elevated concentrations of antibiotics and antibiotic resistant bacteria [113]. Therefore unsurprisingly, urban wastewater from combined veterinary, urban, clinical and environmental sources is a good measure for antibiotic resistance in communities [114]. It has been suggested, that source control of antibiotics and ARGs via pre-treatment of urban wastewaters and manures is an efficient way to diminish the impact of antibiotics in ecosystems [115].

Urban wastewaters are, however, not our only problem. Two other crucial sources for antibiotics and ARGs in the environment are pharmaceutical manufacturing sites and combined sewer overflow (CSO). CSO can cause serious environmental problems, as it can lead to contamination of environmental water bodies with untreated manure and sewage. Combined sewer sediments contain high ARG diversity, including clinically relevant ARGs and high concentrations of heavy metals [116]. Heavy metals have been known to have

significant impact on microbial diversity [117] and to benefit antibiotic resistance [118–121], not least through mechanisms of co-conjugation and co-selection [122,123,120,124]. One solution might be de-centralized, local treatment [125]

Although much less impacting in volume, antibiotic concentrations discharged in industrial wastewaters are globally disproportionately higher [126–130] than in urban wastewaters. Antibiotic pollution can increase relative and absolute ARG abundances, induce the emergence of novel ARGs and proliferate ARG diversity. It has also been shown to promote genetic mobility and potential for HGT [128,131–133]. The resulting pollution of receiving environments has detrimental effects [134–136]. High antibiotic pollution from manufacturers is high-risk [132] and recipient environments can become reservoirs for ARGs [131], it can also change microbial community structure in a way that reflects the changes necessitated by strongly elevated antibiotic concentrations, such as elevated HGT and modified microbial interactions [137,131](Huerta et al., 2013; Bengtsson-Palme et al., 2014). A large number of novel MGEs and ARGs (most of which were associated with MGEs) have first been identified in highly polluted waters [138–142]. The question that arises is if there are local differences in risk potential due to high antibiotic concentrations globally. And indeed, unusually high antibiotic concentrations in surface waters were found in Asia, up to 484 µg/L over 20km downstream from the nearest WWTP while Europe and the Americas usually had one-digit or low two-digit concentrations and Africa showed antibiotic concentrations in the mid-two-digit range [104]. Another crucial question that remains unanswered is, how very high antibiotic and ARG concentrations might affect the health and gut microbiome of people living in the area [131].

For both, wastewater from communes and from pharmaceutical producers, it can thus be said that adequate (preferably de-centralized local to account for CSO) wastewater treatment is of utmost importance and that inadequate treatment reflects in poor regional water quality and increases in AR [143]. (Un-)Treated wastewater effluents are the biggest contributor of antibiotics and other pollutants in the aquatic environment [73,144]. More than the main source for anthropogenic pollution, wastewater treatment plants can also act as a "hot spot" for horizontal gene transfer [145–148] and they can break antibiotics into uncountable degradation products with unknown and potentially increased potency [149–151]. A vicious circle of antibiotic resistance transfer and propagation is thus created, its risk potential can be demonstrated by environmentally derived antibiotic resistance gene classes such as qnr, conferring resistance to fluorquinolones [152]. This cycle needs to be broken and risk associated with wastewater-derived pollution reduced to be able to control and decrease the clinical and economic burden of antibiotic resistance [29].

THE EFFECTS OF WASTEWATER TREATMENT ON ANTIBIOTICS AND RESISTANCE GENES

Secondary wastewater treatment

While traditional wastewater treatment using sludge digestion can significantly reduce the total concentration of ARGs in sewage to a certain extent [153], it has been proven to be subpar for sufficiently removing ARGs from wastewaters with relative abundances often increased in the WWTP effluent [154,104,155,113]. Moreover, it could be shown that ARGs can be enriched during certain phases of WWT and that the usage of high levels of activated sludge favour ARGs and ARB proliferation [156,157]. Further, biotransformation rates and mechanisms varied for different antibiotics and across different WWTPs resulting in unpredictable antibiotic potential after treatment [158]. Overall, it can be said, that there is ambiguity about the effect of regular WWT [159]. Tertiary or advanced wastewater treatment methods have therefore extensively been studied in recent years.

Advanced Wastewater Treatment

Chlorination, granulated activated carbon (GAC), membrane bioreactors (MBR), Ozonation and treatment with ultraviolet light (UV) have been utilized to treat wastewaters. Ambiguous effectiveness was reported for different methods in different studies. GAC has been shown to increase relative ARG abundance [160,161]. Chlorination has been reported to promote ARG increase [154,160] or to have no effectiveness regarding AR [162]. Similarly, UV treatment did not adequately/ sufficiently degrade ARGs in one study [154], but could be shown to be efficient depending on the parameters used in another study [163]. However, a combination of UV treatment with different catalysts (including chlorine) was able to achieve improved antibiotic and ARG removal results and worked best in alkaline environments [163,164] and increased doses of UV and chlorine enhanced efficiency [165]. Ozonation was shown to be less effective in reducing antibiotic resistance genes than UV and chlorine [166] and MBR showed good removal of antibiotic resistance genes [159]. Ozone and UV-treatment could be shown to release ARGs from cells due to apoptosis [165]. Treatment types which were used with lower frequency were pre-flocculation/sediment/sand filtration which reduced ARGs by reducing total the biomass [160] and constructed wetlands which were shown to remove antibiotics and ARGs "efficiently" [167].

Comparability between different studies and treatment methods was partly hindered by widely varying experimental conditions and, more importantly, by the vaguely or undefined state of the term "removal efficiency". Some studies used the term to indicate that antibiotic resistant genes and bacteria were significantly decreased in number, while others used it to express if antibiotic resistance was still detectable after treatment. The optimal method for ARG and ARB removal does therefore see to strongly depend on the type of wastewater and the conditions used during treatment. More studies that are comparable are most definitely needed.

Wastewater Treatment Plant Selectors of ARGs?

Wastewater treatment plants have been described as the optimal place for ARG dissemination between microorganism, a paradise for resistant bugs, so to speak [168]. WWTPs have high number of potential co-selectors and often sub-inhibitory antibiotic concentrations [169]. While it is not sure if concentrations found in WWTPs are always sufficient to serve as selectors [170], it has been shown that sub-clinical antibiotic concentration can promote antibiotic resistance [171]. Further, non-antibiotic pollutants with the potential to co-select for ARGs (such as heavy metals) are widely present in wastewater and WWTPs. High amounts of ARG are likely to persist in the environment, which may have consequences [120,122–124]. To add insult to injury, microplastics, which can serve as vehicle for antibiotics and ARGs in the environment, are often present in wastewaters [172–174]. MGEs are major drivers for shifting ARG patterns [175], they are abundant in wastewater and treatment plants and not all MGEs are easily removed by treatment [160]. Co-selection can occur due to location on same MGE [176,131], as frequently happens when heavy metals are involved [122,123,120,124]. Chlorination has been shown to increase AR during waste water treatment [177] likely due to stress-mediated molecular mechanisms [178]. Both UV and chlorine have been shown to incompletely degrade ARGs after relatively fast inactivation of bacterial cells, thus creating additional potential for HGT [179].

While WWTPs do remove ARBs and ARGs to a certain extent, they have been shown to do so insufficiently and in addition release tremendous volumes of effluent into the environment thus potentially converting the environment to another AR hotspot [180]. Activated sludge in traditional WWTPs benefits antibiotic resistance selection and propagation and could thus be utilized as a model for the "worst case" scenario possible in the environment [132]. In general it can be said that processes of AR selection and propagation in activated sludge need to be further studied [132].

PERSISTENCE AND DISSEMINATION OF ANTHROPOGENICALLY INTRODUCED ARGS IN THE ENVIRONMENT

Elevated levels of ARG in the environment can largely be explained by fecal pollution. One exception to this rule are highly polluted environments impacted for example by the pharmaceutical industry [180]. This source tracking of ARGs in aqueous environments is crucial in order to classify and mitigate risk. qPCR methods are often not sensitive enough to detect fecal marker bacteria [181]. Using specific bateriophages for source tracking have therefore recently been suggested [181,182,180,183]. The most promising is crAssphage a bacteriophage first identified from human fecal substance, not physically linked to antibiotic resistance genes, so that the risk for correlation from co-selection is small [184]. crAssphage has been successfully used for source tracking [181,185], thus facilitating an important step in investigations regarding the origin, dissemination and fate of anthropogenic ARGs in the environment.

Equally important are investigations into the fate of antibiotic and ARGs after their introduction into the environment. Even in water environments, there are vast differences in the dynamics of pollutants depending on the type of water body, indicating that the potential risk from AR differ across different water bodies. Pollutant circulation in lakes is slower than for example in rivers thus providing conditions for cumulative pollutant build-up [186] which can in turn lead to ARG accumulation, especially in combination with increased input from WWTPs [106]. One study did not find tempo-spatial differences of AR in a Chinese lake [187]. Rivers transport pollutants away from the source of pollution but also disseminate pollution more widely [188–191], however pollution levels decreased with distance from the pollution input location [191,192]. Elevated ARG levels caused by WWTP effluent discharge do not stay elevated and increased antibiotic levels do not seem to have a an impact on selection despite sorption to sediments (in bioreactor experiments modelling natural environments) [193]. Maintaining elevated ARG levels after WWTPs in rivers requires continuous pollutant input, but might persist in biofilms [107], as other studies identified aquatic environments as reservoir for AR [159]. Presence and concentration of different ARGs is also distinct in water and sediments [194].

RISK FROM ANTIBIOTIC RESISTANCE IN AQUEOUS ENVIRONMENTS

The dissemination, propagation and potential persistence of antibiotic resistance genes in aqueous environments raises a number of questions, the most important of which is: So what? – Or, to phrase it more eloquently: "What is the risk to the environment and human health from ARGs in water environments?".

ARGs can transfer between environmental bacteria and human pathogens [115,195] and water environments create a number of different pathways for contact between the two. Surface water bodies (even those with increased levels of AR) are often used as source for drinking water [108,109,111,160,187,196]. Antibiotic resistance can enter the food (supply) chain via water as it is a connection between the environment, animals and humans (e.g. in aquaculture, vegetable farming etc.) [197,198,194].

Rainfall events can lead to strongly (up to 100-fold) elevated ARG levels, which can persist up to 2 weeks. These astounding increases and associated presence of potential pathogens can stem (at least partly) from sewer overflow [199]. ARGs can then transfer from environmental bacteria over intermediaries to human pathogens, which can persist in recreational water body habitats [200]. A particularly high risk can be identified at locations where humans or animals interact with polluted water [104].

Ingestion of water from recreational water bodies represents a direct, quantifiable risk as it leads to direct exposure with predictions of up to 345.09 cfu of antibiotic resistant E. *coli* ingested 100 ml swallowed water [201,202], certain water sports, such as wakeboarding may therefore be particularly risky due to the characteristic high quantities of water ingested. More research is needed to investigate concrete health implication and for complete risk assessment and quantitative microbial risk assessment (QMRA) has been suggested as "a suitable method to evaluate and quantify this health risk." [203]. Nevertheless, uncertainty about HOW exactly to quantify the risk from ARG and ARB remains, as the risk of both antibiotic concentrations and ARGs is strongly context dependent [146,159]. In regards to antibiotics, more information on minimum inhibitory concentrations, predicted effect concentrations and the effect of sub-inhibitory concentrations under realistic conditions is required [104,204] as regular lab conditions are not necessarily meaningful to build models regarding the environmental setting [104].

Better monitoring of recreational water bodies (regarding AMR and pathogens) [201,205] and location choice [206] have been called for and are imperative.

TECHNOLOGIES USED IN ENVIRONMENTAL AR RESEARCH

Culture-based Methods

Another concern is the difficulty to study and evaluate resistance in environmental bacteria, as approximately 99% of environmental strains cannot be cultured [207]. However, phenotypic expression of genetic sequences and important parameters for risk assessment, such as the minimum inhibitory concentration (MIC), can be investigated. Unfortunately, the small portion of environmental bacteria that can be cultured are non-representative of the phylogenetic diversity for the entirety of indigenous bacterial communities [103,106]. These complications can be partly remedied by employing culture-independent metagenomic methods for investigations of these uncultured microorganisms [80,96,208–210]. Nevertheless, these methods come with their own limitations.

qPCR

Quantitative real-time polymerase chain reaction (qPCR)-based methods introduce a bias by utilizing gene-specific probes and primers and thus selecting for a subset of genes to investigate, they are further prone to potential PCR inhibitors [211]. Regular qPCR is further not a high-throughput method, strongly limiting the potential of information that can be gained from samples in a timely manner. Higher-throughput qPCR-based methods, such as microarrays exhibit batch-to-batch variability and are considered less sensitive and specific [212]. HT-qPCR suffers from instrumental sensitivity and analytical differences which can significantly impact results and individual assays tested cannot effectively be optimized during the experimental run [212]. All qPCR-based technologies require the creation of sequence-specific primer thus limiting exploration and analysis to already known genetic sequences; they further have the attribute that they are independent of gene expression, which can be seen as an advantage or a disadvantage [213] which could lead to an overestimation of the real risk from present ARGs [214].

Next-generation Sequencing

Next-generation shotgun sequencing is not fully quantitative and is strongly impacted by sequencing depths, database integrity and varying bioinformatic pipelines [215–217,181]. A multitude of analysis methods [218] and potential parameters for those methods [181] exist and not all suitable for ARG research or for assessment of human risk from ARGs, [181]. Single-cell sequencing allows for capturing the host of genes of interest, but is not sufficiently high-throughput [219,220]. However, NGS is high-throughput (at rapidly declining cost) and is able to capture, detect and identify novel hitherto unknown genes [181]. Further, both qPCR and NGS allow for retrospective data analysis [181], which can be deciding for some research questions as well as for collaboration and comparability as results can be used and analyzed by colleagues and other researchers if made available.

To adequately investigate antibiotic resistance in co-cultivable environmental microorganisms, a combination of different culture-independent methods would therefore be most appropriate. Similar to qPCR, NGS is independent of gene expression and gives no information about important parameters for risk assessment.

1.2.5 Research methods

Due to the limitations imposed by working with mainly uncultivable microorganisms [7,221], the main research methods used in this thesis are qPCR, next-generation sequencing and metagenomic data analysis as well as the application of microcosms experiments.

1.3. The Problem at Hand

Reigning antibiotic resistance in, will require a joint global effort. Such an effort, in turn, requires clear policies, regulation and guidelines. One of the main issues is that due to critical gaps in knowledge, such policies are often only available regionally, insufficient or even non-existent in regards to many issues [85]. Researchers need to make a collective effort to provide the necessary information in order for policy makers to start taking the right steps into a future in which antibiotic resistance does not have the potential to threaten the economy, the stability of the medical establishment and human lives. This thesis is aimed at advancing this cause.

1.3.1 Knowledge Gaps

A number of topics in the field that have not yet adequately addressed by scholars stand out and will be addressed in this work:

The impact of anthropogenic sources on aquatic environments, especially the role of high-risk point sources and associated risk are not well addressed. Further, the transfer of antibiotic resistance genes in the environment is not sufficiently investigated, as only a small number of studies exist which trace the origin of antibiotic resistance in and to environmental bacteria and their transfer to clinically relevant bacterial strains [42,62,222,223]. Persistence of antibiotic resistance genes that were introduced to natural environments due to anthropogenic pollution is also not sufficiently studied [224–226].

Insufficient information also exists on the impact of physico-chemical stressors, such as UV radiation, chlorination, ozonation [227–229] but also sub-inhibitory antibiotic concentrations and their degradation products [228,229]. Information on the consequences of different concentrations/doses of such stressors under different conditions (e.g. laboratory versus natural environmental conditions) is even scarcer. Additionally, the benefits of employing more aggressive wastewater treatment technologies on antibiotic resistance and antibiotic concentrations within anthropogenic (waste-) water systems and the subsequent environment have not been comprehensively studied.

Finally yet importantly, potential variation and biases introduced by next-generation data analysis have not been studied in regards to antibiotic resistance gene research. While an increasing number of environmental studies employ NGS and it is known that sequencing depth and bioinformatic analysis pipelines strongly impact NGS results [216], studies comparing NGS results to qPCR results (which is currently seen as the gold standard in ARG detection and quantification[230]) are missing [231,232]. However, such studies are crucial to create data that is comparable and subsequently a reliable knowledge base.

1.3.2 Research Question

Main Objective

This thesis will generate information that helps to decrease the knowledge gaps introduced in *1.3.1* .

The main objective is to generate knowledge about antibiotic resistance and its propagation, spread and impact in the aquatic environment. Further, conditions and measures that can create or mitigate increased risk will be elucidated.

Four main objectives can be expressed as follows:

1. Investigate the impact of anthropogenic pollution on environmental antibiotic resistance levels and the degree of persistence
2. Explore the impact of high-risk point sources and the benefits of on-site treatment of high-risk wastewater
3. Study horizontal gene transfer of antibiotic resistance under different conditions and evaluate the role of sub-inhibitory antibiotic concentrations and different matrices
4. Evaluate the impact of different analysis methods on antibiotic resistance gene detection and quantification and their comparability

Specific Objectives and Research Questions

To address the main objectives, research questions are introduced that answer the following queries:

- What is the impact of high-risk point sources? Does on-site wastewater treatment of high-risk point sources provide benefits? Are secondary urban wastewater treatment plants efficient enough to significantly decrease antibiotics and antibiotic resistance gene concentrations? How much more efficient is tertiary wastewater treatment?

  - Chapter 2 answers these questions by sampling hospital wastewaters at two locations in the Netherlands, the receiving urban wastewater system and the urban wastewater treatment plant effluent released into the environment. Only one of the two locations is treated on-site using advanced tertiary wastewater treatment

- Does large-scale anthropogenic pollution have a significant and lasting impact on large water bodies? Do antibiotic resistance gene concentrations increase with steadily increasing anthropogenic pollution?

  - Chapter 3 answers these questions by monitoring the Rhine river in a tempo-spatial manner. Additionally, relationships between antibiotic resistance gene concentrations and different proxies for ARG pollution of anthropogenic origin were compared.

- How do different antibiotic concentrations impact horizontal gene transfer of antibiotic resistance between a donor and a potentially pathogenic recipient under laboratory conditions? How do these concentrations impact bacterial communities under environmental conditions? Do sub-inhibitory antibiotic concentrations play a special role?

  - Chapter 4 answers these questions by transfer experiments between $E.coli_{DH5\alpha}$ carrying a multi-resistance plasmid and a recipient organism or a recipient bacterial wastewater effluent community. Transfer experiments were organized either on filters or as microcosm experiments to investigate the impact of different matrices on the outcome

- Are NGS results (and more specifically whole-genome sequencing results) comparable to qPCR results? What is the impact of different analysis methods and parameters on NGS results? Which bioinformatic pipelines generate results most similar to qPCR?

  - Chapter 5 answers these questions by analysis of 11 DNA extracts by qPCR and NGS and subsequent detection and (semi-)quantification of antibiotic resistance genes. NGS data is subsequently analyzed by a machine learning algorithm, a traditional bioinformatic pipeline or in-silico qPCR scripts. Different parameters are evaluated, including: use of databases, the impact of assembly and levels of abstraction.

2. The Impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes

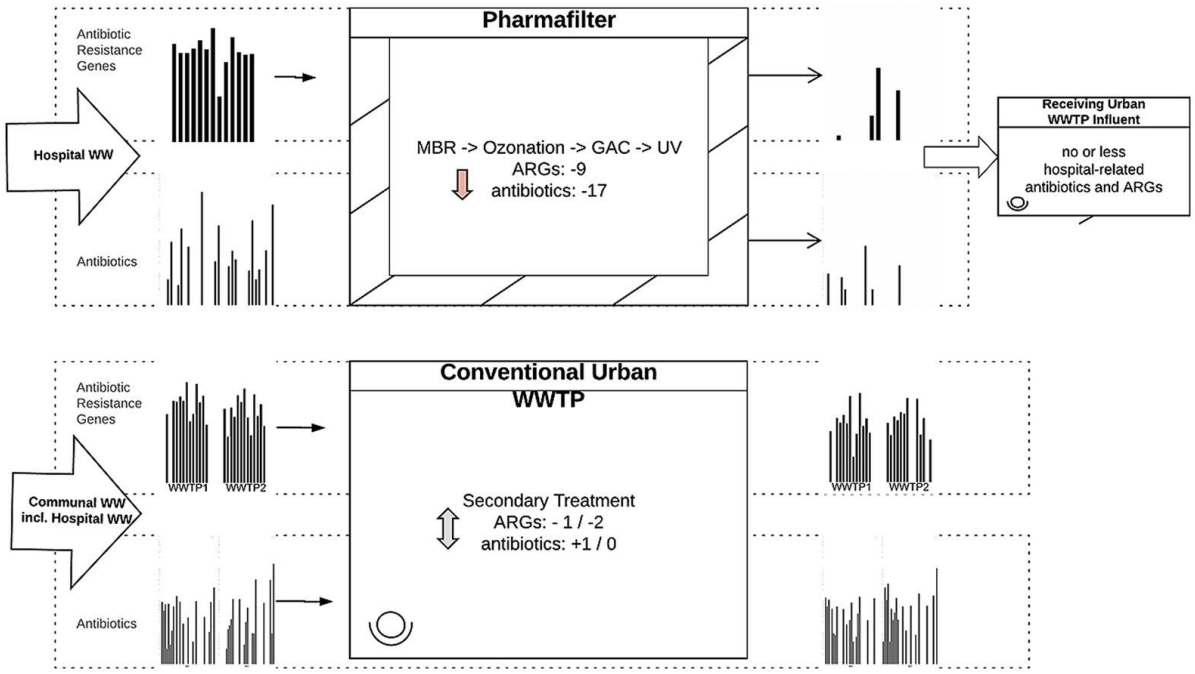

Figure 2-1: Graphical Abstract; Chapter 2

Keywords: Advanced Wastewater Treatment, Contaminants of Emerging Concern, Pharmafilter, Antibiotic Resistance

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2.1. Abstract

This study quantified antibiotic and antibiotic resistance gene (ARG) concentrations in hospital and communal wastewaters as well as the influents and effluents of the receiving urban wastewater treatment plants (UWWTP) in two Dutch cities. In only one city, hospital wastewater was treated on-site using advanced technologies, including membrane bioreactor treatment (MBR), ozonation, granulated activated carbon (GAC) and UV-treatment.

On-site hospital wastewater (HWW) treatment reduced gene presence of hospital-related antibiotic resistance genes and antibiotic concentrations in the receiving urban wastewater treatment plant. These findings support the need for on-site treatment of high-risk point sources of antibiotic resistance genes.

13 antibiotic resistance genes, Integrase Class 1 and 16S rRNA concentrations were quantified using multiplex quantitative real-time PCR (qPCR) assays and the presence and/or concentration of 711 antibiotics were analyzed.

Hospital wastewater contained approximately 25% more antibiotics and gene concentrations between 0.4 log to 1.8-fold higher than communal wastewater (CWW). *bla*$_{KPC}$ and *van*A could be identified as hospital-related genes and were reduced to under the limit of detection (LOD) during on-site treatment. Advanced on-site treatment removed between 0.5 and 3.6-fold more genes than conventional biological urban wastewater treatment (activated sludge). Advanced on-site treatment was able to eliminate 12 out of 19 detected antibiotics, while urban wastewater treatment eliminated up to 1 (out of 21 detected). Different advanced treatment technologies were able to target different pollutants to varying extents, making sequential alignment more effective. MBR treatment was most efficient in antibiotic resistance gene reduction and ozonation in antibiotic reduction.

bla$_{KPC}$ could only be detected in the influent of the urban wastewater treatment plant receiving untreated hospital wastewater. Similarly, *van*A was only consistently detected in this treatment plant. These results indicate a positive effect of on-site treatment of hospital wastewater on the communal sewage system.

2.2. Introduction

Antibiotic Resistance (AR) is a growing global threat which will require worldwide joint efforts to be conquered [39,233]. Hospitals have been in the focus of AR research as one of the high-risk point sources of antibiotics [234–236] and ARGs [146,236–240]. Although, the release of untreated HWW might be posing a hazard to the environment and human health, there are still few studies investigating the release and direct impact of HWW into the environment or communal sewage system [241,242]. Due to this gap in information, regulations for the treatment of HWW are absent in most countries [243–245].

The release of untreated HWW could increase ARG prevalence in environmental water bodies. Antibiotic resistant bacteria were shown to survive in the HWW, in the UWWTPs and, subsequently, the UWWTP effluent [246]. The risk potential of HWW is further increased by the fact that hospitals use last-resort antibiotics (e.g. piperacillin and vancomycin) more frequently and thus their ARG profiles might be different when compared to other wastewaters [247]. Overall, conventional wastewater treatment renders limited results in terms of antibiotic and ARG removal and might even increase the concentration of certain ARGs [146,248–251].

The present study investigates the impact and efficiency of antibiotic, ARG and bacterial removal of advanced on-site treatment compared to urban wastewater treatment. The effect of different advanced treatment steps and their impact on the downstream urban wastewater system are studied. ARG occurrence and concentrations in HWWs and CWWsin the Netherlands are compared to identify potential differences. To this end genes conferring resistance to aminoglycosides (*aph*(III)a), β-Lactam Antibiotics (bla_{KPC}, bla_{SHV}, bla_{OXA}, *mec*A), macrolides (*erm*B, *erm*F), quinolones (*qnr*S), sulfonamides (*sul*1), tetracyclines (*tet*B, *tet*M) and vancomycines (*van*A, *van*B) as well as a class 1 Integrase (*IntI1*) were screened for and quantified. A total of 711 antibiotics were investigated, out of which 41 were quantified and 670 were screened for presence in the samples. Further, correlations between antibiotic and ARG concentrations were studied.

2.3. Methods and Materials

2.3.1 Sampling

Samples were taken from two cities in the Netherlands, namely from Delft (location 1) and Nieuwegein (location 2). At location 1 HWW was treated on-site. The following samples were taken from each location: hospital wastewater, communal sewage (at a location not impacted by HWW), on-site hospital wastewater treatment plant (Pharmafilter, location 1only) and samples from the receiving UWWTPs. Two sampling rounds were conducted at all sampling locations with at least 6 months in between sampling rounds. The first sampling round took place in spring and the second in winter.

HOSPITALS

Samples were taken from combined HWW at location 1 (H1) and location 2 (H2). All samples were composite sample (12h-composites – 1st sampling round; 8h-composites – 2nd sampling round).

Both hospitals contained wards that typically have high antibiotic use.

COMMUNAL WW

CWW samples were taken from the urban sewage system, which was accessed by street manholes. Samples were taken at a location at which the sewage system was not impacted by HWW. Samples were combined grab samples consisting of at least 3 subsamples taken approx. 3 h apart, which were pooled together before analysis.

URBAN WWTPS

Samples were taken from two UWWTPs: 1) W1 (location 1) and 2) W2 (location 2).

W1 (built in 2006): The treatment plant processes a quantity of water, which compares to a population equivalent of 1.260.000 (PE) and has an average in- and outflow of 180.000 m^3/d. W1 receives CWW including wastewater from H1. Wastewater treatment consists of primary and secondary treatment, including: influent screening (6 mm bars), primary sedimentation, biological (activated sludge) treatment, final clarification and biological phosphorus removal.

W2 (built in 1975 and renovated in 2010): The treatment plant has a volume capacity, which compares to 144.000 PE and an average in- and outflow of 25.700 m^3/d. W2 receives CWW including wastewater from H2. Wastewater treatment consists of primary and secondary, including influent screening, primary sedimentation, biological (activated sludge) treatment and biological Nitrogen and phosphorus removal.

24h-composite samples (taken by automatized composite samplers) were obtained from each UWWTP (influent and effluent wastewater).

PHARMAFILTER

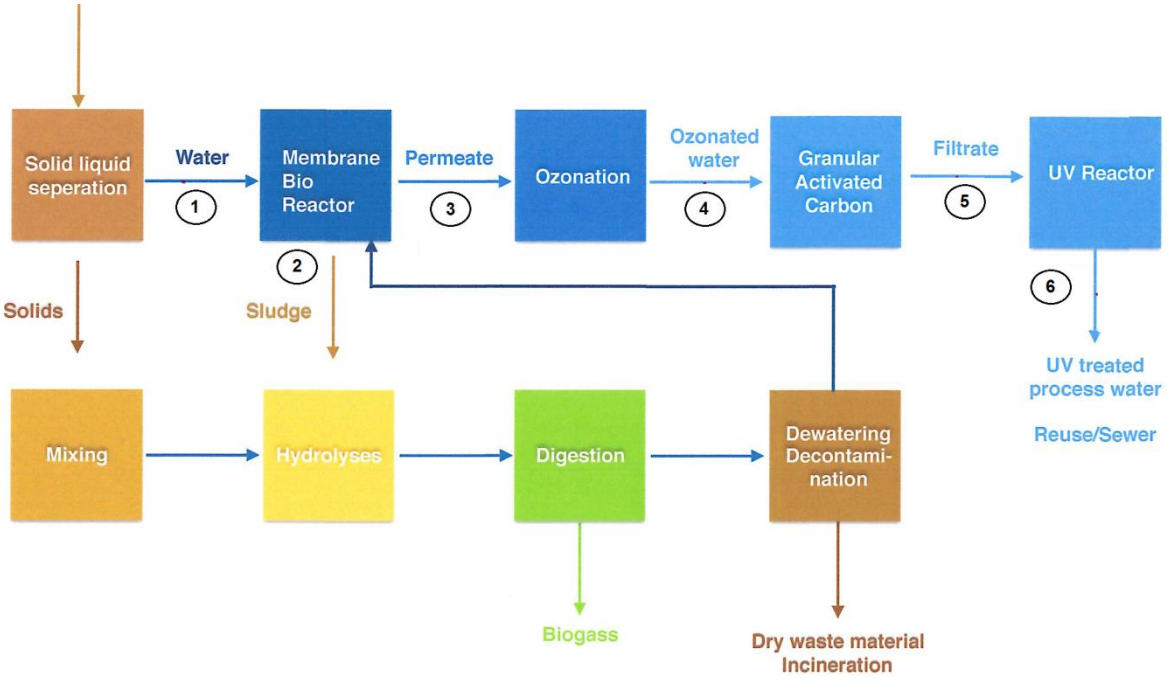
HWW at location 1 was treated on-site by an installation called the Pharmafilter. Pharmafilter treatment consists of four sequentially aligned treatment steps (**Figure 2-2**):

Membrane Bioreactor (Microfiltration) (**MBR**)

Ozonation (**Ozon.**)

Granulated Activated Carbon (**GAC**)

UV Treatment (**UV**)



1 Figure 2-2: Pharmafilter Installation and Process Steps*; samples taken (1)-(6): (1) Untreated HWW, (2) Sludge, (3) MBR, (4) Ozonation, (5) GAC, (6) UV Treatment/ Effluent*

24h-composite samples were taken after each treatment step, as well as from the MBR-Sludge (**Figure 2-2**).

2.3.2 Sample Preparation

Samples were processed immediately after arrival to the laboratory.

2.3.3 Biological Analysis

Samples were filtered using 0.22-µm-pore-size polycarbonate track-etch filter membranes (Sartorius). DNA was extracted from the filters using DNeasy PowerSoil Kit (QIAGEN Benelux B.V). Extraction was performed according to manufacturer instructions, with one exception:

An internal control (IC) plasmid was added to the samples (concentration: $2.5x10^4$ gene copies/µL) to quantify the DNA loss caused by the extraction process [252].

Extraction blanks yielded negative results. DNA loss was corrected for, based on IC concentrations measured by qPCR.

2.3.4 Chemical Analysis

The sample preparation protocol involved clean-up and 4000x pre-concentration on an Atlantic HLB-M Disk, using a HORIZON SPE-DEX 4790 (USA) with 47 mm disk holder. Conditioning and extraction programs used for the preparation of the wastewater samples

can be found in the *SI Table 2-11*. The extract was evaporated using a gentle stream of nitrogen and was reconstituted with 250 µl of 50:50 methanol:water mixture for instrumental analysis. Before analysis, extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 µm pore size (Phenomenex, USA). *See SI (SI 2-10*) for information on chemicals and reagents.

2.3.5 ARG Detection and Quantification – Biological Analysis

Multiplex qPCR Assays

DNA extracts were stored at -20 °C prior to qPCR analysis. All qPCR assays were performed at least twice using technical duplicates each time. 16S rRNA was quantified using a SYBR Green qPCR assay. The following genes were quantified by qPCR: *aph*(III)a, bla_{KPC}, bla_{OXA}, bla_{SHV}, *erm*B, *erm*F, *intI1*, *mec*A, *qnr*S, *sul*1, *tet*B, *tet*M, *van*A and *van*B. Multiplex qPCR assays were performed under the conditions described in *SI 2-1, SI 2.-2 and SI 2-3*. Standards, a positive and a negative control were included in every assay to confirm multiplex qPCR quality. Standards were made up of 5 subsequent dilutions with concentrations ranging from $2.5x10^4$ to $2.5x10^0$ gene copies/µL. Multiplex qPCR assays were performed using the iQ™ Multiplex Powermix (Bio Rad, München, DE) and qPCR reactions were performed using a CFX96™ Real-Time PCR Detection System (Bio Rad, München, DE). CFX96™ Real-Time PCR Detection System data was interpreted by CFX Manager v.3.1.1517.0823.

Data Analysis

Python 3.6.0 [253,254] executed in Jupyter Notebooks was used to clean and analyze raw data, to calculate descriptive statistics and correlations and to create data visualizations. R version 3.5.0 was used to perform inferential statistical analysis. Significant differences between experiments and/or measurements were detected by employing paired or unpaired Student's t-Tests, or Welch's t-Tests for the case that the sample variances were not comparable and data transformation not possible. Two samples/measurements were defined to be significantly different from each other for $p<0.05$. Correlations between antibiotic and ARG concentrations were calculated using Pearson's rank correlation coefficient. An ARG and antibiotic were considered correlated for $R^2>0.5$, $p<0.05$ and if there were ≥4 common data points available. Relatedness with values of $0.5<R^2<0.7$ was considered a 'moderate correlation', while $R^2>0.7$ was considered a 'strong correlation'.

2.3.6 Antibiotic Detection and Quantification – Chemical Analysis

Instrumental Analysis

Instrumental analysis was performed with a Thermo UHPLC Accela system connected to a TSQ Quantum Access triple quadrupole mass spectrometer from Thermo Electron Corporation (San Jose, CA, USA) equipped with an electrospray ionization source (Thermo IonMAX) in positive mode. Chromatographic separation was achieved on an Atlantis T3 C18 (100 mm x 2.1 mm, 3 µm) column from Waters Corporation (Milford, MS, USA) at a constant flow rate of 100 uL/min. The mobile phase, the gradient elution programs and the

ESI parameters are presented *(SI Table 2-12)*. Identification and quantification were performed under selected reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte, thus achieving 4 identification points per compound (2002/657/EC). SRM transitions for each compound were optimized by infusion of standard solutions at mean concentration 1 mg/L. The optimized ionization mode, fragmentation voltages, collision energies for each antibiotic (41 in total) are summarized in *(SI 2-13)*. To assure that as many antibiotics as possible were captured, extracts were also injected in a UHPLC-QTOF-MS system, equipped with a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 x 100 mm, 2.2 µm) from Thermo Fisher Scientific (Dreieich, Germany) preceded by a guard column of the same packaging material, kept at 30°C. Gradient program, ESI parameters and mobile phases are summarized in *(SI 2-14)*. See *SI 2-17* for detailed data analysis.

2.4. Results and Discussion

2.4.1 Antibiotics and ARGs in the Urban WW Cycle

Hospital Wastewater Had Higher Prevalence and Concentrations of Antibiotics and ARGs than Communal Wastewater

HWW samples showed 0.4 to 1.8-fold higher relative ARG concentrations than CWW samples (**Figure 2-3**). No ARGs were observed in significantly higher concentration in CWW samples. Similarly, absolute ARG concentrations (meaning: ARG concentrations per mL sample) which significantly differed from each other between HWW and CWW samples showed between 0.8 and 2.3-fold increase in HWW samples (*SI Figure 2-9*). The higher ARG pollution of HWWs suggests higher incidences of AR and can potentially suggest multi-drug-resistant bacteria, as has been found previously in several studies [255–257] or a larger proportion of resistant organisms compared to CWW.

*bla*KPC and *van*A were not found in any of the analyzed CWW samples, suggesting that these genes are hospital-related ARGs and that occurrences at other location of the urban wastewater cycle originate from health care facilities. *Van*A has previously been suggested as an indicator gene to monitor AR of anthropogenic origins in the environment [258]. *Van*A and bla_{KPC} have repeatedly been detected in HWW [259–267]. Occurrences of bla_{KPC} in the environment were only recently and rarely shown [268]. Some of these occurrences could be traced back to hospital-associated bacterial strains [269]. The assumption of association of HWW with bla_{KPC} and *van*A is strengthened by previous findings that these genes are more prevalent in hospitals which use more carbapenems [270] or vancomycin [263]. *Van*A, is found downstream of hospital sewage release with higher prevalence [265]. The potential risks of these specific genes would be exacerbated by the possibility to be transferred horizontally between strains. At least in case of bla_{KPC}, transconjugants were detected after horizontal gene transfer (HGT) [262], suggesting a heightened transfer risk potential of this gene.

Ciprofloxacin (2706 ng/L – H1, 3752 ng/L - H2) and sulfamethoxazole (367 ng/L - H1, 269 ng/L - H2) were detected at concentration levels of up to several orders of magnitude higher

than in CWW samples. Metronidazole with a frequency of detection of 92% across all samples, reached concentrations as high as 4 ng/L (H1) and 7500 ng/L (H2) (*SI Table 2-15*). While antibiotic concentrations in HWWs can vary widely [236], concentrations within the same dimensions have been previously recorded, with ciprofloxacin, sulfamethoxazole and metrondazole frequently being detected [236,271–275]

Some antibiotics could only be detected in HWW (Fluconazole, Sulfaclozine, Trimethoprim) or were detected in HWW with disproportionally higher concentration than in CWW (Sulfamethoxazole, Ciprofloxacin; both detected at concentrations over 2-fold higher in HWWs). Ampicillin and Amoxicillin, on the other hand, were only detected in CWW. These findings are consistent with previous reports, that β-lactam antibiotics are largely used inside and outside hospitals with Amoxicillin being one of the most frequently used antibiotics for outpatient prescription [276–278]. Quinolones and sulfonamides are more frequently used in hospitals than for outbound patients in the Netherlands [278]

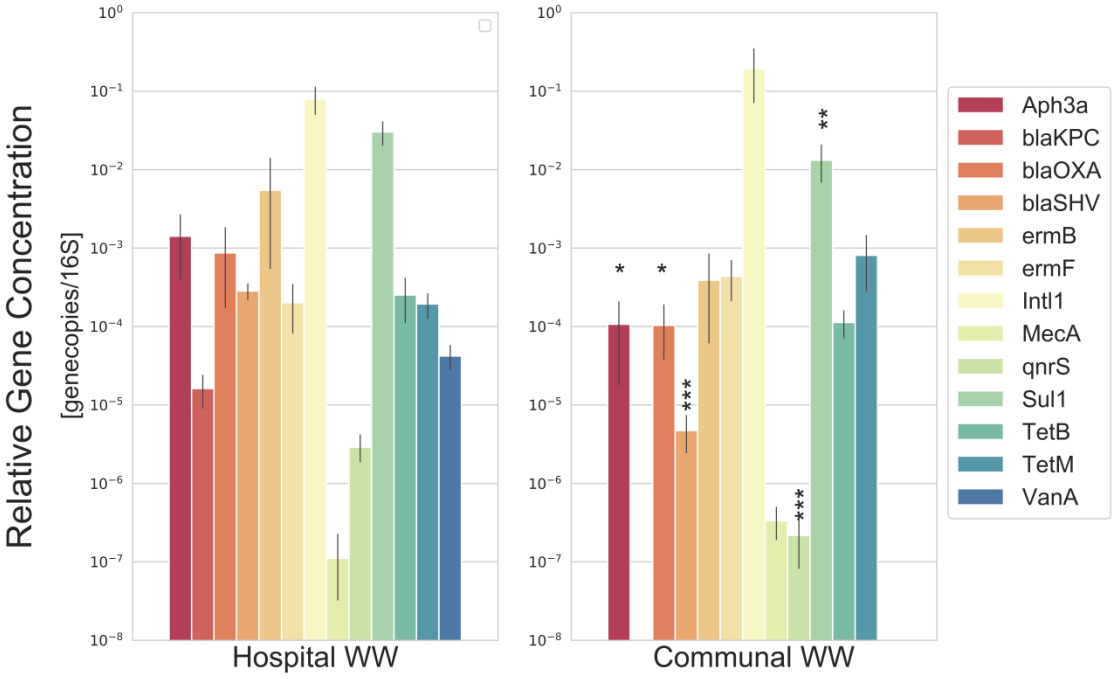


Figure 2-3: Relative Gene Concentrations in Hospital and Communal Wastewater Samples; values mean ± std of all four hospital or communal WW samples combined; * = ARG concentrations significantly (p≤0.05) lower in CWW, ** = ARG concentrations significantly (p≤0.01) lower in CWW, *** = ARG concentrations significantly (p≤0.001) lower in CWW

2.4.2 Reduction of Antibiotics and ARGs During Communal and On-Site Treatment

On-Site Treatment Eliminated Antibiotics and ARGs Efficiently

Bla$_{KPC}$, *bla*$_{SHV}$, *mec*A, *qnr*S, *tet*B, *tet*M and *van*A were reduced <LOD from HWW during MBR treatment (**Figure 2-4**). The following genes could not be detected in the MBR permeate but were detected in the MBR sludge: b*la*SHV, *tet*B, *tet*M and *van*A (*SI 2-5*). No genes were consistently eliminated during the ozonation treatment step. GAC treatment showed some variation between the two sampling rounds, with some genes being significantly reduced or

increased. *IntI1* and *sul*1 were consistently detected in the highest and second-highest concentration, respectively.

Overall changes in gene concentrations showed high consistency between the two sampling rounds. All detected genes were significantly reduced in absolute concentration and most also in relative concentration during the Pharmafilter treatment (**Table 2-1**). 9 out of 13 initially detected ARGs in HWW were reduced <LOD during Pharmafilter treatment, including *bla*$_{KPC}$, *bla*$_{OXA}$, *bla*$_{SHV}$, *erm*B, *mec*A, *qnr*S, *tet*B, *tet*M and *van*A. *Aph*(III)a, *erm*F, *intI1* and *sul*1 stayed detectable but were significantly reduced.

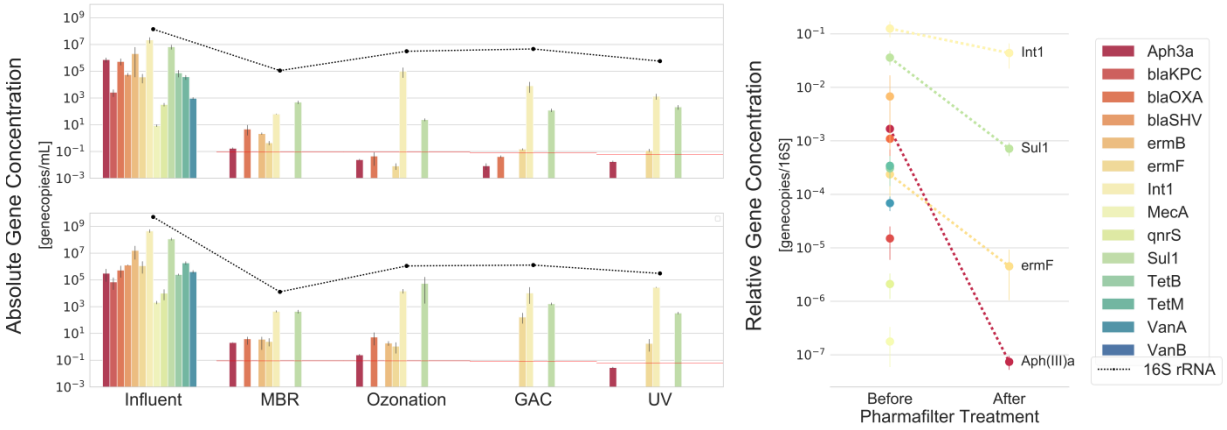


Figure 2-4: Gene Reduction During Pharmafilter Treatment; left: impact of different treatment steps (1st (top) and 2nd (bottom) sample rounds) , dotted black line – 16S rRNA concentration; right : overall impact of Pharmafilter on HWW; red line – LOQ

Notably, the bacterial load increased during ozonation treatment. This can be explained by hydraulic retention times up to 2 hours before ozonation, during which the microbial community has time to adjust to the new conditions and propagate.

Table 2-1: Mean and Standard Deviation for ARG Reduction and Increase (log10) in W1, W2, and Pharmafilter (combined data from two sampling rounds); [1] – only detected in first sampling round, [2] – only detected in second sampling round, [3] – reduced to < LOD, [4] –only detected during 2nd sampling round of W2 effluent, [5] – non-quantifiable reduction from <LOQ to <LOD, * - Pharmafilter reduction significantly higher than UWWTP reduction, bold: gene concentrations significantly increased

ARG	W1	W2	Pharmafilter
***aph*(III)a**	0.4±0.9	0.5±0.1	> 3.8 *, 3
***bla*$_{KPC}$**	n.s	**1.0±2.6**	> 1.7 *, 3
***bla*$_{OXA}$**	0.7±0.1	n.s	> 3.6 *, 3
***bla*$_{SHV}$**	1.0±0.1	**0.6±0.1**	> 3.1 *, 3

***erm*B**	0.8±0.1	n.s	> 4.4 ***, 3**
***erm*F**	1.1±0.3	n.s	1.7±0.8
intI1	n.s	n.s	0.5±0.9 *****
***mec*A**	> 1.7 **3**	> 2.0 **2, 3**	**5**
***qnr*S**	1.0 **1**	> 1.3 **3**	> 0.9 **3**
***sul*1**	n.s	**0.5±0.1**	1.7±0.4 *****
***tet*B**	1.3±0.1	0.9±0.1	> 3.1 ***, 3**
***tet*M**	1.2±2.2	0.5±0.2	> 3.1 **3**
***van*A**	n.s **1**	> 2.2 **3**	> 2.4 **3**
***van*B**	-	**4**	-

Pharmafilter treatment reduced ciprofloxacin from 2706 ng/L to 62 ng/L. Sulfamethoxazole was reduced from 367 ng/L to 0.9 ng/L (**Figure 2-6**). Ozonation was the crucial treatment step for the elimination. MBR treatment seems to release cleavage forms of certain types of antibiotics thus increasing concentrations of certain antibiotics such as metronidazole, which is increased from 4 ng/L to 1203 ng/L during this step. The same trend was observed for other compounds: sulfamethoxazole (concentration difference after MBR treatment: +96 %), ofloxacin (+110 %), fluconazole (+289 %) and erythromycin (17-fold difference). In some cases, concentrations of pharmaceutical residues appear to increase through MBR treatment, a documented phenomenon [279], and might be explained by the cleavage of conjugated residues. For example, sulfamethoxazole can be generated during treatment by cleavage of its human metabolite N4-acetylsulfamethoxazole in WWTPs [280]. Moreover, it is known that antibiotics are absorbed onto negatively charged surface of sewage sludge through ionic interactions. In case of malfunction of membranes or poor maintenance it is possible that desorption phenomena may happen [280].

URBAN WASTEWATER TREATMENT PLANTS SHOW LOW EFFICIENCY IN ANTIBIOTIC REDUCTION AND VARYING EFFICIENCY IN ARG REDUCTION

ARG concentrations did not uniformly show significant decrease during urban wastewater treatment (**Figure 2-5**). Significant gene reductions varied between 0.5±0.1 (*aph*(III)a) to > 2.2-fold (vanA) in UWWTPs (**Table 2-1**). ARG reduction efficiency varied between the two UWWTPs. Significant changes in relative gene concentration were uniformly reductions in W1, while three ARGs significantly increased in concentration in W2. Genes which did not significantly decrease in concentration were intI1 and bla_{SHV} (during both sampling rounds), bla_{KPC}, bla_{OXA} and *sul*1 (during the 2nd sampling round). On the other hand only one ARG was reduced <LOD in W1; *mec*A and *tet*M during the 1st and 2nd sampling round, respectively; while 3 ARGs were reduced <LOD in W2; *qnr*S and *van*A (in both sampling rounds) and *mec*A (1st round) or bla_{KPC} (2nd round).

In W1 50-67% of present ARGs could be significantly reduced, while only 23-36% of present ARGs were significantly reduced in W2. A large proportion of genes did not show a significant change in relative concentration after treatment at W2. *IntI1* is the only gene that does not show any significant changes in relative concentration in any of the different WWTPs and sampling rounds (**Figure 2-5**).

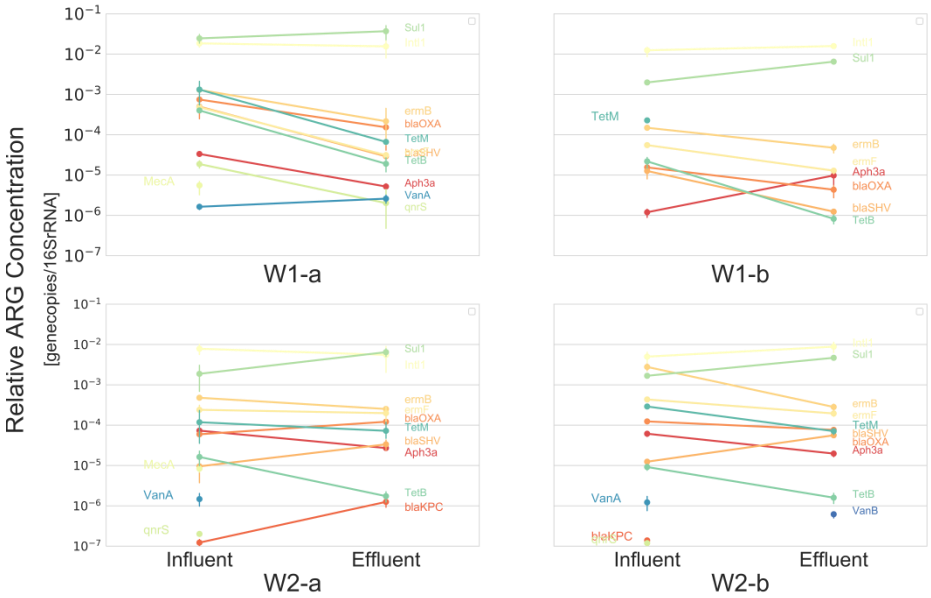


Figure 2-5: Change in Relative Gene Concentration During Communal Wastewater Treatment in W1 and W2 at Different Times, (a – 1st sampling round in spring, b – 2nd sampling round in winter)

Previous studies showed that secondary wastewater treatment decreased half of 78 detected ARGs by <94% in concentration [281], while tertiary treatment has been found to retain 2%-50% of ARG raw influent concentrations [282]. Generally UWWTPs were shown to have varying effects on ARG concentrations depending on wastewater treatment conditions and the type of ARG, even for wastewater treatment plants with tertiary treatment steps [245,283].

Conventional urban wastewater treatment was not capable of removing ciprofloxacin effectively (removal efficiency: 41% for W1, 39% for W2). Similarly, sulfamethoxazole could not be eliminated effectively (removal efficiency: 25% for W1, 19% for W2). Both investigated UWWTPs fail to remove most of the detected antibiotics. Other antibiotics with poor removal efficiency were ofloxacin, trimethoprim, clarithromycin, sulfachloropyridazine, fluconazole, azithromycin, erythromycin and lincomycin (**Figure 2-6**). Low biodegradability of many antibiotics might explain the inefficient antibiotic removal[284].

Conventional urban wastewater treatment might therefore not be the most efficient method to reduce antibiotic and ARG concentrations from CWW, contaminated with HWW, prior to release into the environment. Due to substantial fluctuations in antibiotic and ARG concentrations and CWW quality, the resulting effluent will be of variable quality with unknown environmental impact.

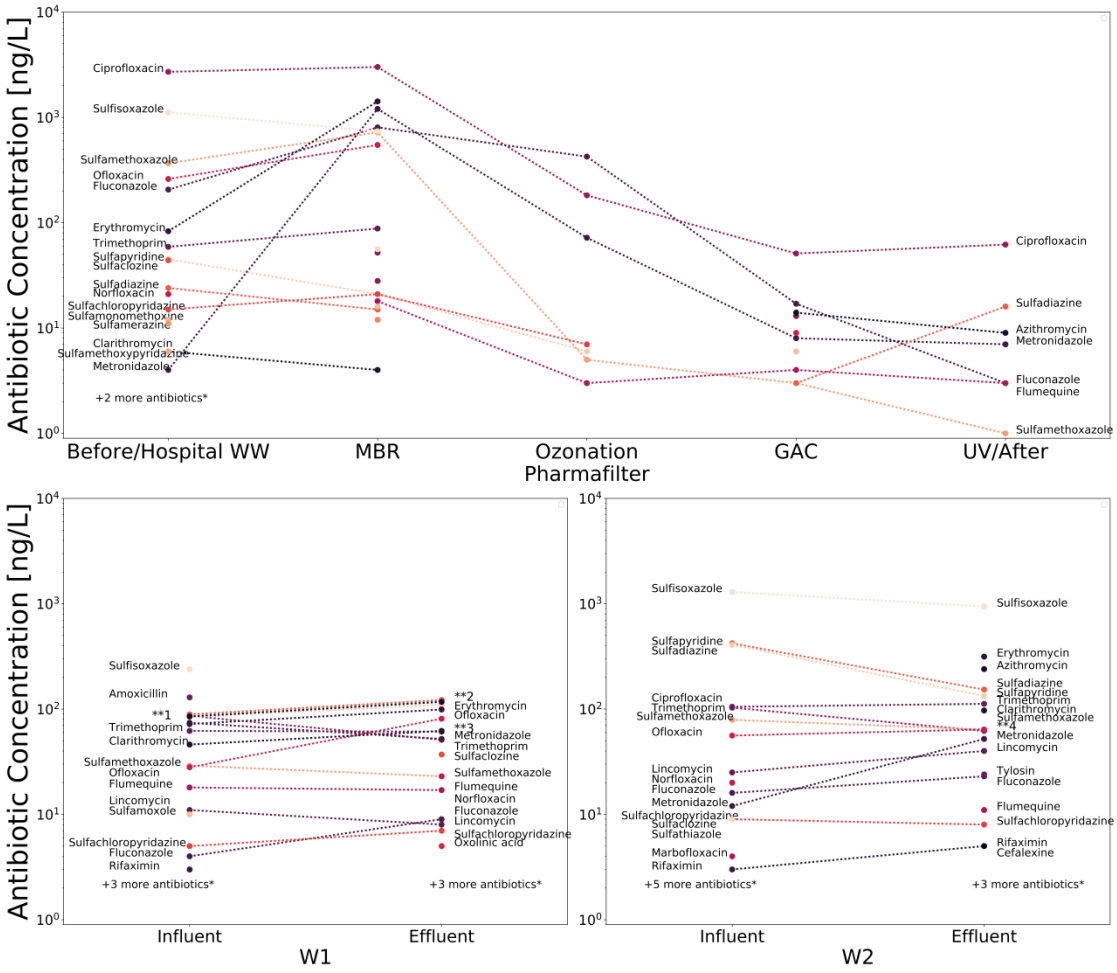


Figure 2-6: Antibiotic Concentrations Compared During Pharmafilter Process and During Conventional Urban Wastewater Treatment; * - number of antibiotics detected but not quantified; **1 – Azithromycin, Erythromycin, Metronidazole, Ciprofloxacin, Sulfadiazine, Sulfapyridine (72-89 ng/L); **2 – Azithromycin, Sulfapyridine, Sulfadiazine (110-120 ng/L); **3 – Ciprofloxacin, Clarithromycin (51-62 ng/L); **4 – Ciprofloxacin, Ofloxacin (62-62 ng/L)

ADVANCED ON-SITE TREATMENT IS MORE EFFICIENT AND CONSTANT THAN REGULAR URBAN WASTEWATER TREATMENT

While relative ARG concentrations did not uniformly decrease in UWWTPs and increased for approximately 10-30 % of all ARGs detected, all relative ARG concentrations were consistently significantly reduced during the Pharmafilter process (**Figure 2-4**). Only *Intl*1 was not consistently significantly removed during Pharmafilter treatment. Pharmafilter treatment reduced approx. 70% of all detected ARGs to <LOD, while regular urban wastewater treatment reduced between 10% (W1) and 22% (W2) of detected ARGs to <LOD. Furthermore, the reduction of ARG concentrations, of genes which were still quantifiable after the respective treatments, was 0.5 – 4.4-fold during Pharmafilter treatment and 0.5 – 2.2-fold during UWWT. Pharmafilter reduces individual genes with efficiencies between 0.5-fold (*intI*1) to more than 3.6-fold (*ermB*) higher than that of UWWTPs. This discrepancy in efficiency is further increased considering that UWWTPs could increase certain ARG concentrations more than 1-fold.

The increased ability of the Pharmafilter treatment compared to urban wastewater treatment is, with high probability, due to several interconnected factors: Conventional

wastewater treatment has a limited capacity to remove resistance genes [249,251,285] while advanced wastewater treatment (including MBR, Ozone and UV treatment) has been shown to have a better efficiency [286,287]. The sequential set-up of the Pharmafilter treatment steps seems to be of importance, as single treatment steps, seem to have the potential to increase the relative ARG concentrations when applied alone [154,282]. This study showed similar findings. While MBR seems to be the single most effective treatment step to eliminate ARGs, only 7 out of 13 detected ARGs were reduced <LOD during this step. Two ARGs (bla_{OXA}, *erm*B) were reduced <LOD, aph(III)a was significantly reduced in concentration in the subsequent treatment steps. The subsequent treatment steps therefore accounted for approximately 1/4 of the overall removal efficiency of the Pharmafilter. UV treatment had the least positive impact. Each of these advanced treatment types have their benefits and disadvantages [243] with ARG removal efficiency strongly depending on the type of ARGs present, the quality of wastewater influent and the applied treatment processes [288,289]. The high efficiency of MBR treatment is likely to be due largely to size exclusion, thus filtering out ARG-carrying microorganisms [290,291]. MBRs have been shown to develop characteristic communities, which differ from the influent community [291]. Subsequent partial detachment of microorganisms from this characteristic community might explain why some ARGs are eliminated to a higher extent than others during this treatment step.

Further, antibiotics and other pharmaceutical compounds which might exert selective pressure and increase HGT of ARGs [195,292,293] are thoroughly eliminated by the Pharmafilter process. Correlations between antibiotic and ARG concentrations have been shown [282,294]. Elevated concentrations of β-lactam antibiotics, glycopeptides and trimethoprim were detected in untreated HWW [251]. In contrast to the Pharmafilter, UWWTPs were shown to eliminate a much lower percentage of chemicals, including antibiotics. Elimination of antibiotics can be as low as 20 % for sulfamethoxazole, 69 % for trimethoprim and 70 % for ofloxacin [234].

Correlations between antibiotic and ARG concentrations were detected during the present study. Of the 41 quantified antibiotics, concentrations of two antibiotics correlated strongly with ARG concentration ((rifaximin, metronidazole), two correlated moderately (azithromycin and norfloxacin) and ciprofloxacin correlated moderately to strongly (depending on the ARG). Antibiotics correlated with different numbers of ARG (azithromycin(3), rifaximin(7), metronidazole(6), ciprofloxacin(3), norfloxacin(5) (*SI Table 2-8*). While most correlations were observed between unrelated antibiotic-ARG pairs, azithromycin (a macrolide) and *erm*F (R^2=0.66), ciprofloxacin (a fluorquinolone) and *qnr*S (R^2=0.56) and norfloxacin (a quinolone) and *qnr*S (R^2=0.64) correlated moderately. Interestingly, rifaximin and metronidazole concentrations correlated with ARG concentrations of a large number of unrelated ARGs. This could indicate that selective pressure of antibiotics on unrelated ARGs might be an larger problem than selective pressure on related ARGs. Antibiotics like metronidazole which do not largely cause resistance [295,296] might then have a larger impact on AR. Another explanation for these correlations could be co-selection. Co-selection of related and unrelated genes can be caused by co-occurrence on plasmids or other mobile genetic elements [294,297–299]. Finally, (non-antibiotic) pharmaceuticals which have not been investigated but are largely present in wastewaters, could be further driving HGT thus increasing AR [300,301]. It is to be noted that correlation does not necessarily imply causation and that further research will be needed to conclude if one of the described mechanisms are responsible for the observed correlations. Nevertheless, these observations are of interest, in case future research can find similar relationships between the respective antibiotics and ARGs.

2.4.3 The Impact of Pharmafilter Treatment on ARG Concentration in Hospital Wastewater Effluents and the Urban Wastewater System

On-site wastewater treatment with the Pharmafilter reduces the number of quantified ARG present in hospital wastewater discharge from 13 to 4 and the number of quantified antibiotics from 17 to 7. ARG concentrations of the four genes still detectable after treatment are reduced between 0.5±0.9 to >3.8-fold (**Figure 2-4**). Similarly, relative gene concentrations are reduced for genes detectable after treatment. *Intl*1 has been identified as a measurement of HGT potential and gene acquisition [249] and it has been proposed as an indicator for anthropogenic pollution [302]. *Intl*1 was found to show the highest relative concentrations of all genes in all analyzed HWW samples. A study had previously found that high antibiotic concentrations increase intI1 rearrangement, thus increasing the likelihood of HGT [303]. The overall discharge of ARGs concentrations, including intI1, from HWW to the communal sewage system is therefore greatly reduced by Pharmafilter treatment, decreasing the potential for HGT events induced by this otherwise high pollution point source for ARGs.

There are indications of a positive impact of the Pharmafilter treatment on the downstream urban wastewater system and, as a consequence, a benefit in terms of downstream environmental pollution. A lower number of genes was detected in influent samples of W1 (receiving treated HWW) than of W2 (receiving untreated HWW). Interestingly, hospital-related genes (not found in CWW) eliminated during Pharmafilter treatment could rarely be detected in W1. bla_{KPC} could not be detected in W1 samples and *van*A could only be detected during one of the two sampling rounds (**Figure 2-7**). Both genes were consistently detected in W2 samples (**Figure 2-7**). Similar results could be found for hospital-related antibiotics, which were consistently detected at elevated concentrations in W2, with concentrations up to > 5-fold higher (**Figure 2-7**). Antibiotics only detected in W1 influent (amoxicillin, azithromycin clarithromycin, erythromycin, flumequine and sulfamoxole) were not detected in treated HWW (location 1) and must therefore originate from other sources. Antibiotics detected only in W2 influent (marbofloxacin, norfloxacin and sulfathiazole) were similarly only detected in H2, with the exception of norfloxacin, which was also detected in CWW, albeit at low concentrations.

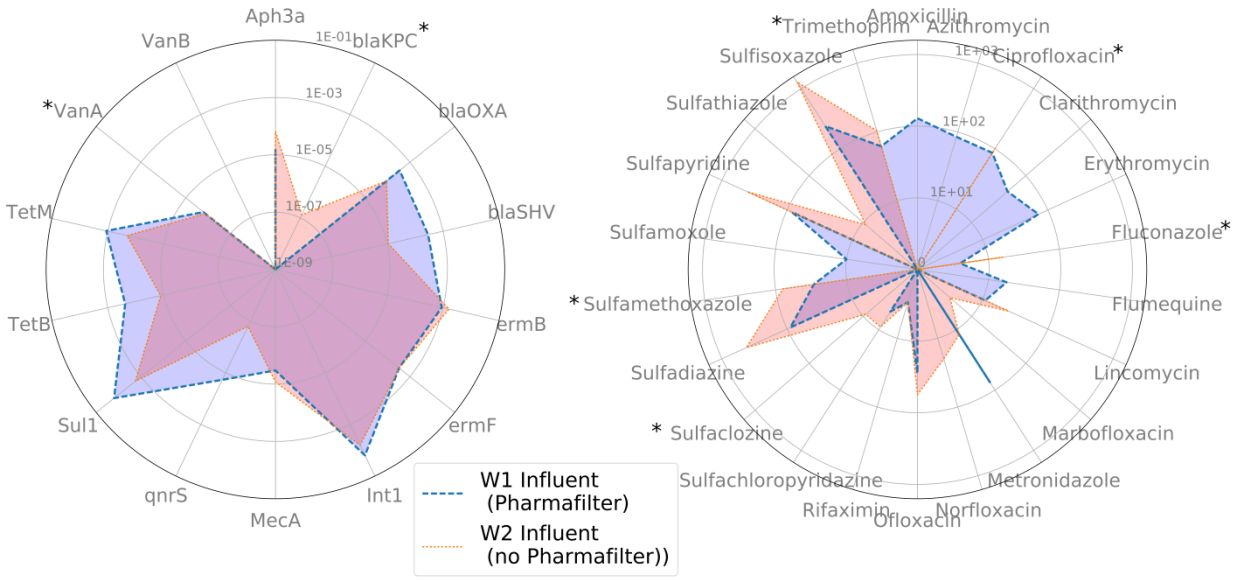


Figure 2-7: Antibiotic and ARG Presence and Concentration in UWWTP Influents; left - ARG concentrations in Influents; right – antibiotic concentrations in Influents; * - hospital-related antibiotics or ARGs found in HWW of both location (H1 and H2) at comparable concentrations

2.5. Conclusion

On-site treatment was substantially more efficient in reducing antibiotic and ARG concentrations than UWWTPs. On-site treatment of HWW did also reduce UWWTP influent loads with hospital-related pollutants. *Intl*1 concentrations were reduced to a considerably larger extent, which could subsequently reduce HGT potential in wastewaters. Combining these findings with elevated levels of antibiotics and ARGs in HWWs (compared to CWW), on-site treatment of HWWs with sequentially aligned advanced treatment technologies is an important step to decrease the risk potential of HWWs and to decrease the impact of wastewater effluents on the environment and subsequently on human health. Alternatively, upgrading existing UWWTPs to include more advanced treatment technologies could mimic the benefits of on-site wastewater treatment of high-risk point sources.

Pharmafilter treatment results in the reduction of pharmaceuticals, including antibiotics, in the treated wastewaters. Correlations between antibiotic and ARG concentrations, suggest potential interactions between these two factors. This reduction could further decrease HGT events as potential sources of selective pressure are diminished, especially for last-resort antibiotics frequently used in hospitals.

Summarizing it can be said that on-site treatment of high-risk wastewater sources was proven highly advantageous in regard to antibiotic and ARG reduction. Legislative guidelines and requirements would be conducive to create incentives and increase practical implementation of on-site wastewater treatment.

2.6. Supplementary Material

BIOLOGICAL ANALYSIS

Si Table 2-1: qPCR Program Used for Multiplex Assays

1	**95.0 C for 5:00**	
2	**95.0 C for 0:30**	
3	**[change temp. according to multiplex assay used] for 0:30**	
4	**72.0 C for 0:45**	
	+ Plate Read	10 > Cq > 36 were considered out of range for precise quantification
5	**GOTO 2, 40 more times**	
	END	
	machine used: CFX96	
	software used: CFX Manager Version : 3.1.1517.0823.	

Si Table 2-2: Triplex Master Mix Preparation

Reagent/Component	**%**	**MM/well**
iQ Mix	**62.5**	25
Water	**17.5**	1
BSA	**12.5**	5
3xForward Primer	**2.5**	3x 1
3xReverse Primer	**2.5**	3x 1
3xProbe	**2.5**	3x 1

Volume per well: **40µL**

Sample Volume per well: 10µL

Si Table 2-3: Multiplex Assay information

Multiplex	**Gene**	**Annealing Temp (°C)**	**FW primer***	**RV Primer***	**Probe Sequence**	**Probe Dye**
SIQ	**sul1***	58	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG	CGAACCTTCAAAAGCTGAAGTCGGCGT	HEX
	qnrS*		TACGACATTCGTCAACTGCAAGT	GACGTGCTAACTTGCGTG	TACGACATTCGTCAACTGCAAGT	FAM
	IC (optional)		ATGACAGCCACTCCTCCG	GGAACGAACCAAACAGTCTTC	AGCAGAGACCCATTCCCTCAGAGC	Texas Red
TSI	**tetB***	60	ACTGCCGTTTTTTCGCC	CCTTATCATGCCAGTCTTGC	TATTCTTCCTGCCACAAAGGCTTGGA	HEX
	bla_{SHV}*		TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	TTGAGCAAATTAAACAAAGCGA	FAM

	Int1*		CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	TCGTGATGCCTGCTTGTTCTACGGCA	Texas 615
MOA	**mecA***	57	CTTCCACATACCATCTTCTTTAAC	GTTGTAGTTGTCGGGTTTGG	ACGTTGCGATCAATGTTACCGT	HEX
	bla$_{OXA}$*		CACTTACAGGAAACTTGGGGTCG	AGTGTGTTTAGAATGGTGATC	ACATCAAGCATAAAAGCCAAGA	FAM
	aph(III)a*		ACATATCGGATTGTCCCTATACGAA	TCGGCCAGATCGTTATTCAGTA	TAGCTTAGACAGCCGCTTA	Tex615
EEK	**ermB***	59	GATACCGTTTACGAAATTGG	GAATCGAGACTTGAGTGTGC	ACAGGTAAAGGGCATTTAACGA	Texas 615
	ermF*		CGACACAGCTTTGGTTGAAC	GGACCTAACTCATAGACAAG	AATTATTTTCTGATGCCCGA	FAM
	bla$_{KPC}$*		TCGAACAGGACTTTGGCGGCT	GGACAGCTCCGCCACCGTCATG	CGCTGGTTCCGTGGTCA	HEX
BAM	**vanB***	58	CCGCCATCCTCCTGCAAAAAA	GTGACAAACCGGAGGCGAGGA	ACACGAGCAAGCCCTCTGCA	TexRed
	vanA*		TCTGCAATAGAGATAGCCGC	GGAGTAGCTATCCCAGCATT	ATACGAGCCGTTATACAT	HEX
	tetM*		GCAATTCTACTGATTTCTGC	CTGTTTGATTACAATTTCCGC	AAAGATGGCGTACAAGCAC	FAM

*primers as suggested by ANSWER-ITN supervisory board (see http://www.answer-itn.eu/ for more information)

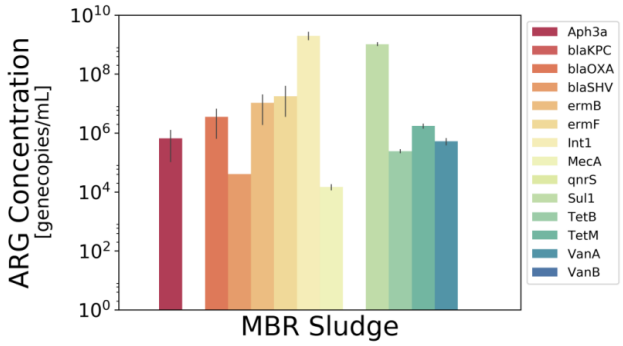
Multiplex qPCR Standard Curves

Standard curves were obtained by using gBlocks at increasing concentrations (2.5E00 – 2.5E04 gene copies/µL). The gBlock sequences contained the target sequences (=sequence between FW and RV primers) for all genes quantified within one multiplex assay.

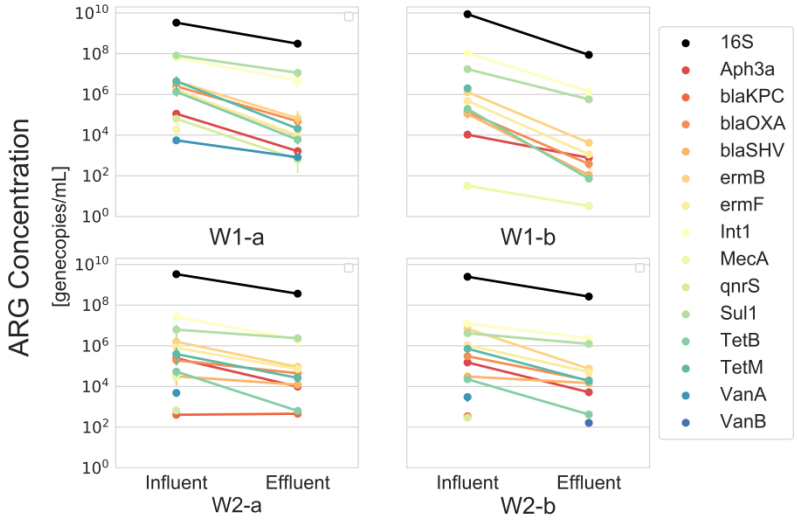
Si Table 2-4: bsolute Gene Concentrations Within Separate Communal WW Samples

	W1-b	W1-a	W2-b	W2-a
Aph3a	9.2E+04±1.3E+05	2.3E+03±3.3E+03	9.0E+05±1.2E+06	1.4E+5±1.9E+5
blaKPC	-	-	-	-
blaOXA	1.4E+05±1.8E+05	5.8E+04±8.2E+04	1.1E+05±1.9E+05	3.9E+4±2.9E+4
blaSHV	1.3E+04±3.1E+03	2.6E+03±2.2E+03	3.0E+03±5.1E+03	2.3E+3±1.9E+3
ermB	7.7E+06±8.0E+06	3.1E+04±3.8E+04	9.1E+04±3.3E+04	2.3E+5±2.4E+5
ermF	3.3E+06±3.3E+06	1.3E+05±1.5E+05	9.6E+05±9.5E+05	4.6E+5±6.3E+5
Int1	5.4E+07±2.6E+07	1.2E+08±1.4E+08	2.4E+07±2.5E+07	2.6E+8±2.4E+8
MecA	5.6E+02±4.8E+02	1.7E+01±1.6E+01	9.0E+02±6.1E+02	3.7E+3±3.6E+3
qnrS	3.2E+03±6.1E+02	-	6.9E+01±4.1E+01	2.1E+2±8.0E+1
Sul1	7.5E+06±2.4E+06	8.2E+06±5.2E+06	3.7E+06±3.6E+06	1.6E+7±1.0E+7
TetB	9.2E+05±3.6E+05	5.2E+04±2.9E+04	1.6E+03±7.2E+02	4.3E+3±2.6E+3
TetM	2.2E+06±2.4E+06	1.2E+06±2.8E+05	9.8E+04±8.3E+04	3.3E+5±3.2E+5
VanA	-	-	-	-
VanB	-	-	-	-

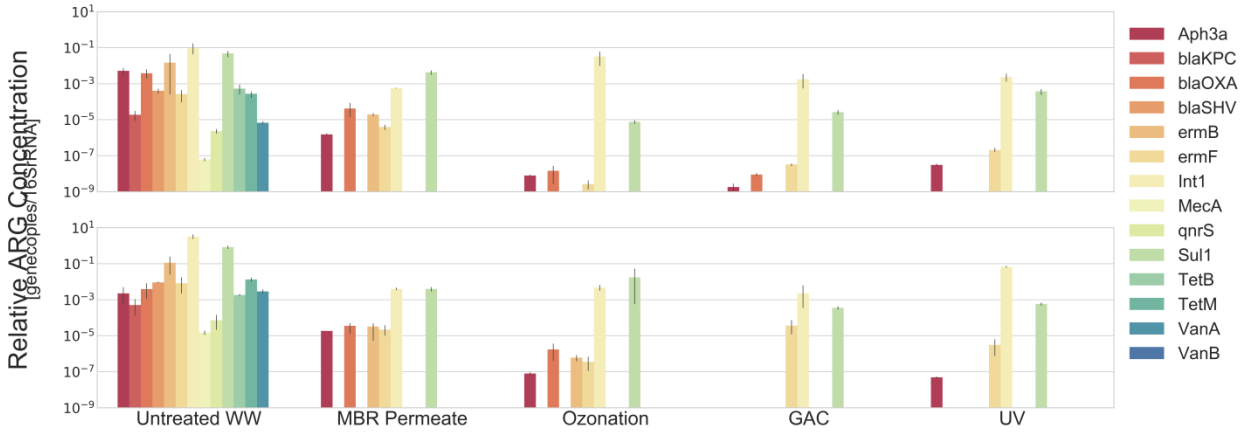
SI Figure 2-5 Absolute Gene Concentration in MBR Sludge Sample (Pharmafilter)



SI Figure 2-6 Absolute Gene Concentrations including 16S rRNA Concentrations: *in Urban WWTPs (W1, W2) during different sampling rounds (a, b)*



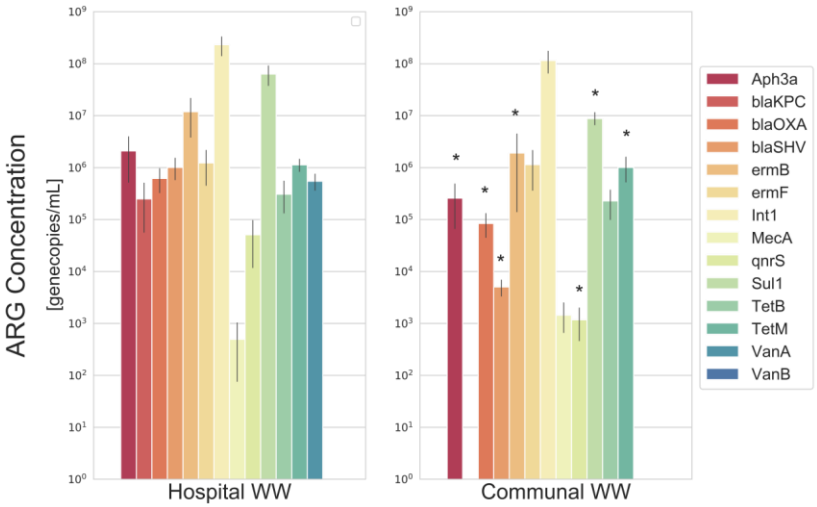
SI Figure 2-7 Relative Gene Reduction during Pharmafilter Processes*; top: 1st sampling round, bottom: 2nd sampling round*



SI Table 2-8 Correlation between A and ARG Concentrations Expressed by the Coefficient of Determination (R^2); significant correlations marked *red: R^2>0.5 with p<0.05 and >5 common data points; black: R^2>0.5 and p>0.05; grey: R^2<0.5; antibiotics without correlations R^2>0.5 not shown (Trimethoprim, Amoxicillin, Ofloxacin, Sulfadiazine, Sulfapyridine)*

	Azithro-mycin	Clarithro-mycin	Erythro-mycin	Rifaximin	Metro-nidazole	Fluco-nazole	Linco-mycin	Ciproflo xacin	Norflo xacin	Sulfach loro-pyridazine	Sulfacl ozine	Sulfam etho-xazole	Sulfis o-xazole
Aph 3a				0.998 996	0.971 367				0.6492 94				
IC	0.582 158	<0.5	<0.5	0.699 297	0.645 689	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Int1	0.589 325			0.971 250							0.5845 13		
Mec A	0.987 371	0.716 079	0.525 182	1	<0.5	0.668 912	0.553 194	<0.5	<0.5	0.9045 70	0.6155 29	<0.5	0.576 693
Sul1	0.599 297			0.977 817									
Tet B	0.551 858	<0.5	<0.5	0.750 341	0.721 534	<0.5	<0.5	<0.5	<0.5	<0.5	0.5558 34	<0.5	<0.5
Tet M						0.835 294							
Van A	0.548 800	<0.5	<0.5	0.999 997	0.899 329	<0.5	<0.5	0.7459 52	0.7291 99	<0.5	0.6409 94	0.5186 14	<0.5
Van B	1	1	<0.5	<0.5	<0.5	<0.5	1	1	<0.5	<0.5	<0.5	1	1
blaK PC	<0.5	1	1	1	0.998 528	<0.5	1	0.5406 91	0.8587 33	<0.5	0.5716 26	<0.5	<0.5
bla OXA				0.971 815	0.814 553				0.6549 45		0.6011 37		
blaS HV	<0.5	<0.5	<0.5	0.999 414	0.884 524	<0.5	<0.5	0.8350 77	0.7150 63	0.7818 65	0.6268 08	<0.5	<0.5
erm B				0.861 844	0.545 556						0.5789 00		
erm F	0.663 233	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
qnr S	0.540 792			0.999 841	0.998 023			0.5561 39	0.6425 30				

SI Figure 2-9 Absolute Gene Concentration in HWW and CWW; ** - ARG concentration significantly higher in HWW*



I. Chemical Analysis

SI 2-10

Chemicals and reagents

Acetonitrile (ACN) and Methanol (MeOH) LC–MS grade was purchased from Merck (Darmstadt,Germany) and formic acid (FA) 99% was obtained from Sigma–Aldrich, Fluka (Buchs, Switzerland). Distilled water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Oasis-HLB disks were purchased from (Waters, Milford, MA, USA) and RC syringe filters (4 mm diameter, 0.2 µm pore size) from Phenomenex (Torrance, CA, USA).

Sample preparation automatic SPE (SPEDEX) program

SI Table 2-11: Conditioning and extraction program used for sample preparation of wastewater samples by HORIZON SPE-DEX 4790.

		Solvent	Soak Time (sec)	AirDry Time (sec)
HLB conditioning	PreWet Cycle	Isopropanol	-	5
		Isopropanol	-	5
		Milli-Q Water	-	5
		Methanol	-	5
		Ethyl Acetate	-	5
	Rinse Cycle	Methanol	-	5
		Ethyl Acetate	-	5
Extraction	PreWet Cycle	Ethyl Acetate	120	30
		Ethyl Acetate	120	30
		Ethyl Acetate	90	30
		Methanol	120	30
		Methanol	120	30
		Methanol	60	30
		Milli-Q water	120	30
		Milli-Q water	60	30
		Milli-Q water	60	30
	Sample AirDry Cycle	Ethyl Acetate	150	60
		Ethyl Acetate	90	30
		Ethyl Acetate	90	30
		Methanol	150	60

		Methanol	90	30
		Methanol	90	30

Instrumental analysis parameters

SI Table 2-12: LC-MS/MS conditions in positive ionization mode and gradient elution program.

Positive Ionization Mode

Gradient Program		**ESI (+) Parameters**	
Time (min)	% B	Spray Voltage	3500V
0	2	Capillary temperature	270 °C
3	2	Sheath gas	30 psi
20	100	Auxiliary (drying) gas	10 a.u.
29	100	(A) H_2O 0.01% v/v HCOOH	
30	2	(B) MeOH	
45	2		

SI Table 2-13: Selected reaction monitoring transitions of the targeted analytes, precursor ions, product ions, collision energies and tube lens.

Analyte	**Precursor Ion**	**Product Ion 1**	**CE (eV)**	**Product Ion 2**	**CE (eV)**	**Tube Lens (V)**
Cefalexin	348	158	6	106.1	30	113
Cefaclor	368	178	31	106	27	93
Clarithromycin	749	158	30	590.5	20	123
Azithromycin	749	591.1	29	157.9	37	127
Erythromycin	734	158.1	30	576.3	20	130
Metronidazole	172	128	13	82.3	25	69
Trimethoprim	291	230	25	122.9	30	87
Lincomycin	407	126.2	30	359.2	17	99
Tylosin	917	173.9	36	772.2	28	148
Tiamulin	494	192	21	119	33	101
Amoxicillin	366	348.9	8	114	22	68
Ampicillin	350	106	27	160	14	98
Dicloxacillin	468	327	15	424	11	91
Ciprofloxacin	332	288	18	314	22	85
Ofloxacin	362	317.9	19	260.9	27	120
Norfloxacin	320	276	16	233	23	91
Flumequine	262	244	20	201.9	30	85
Oxolinic acid	262	244	18	158	31	79
Sarafloxacin	386	342	18	299	27	85
Difloxacin	400	356	20	299	27	85
Enrofloxacin	360	245	25	316.8	20	85
Marbofloxacin	363	320	15	71.9	20	85
Sulfamethoxazole	254	155.8	16	108	25	87
Sulfadiazine	251	156	15	92.2	27	87
Sulfapyridine	250	156	15	184	17	87
Sulfisoxazole	268	156	13	92.2	27	87
Sulfachloropyridazine	285	92.1	28	155.9	14	87
Sulfaclozine	285	92.1	28	156	15	87
Sulfathiazole	256	155.9	15	92.2	26	87

Sulfamonomethoxine	281	156	13	92.2	29	87
Sulfamoxole	268	156	13	92.2	28	87
Sulfamerazine	265	156	16	172	16	87
Sulfadimethoxine	311	156	17	108.1	29	87
Sulfadimidine	279	185.9	17	124.1	26	87
Sulfadoxine	311	156	17	108.1	27	87
Sulfamethoxypyridazine	281	156	13	92.2	29	87
Sulfaguanidine	215	156	14	92.2	14	87
Sulfamethizole	271	155.9	14	92.2	28	87
Sulfaquinoxaline	301	156	18	92.2	30	87
Chlortetracycline	479	444	20	462	15	90
Minocycline	458	441	19	352	29	105

SI Table 2-14: LC-MS/MS conditions in positive and negative ionization modes and gradient elution program.

Positive Ionization			
Gradient Elution Program		**ESI (+) Parameters**	
Time (min)	% B	Capillary Voltage	2500V
0	1	End plate offset	500V
1	1	nebulizer	2 bar
3	39	Drying gas	8 L min^{-1}
14	99.9	Drying temperature	200°C
16	99.9	(A) Water : Methanol 90:10 5mM $HCOONH_4$ with 0.01% HCOOH	
16.1	1	(B) Methanol 5mM $HCOONH_4$ with 0.01% HCOOH	
20	1		
Negative Ionization			
Gradient Elution Program		**ESI (-) Parameters**	
Time (min)	% B	Capillary Voltage	3500 V
0	1	End plate offset	500 V
1	1	nebulizer	2 bar
3	39	Drying gas	8 L min^{-1}
14	99.9	Drying temperature	200°C
16	99.9	(A) Water : Methanol 90:10 5mM $CH3COONH_4$	
16.1	1	(B) Methanol 5mM $CH3COONH_4$	
20	1		

SI Table 2-15. Antibiotics, class of antibiotics, limit of detection (LOD), frequency of detection and concentration of the antibiotics (ng/L) in the wastewater samples

Antibiotics	Class of Antibiotic	LOD	H1 untreated	H1 treated	W1 Influent	W1 Effluent	H2	W2 Influent	W2 Effluent	Communal WW	H1 - station1	H1 - station2	PharmaMBR	PharmaGAC	PharmaOzone
Ciprofloxacin	Antibiotics (Quinolones)	5.6	2706	62	86	51	3752	103	62	16	1843	264	3006	51	182
Sulfamethoxazole	Antibiotics (Sulfonamides)	0.1	367	1	29	23	269	79	64	1	5	2	721	3	5
Metronidazole	Antibiotics (Nitroimidazole)	1.1	4	7	74	52	7567	12	52	5	18	<LOD	1203	8	72
Sulfadiazine	Antibiotics (Sulfonamides)	3.0	24	16	89	122	233	421	153	3	<LOD	3254	15	3	<LOD
Ofloxacin	Antibiotics (Quinolones)	4.7	260	<LOD	28	81	30	56	64	7	8	<LOD	547	9	<LOD
Sulfapyridine	Antibiotics (Sulfonamides)	4.5	45	<LOD	84	110	253	408	133	5	<LOD	3215	21	<LOD	6
Fluconazole	Antibiotics (Other)	2.2	206	3	4	9	60	16	23	<LOD	<LOD	<LOD	803	17	424
Sulfisoxazole	Antibiotics (Sulfonamides)	2.4	1117	<LOD	239	<LOD	1147	1292	942	684	1603	689	737	<LOD	<LOD
Trimethoprim	Antibiotics (Other)	7.5	59	<LOD	62	61	259	105	112	<LOD	412	<LOD	88	<LOD	<LOD
Sulfachloropyridazine	Antibiotics (Sulfonamides)	5.1	15	<LOD	5	7	<LOD	9	8	<LOD	15	<LOD	21	<LOD	7
Clarithromycin	Antibiotics (Macrolides)	2.9	6	<LOD	46	62	<LOD	<LOD	97	<LOD	7	264	4	<LOD	<LOD
Azithromycin	Antibiotics (Macrolides)	4.6	<LOD	9	85	117	406	<LOD	240	<LOD	<LOD	323	<LOD	14	<LOD
Amoxicillin	Antibiotics (Penicillins)	6.1	<LOD	<LOD	129	<LOD	7	<LOD	<LOD	111	113	8	52	13	<LOD
Norfloxacin	Antibiotics (Quinolones)	4.5	21	<LOD	<LOD	23	41	20	<LOD	8	28	18	<LOD	<LOD	<LOD
Flumequine	Antibiotics (Quinolones)	2.6	<LOD	3	18	17	<LOD	<LOD	11	<LOD	<LOD	<LOD	18	4	3
Erythromycin	Antibiotics (Macrolides)	9.0	83	<LOD	72	99	<LOD	<LOD	315	<LOD	603	<LOD	1418	<LOD	<LOD
Lincomycin	Antibiotics (Other)	5.3	<LOD	<LOD	11	8	15	25	40	<LOD	<LOD	23	<LOD	<LOD	<LOD
Rifaximin	Antibiotics (Macrolides)	0.9	<LOD	<LOD	3	<LOD	84	3	5	3	<LOD	<LOD	<LOD	<LOD	<LOD
Sulfaclozine	Antibiotics (Sulfonamides)	1.7	44	<LOD	<LOD	37	56	9	<LOD	<LOD	22	<LOD	<LOD	<LOD	<LOD

Ampicillin	Antibiotics (Penicillins)	21.0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	45	60	<LOD	28	<LOD	<LOD
Sulfathiazole	Antibiotics (Sulfonamides)	6.3	<LOD	<LOD	<LOD	<LOD	14	9	<LOD	<LOD	<LOD	<LOD	56	<LOD	<LOD
Sulfamonomethoxine	Antibiotics (Sulfonamides)	5.3	12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6	7	<LOD	<LOD	<LOD
Sulfamoxole	Antibiotics (Sulfonamides)	3.2	<LOD	<LOD	10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	16	6	<LOD
Cefalexine	Antibiotics (Cefalosporines)	4.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5	5	<LOD	<LOD	<LOD	<LOD	<LOD
Oxolinic acid	Antibiotics (Quinolones)	2.6	<LOD	<LOD	<LOD	5	<LOD	<LOD	<LOD	<LOD	<LOD	21	<LOD	<LOD	<LOD
Sulfamerazine	Antibiotics (Sulfonamides)	7.6	11	<LOD	<LOD	<LOD	28	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Tylosin	Antibiotics (Other)	6.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sarafloxacin	Antibiotics (Quinolones)	1.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2	<LOD	<LOD	<LOD	<LOD	<LOD
Enrofloxacin	Antibiotics (Quinolones)	14.1	<LOD	<LOD	<LOD	<LOD	22	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Marbofloxacin	Antibiotics (Quinolones)	2.1	<LOD	<LOD	<LOD	<LOD	<LOD	4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sulfadimethoxine	Antibiotics (Sulfonamides)	0.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2	<LOD	<LOD	<LOD	<LOD
Sulfadimidine	Antibiotics (Sulfonamides)	5.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5	<LOD	<LOD	<LOD	<LOD
Sulfadoxine	Antibiotics (Sulfonamides)	6.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	12	<LOD	<LOD
Sulfamethoxypyridazine	Antibiotics (Sulfonamides)	5.1	6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

SI Table 2-16. Detected antibiotics by suspect screening, class of antibiotics and level of identification. Compounds positively detected in wastewater samples are marked with "**+**".

Antibiotics	Class of Antibiotic	Level of Identification	H1 untreated	H1 treated	W1 Influent	W1 Effluent	H2	W2 Influent	W2 Effluent	Communal WW	H1 - station1	H1 - station2	PharmaMBR	PharmaGAC	PharmaOzone
Rifaximin	Antibiotics (Macrolides)	Confirmed structure Level 1			+	<LOD	+	+	+	+					
Fluconazole	Antibiotics (Other)	Confirmed structure Level 1	+	+	+	+	+	+	+				+	+	+
Nalidixic acid	Antibiotics (Quinolones)	2A					+								
Hydroxy-metronidazole (MNZOH)	Antibiotics (Nitroimidazole)	2A	+		+		+	+		+	+		+		
Sulfanilamide	Antibiotics (Sulfonamides)	2A	+		+			+		+	+	+			
Sulfasalazine	Antibiotics (Sulfonamides)	2A				+	+		+				+		
8-Hydroxychinolin	Antibiotics (Other)	2A				+	+	+	+	+	+	+		+	
3-Aminosalicylic acid	Antibiotics (Other)	3								+	+				
Mycophenolic acid	Antibiotics (Other)	3			+	+	+	+	+						

SI 2-17 Chemical Data Analysis

Thermo LCquan 2.7. (CA, USA) was used to analyze the data from LC-QQQ instrument. UHPLC-QTOF-MS data were used for suspect screening of 670 antibiotics, which can be found under the name "S6 ITNANTIBIOTIC" in the website of NORMAN network in Suspect list exchange webpage (https://www.norman-network.com/?q=node/236). Data-independent chromatograms were converted to mzML using msconvert module of Proteowizard software [304] and the collision energy channels were separated using an in-house script [305]. Peak picking using centWave algorithm [306] with optimized parameters [307] for chromatography and mass spectrometry (ppm 17.6, min peakwidth 15, max peakwidth 50, snthresh 10, fitgauss TRUE) was used to find peaks in the data. Isotopic peaks and adduct peaks of MS1 full-scan data were grouped (componentization) using nontarget R-package (Loos, 2016). Suspect list used included the experimental fragments for 152 suspected compounds, while for rest predicted fragments were used [308]. Tentative identification achieved in cases which molecular ion was detected with mass accuracy below 2 mDa and at least 2 library fragment ions were detected. Cases with in-silico predicted fragments were further investigated manually.

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3. International tempo-spatial study of antibiotic resistance across the Rhine River using newly developed multiplex qPCR assays

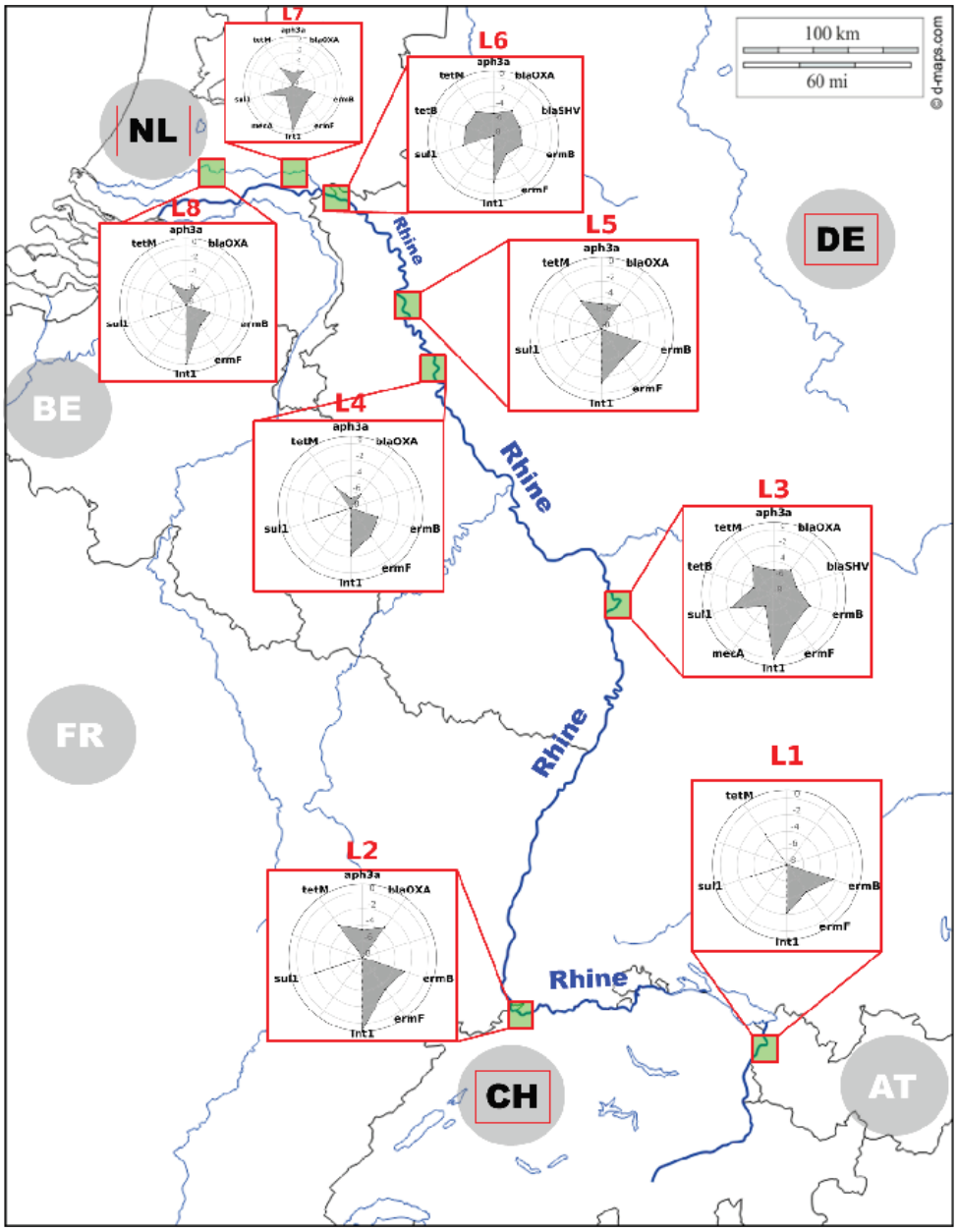


Figure 3-1: Graphical Abstract; Chapter 3

3.1. Abstract

The aim of this study was to capture and explain trends and changes in antibiotic resistance gene (ARG) presence and concentration internationally across the Rhine River. *Intl*1 concentrations and national antibiotic usage were investigated as proxies to predict anthropogenic ARG pollution levels. Newly developed multiplex qPCR assays were employed to investigate antibiotic resistance gene profiles across 8 locations (L1-L8) in three countries (Switzerland, Germany and the Netherlands) and to detect potential local or national causes for variation. Two of these locations were further monitored, over the duration of one month. A total of 13 antibiotic resistance genes, as well as *Intl*1 and 16S rRNA were quantified.

Antibiotic resistance gene presence and concentrations initially increased from L1(Diepoldsau) to L3(Darmstadt). A continuous increase could not be observed at subsequent locations, with the large river volume likely being a major contributing factor for stability. ARG presence and concentrations fluctuated widely across different locations. L2 (Basel) and L3 were the two most polluted locations, coinciding with these locations being well-developed pharmaceutical production locations.

We draw attention to the characteristic, clearly distinct ARG profiles, with gene presence being consistent and gene concentrations varying significantly less over time than across different locations. Five genes were Rhine-typical (*erm*B, *erm*F, *Intl*1, *sul*1 and *tet*M). *Intl*1 and *sul*1 were the genes with highest and second-highest concentration, respectively. *Aph*(III)a and bla_{OXA} were permanently introduced downstream of L1, indicating no source of these genes prior to L1.

We also highlight that strong correlations between *Intl*1 and ARG concentrations (R^2=0.72) were mainly driven by correlation to *sul*1 and disappeared when excluding *sul*1 from the analysis (R^2=0.05). *Intl*1 therefore seems to be a good proxy for *sul*1 concentrations but not necessarily for overall (anthropogenic) ARG pollution. Aminoglycoside usage per country correlated with concentrations of *aph*(III)a and several unrelated antibiotic resistance genes (bla_{OXA}, *erm*B, *erm*F and *tet*M). This correlation can be explained by co-resistance caused by mobile genetic elements (MGEs), such as Tn1545.

3.2. Introduction

Water Pollution and antibiotic resistance (AR) are on the rise globally and the advancing global emergence calls for better and more extensive monitoring of environmental, urban and medical environments [309,310]. While AR has been present since the first microorganisms started producing antibiotics to protect themselves against these toxic compounds [75], a recent sharp increase in resistance of clinically relevant bacteria is evident [29,311–313]. Reasons for this increase include: overuse and inappropriate prescription of antibiotics, extensive use in agriculture and animal husbandry, severe misinformation about when antibiotics are indicated, the absence of coordinated global AR combat strategies and a low number of new antibiotics [29,310,311,314,315]. Global and national action plans to tackle AR are developed in recent years, but they are neither fully coordinated nor incorporated into legislation [316], partly due to gaps in knowledge [317].

It is widely accepted that AR is largely caused by the increased use and misuse of antibiotics [318–320]. However, the exact role of the environment is not well understood [321–323]. It is likely that the environment can act as a reservoir for ARGs [324] and possible that it might facilitate transfer of AR to non-environmental microorganisms, pathogens or human microbiomes [131,318]. Where antibiotic pressure is high in the environment, new ARGs may emerge [195].

A quantitative approach to assess the risk of ARB/ARG in the environment and possible effects on the environment, animals and humans is missing [310]. Increased exposure to AR via the environment will lead to increased risk; with a growing world population, associated water use, discharge of wastewater [325] and the increasing reuse of wastewater [326–329], exposure to environmental ARG/ARB will rise.

Notwithstanding the large number of recent monitoring studies, one of the main gaps in knowledge are definite numbers of ARG concentrations and their fluctuation in the environment at different locations over time and under varying conditions [317,330]. Tempo-spatial studies of water bodies are imperative, especially for substances of emerging concern, such as ARGs [331]. AR baseline levels in the environment and their increase due to varying levels of anthropogenic pollution have not been extensively studied. These numbers are needed to serve as a baseline for knowledge on environmental dissemination of ARG and to estimate the risk of observed ARG concentrations at, for example, ARG hotspots such as strongly polluted surface water bodies [332].

ARGs and MGEs, such as *sul*1 and *Intl,*1 have been suggested as proxies to monitor ARGs (of anthropogenic origin) in the environment. *Intl*1, has been suggested as indicator for bacterial capacity for gene transfer and gene acquisition [249] as well as proxy for ARGs of anthropogenic origin [333]. *Sul*1 has been suggested as indicator for urban and agricultural pollution of ARG [211].

Closely meshed monitoring programs using regular qPCR of more than one target gene require large amounts of time, personnel and material resources. While quantitative high-throughput technologies, such as microarray technologies and HT-qPCR (high throughput qPCR) are increasingly used to determine AR in the environment [212,330,334–338], there are a number of challenges and disadvantages associated with these technologies [212]. Microarray technologies suffer from batch-to-batch variability and are considered less sensitive and specific [212]. HT-qPCR is prone to instrumental sensitivity and analytical differences which can

significantly impact results and individual assays tested cannot be optimized during the experimental run [212]. Our aim was to decrease the necessary resources and to maintain the accuracy of regular qPCR approaches while using a method with a higher throughput and avoiding the shortcomings of high throughput technologies. To this end five multiplex qPCR assays were developed to detect and accurately quantify three genes per qPCR assay simultaneously.

The present work is an international study monitoring a large river from the source to the ocean and is, to the best of our knowledge, the first of its kind for large western European rivers. It monitors spatio-temporal changes in ARG presence and concentration regarding the following: genes conferring resistance to aminoglycosides (*aph*(III)a), β-lactam antibiotics (bla_{KPC}, bla_{SHV}, bla_{OXA}, *mec*A), macrolides (*erm*B, *erm*F), quinolones (*qnr*S), sulfonamides (*sul*1), tetracyclines (*tet*B, *tet*M) and glycopeptides (*van*A, *van*B) as well as a class 1 Integrase (*Intl*1) and 16s rRNA. The study further aims at gaining more information on ARG levels across the river Rhine at different locations as well as changes over time to identify relationships, trends and co-occurrences of environmental ARGs and their concentrations, which will provide insight needed to detect prospective causes. Potential proxies for ARG pollution of anthropogenic origin were compared.

3.3. Methods and Materials

3.3.1 Multiplex Development and Quality Control

Five Multiplex qPCR assays were developed and validated. Development steps included: optimal annealing temperature testing for primers and probes, grouping of ARGs into multiplex qPCR assays based on optimal annealing temperatures and functionality tests using SYBR Green II (for comparison of probe-based multiplex qPCR results) as well as Taqman in combination with the iQ™ Supermix (simplex) or iQ™ Multiplex Powermix (multiplex) (Bio Rad, München, DE).

Quality control steps included: computational interaction tests (for interactions between primers and probes of ARGs within the multiplex qPCR assay) using OligoAnalyzer 3.1 [339], experimental interaction tests and precision tests for accurate quantification (comparing quantification results obtained by SYBR Green II assays to Taqman qPCR assays). Mock samples were prepared in 0.1xTE-buffer or by spiking environmental samples (pond water) with gene concentrations within a specific assay varying up to 1000-fold.

Validation steps included determination of limit of quantification (LoQ) and limit of detection (LoD) [340], calculation of intra- and interassay variation in form of the coefficient of variation (CV) [341,342] and multiplex qPCR assay efficiencies for each of the genes within a multiplex assay [342]. Validation was conducted and reported according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [343]. More specifically: serial dilutions of the standard were prepared for the different genes and quantified multiple times to obtain at least 8 data points per dilution.

3.3.2 SAMPLING

SAMPLING SITES

The Rhine is the second-largest river in Central and Western Europe. It originates at Lake Toma in Switzerland and discharges in the Netherlands into the North Sea after 1230 km and has an average flow rate of 2900 m^3/s. Sampling was done along the Rhine at 8 locations: L1 – L8. The samples were collected by a cooled courier car and were processed within 24h of sampling at the processing facility.

There are six distinctive Rhine regions with different characteristics (**Figure 3-2**).

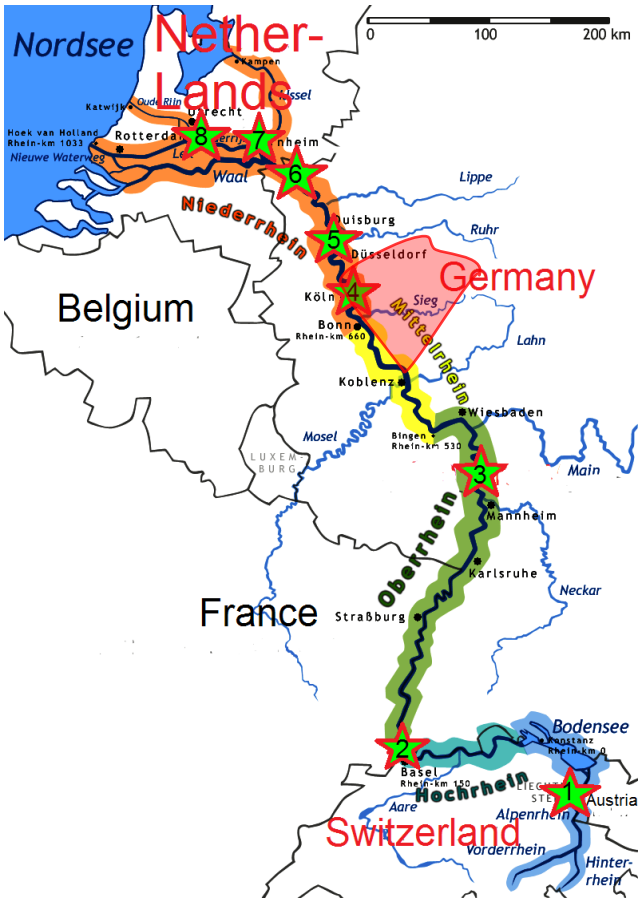


Figure 3-2: Sections and Major Affluents of the Rhine; blues – Alpine Rhine and High Rhine, green - Upper Rhine, yellow – Middle Rhine, Orange – Lower Rhine (up to Dutch-German border) and Rhine Delta; sampling locations indicated by green stars and identified by number; approximate location of the Rhine-Ruhr-region highlighted in red

SAMPLING CONDITIONS

Samples were taken at dedicated river water quality monitoring stations of the International Association of Waterworks of the river Rhine, situated in the streambed of the river. Locations were situated at least 3 km downstream from wastewater treatment plant discharge sites. Samples were taken at a depth of 0-30cm. Four liters (two grab samples of 2 L) of surface water were taken. All samples were stored in identical plastic bottles at 4 °C before, during and after transport to the processing facility. Rainfall data was collected from national weather institutes (see *SI A 3-1*).

Temporal Monitoring

Time-series sampling was performed at the locations Lobith (L6) and Utrecht (L8), approximately six months after the spatial sampling campaign (*3.3.3*). The following samples were taken (day 1 - 24th of October 2017): day 1 (0h, 5h, 10h), day 7 and day 30. Spatial samples were taken into account for temporal analysis. L6 was chosen for its high number of detected ARGs and L8 due to this being the most downstream location where potential fluctuations were hypothesized to be amplified.

Spatial Monitoring

For spatial monitoring, all samples were taken on May 3, 2017. Samples were taken at the following location (increasingly downstream): Diepoldsau (L1, CH), Basel (L2, CH), close to Darmstadt (L3, DE), Cologne, Düsseldorf and Lobith (L4 – L6), Arnhem (L7, NL) and from the Lek at Utrecht/Nieuwegein (L8, NL). L2 and L3 are locations with extensive, well-developed pharmaceutical industries. L4 and L5 fall into a densely populated German region called the Rhine-Ruhr-region. Meteorological conditions were comparable across sampling locations (see *SI A.3-1*).

3.3.3 Filtration and DNA Extraction

Samples were filtered (300 mL) and DNA extracted from sample duplicates. DNA extract duplicates were then pooled for further analysis and stored at -30 °C. Filtration and DNA extraction were performed as previously described [344].

3.3.4 Antibiotic Resistance Gene Quantification

13 ARGs, *Intl*1 and an internal control (IC) were quantified by multiplex qPCR using TaqMan. The IC is a DNA sequence added to the sample before DNA extraction to identify potential DNA loss during/ due to the DNA extraction process, to account for this loss and make data from different samples more comparable [252]. 16S rRNA gene concentrations were quantified using SYBR Green. TaqMan and SYBR Green assays were comparable as previously confirmed by comparison studies conducted using TaqMan and SYBR Green assays for all genes within the multiplex qPCR assays (*chapter 3.3.1*). All qPCR assays were performed on a CFX96 system (Bio-Rad). Each reaction was carried out in a final volume of 50 µL containing 10 µL DNA extract. Primer and probe sequences, as well as cycling conditions, standards and controls used are documented in the supplementary material (*SI Tables 3-1 – 3-3*).

Each sample was analyzed by qPCR at least thrice in separate qPCR runs using duplicate wells.

Primers, standards and probes were obtained from Integrated DNA Technologies, BVBA Belgium.

3.3.5 Antibiotic Consumption and Environmental Data

ARG concentrations were correlated to national human [278] and veterinary [345] antibiotic usage data as well as to agricultural, farming and environmental data (*SI A.3-3*) obtained from European and national surveillance agency reports and databases.

Numbers from human and veterinary antibiotic usage data were added to obtain the antibiotic load used for correlation analysis. Antibiotic data used was country-specific so that the same national antibiotic average was assigned to the locations within a country.

3.3.6 Data Analysis

Python 3.6.0 was used to calculate descriptive statistics and correlations and to create data visualizations. R version 3.5.0 was used to perform inferential statistics, including Student's t-test, Welch's F-test and ANOVA.

Mean and standard deviations (std) were calculated using the results from all qPCR replicates for each sampling location for the (a) spatial samples only, and (b) all temporal samples.

Significant differences between experiments and/or measurements were detected by employing paired or unpaired Student's t-Tests and two samples/measurements were defined to be significantly different from each other when the calculated p-value was lower than 0.05. A test of variance between temporal and spatial data was calculated in a one-sided F-test. When necessary, data was $\log^{10}$-transformed to comply with the assumption of normal distribution of the data.

Regression plots were generated using the .regplot() function in Seaborn 0.9.0 (a Python package for statistical data visualization) [346,347].

Unless otherwise stated, all gene concentrations are relative concentrations normalized to 16S rRNA concentrations.

3.4. Results and Discussion

3.4.1 Multiplex Assays

Gene primers and probes were tested for functionality in SYBR Green II and TaqMan qPCR assays, and for potential incorporation into multiplex qPCR assays (*SI Table 3-1*). Genes were combined into multiplex qPCR assays depending on the optimal annealing temperatures (obtained during annealing temperature tests, *SI Table 3-2*), preliminary computational interaction tests (based on ΔG calculations) and, whenever possible, by amplicon length.

Out of the tested primer-probe combinations, 15 were suitable for multiplex qPCR inclusion and five multiplex assays, quantifying three genes each, were developed (**Table 3-1**).

Table 3-1: Overview of developed multiplex qPCR assays, including ARGs in assay, optimal annealing temperature and limit of quantification (LOQ)

Multiplex	1	2	3	4	5
ARG 1	*sul*1	*tet*B	*mec*A	*van*A	*erm*B
ARG 2	IC	*bla*$_{SHV}$	*bla*$_{OXA}$	*van*B	*erm*F
ARG 3	*qnr*S	*Intl*1	*aph*(III)a	*tet*M	*bla*$_{KPC}$
Annealing Temperature	58 °C	60 °C	58 °C	56 °C	57 °C
Quantification Limit	2.5E00 gene copies / µL DNA extract				

Quality control tests experimentally verified that no interaction took place between primer pair and probe sequences of different genes within an assay, to ensure an independent quantification of the individual ARGs within each of the multiplex assays. All ARGs could be accurately quantified even under conditions where one of the assay genes was present in concentrations up to 1000-fold higher than the other genes. Finally, quantification results obtained by multiplex qPCR assay were compared to quantification results obtained by simplex and SYBR Green II assay. The same quantification results were obtained for multiplex assays 1, 2, 3 and 5, these assays are accurate up to one decimal number (*SI Figure 3-1*). Multiplex assay 4 showed elevated results outside of the margin of error; gene concentrations were estimated up to 3 times higher than results obtained by SYBR Green II assays. The accuracy of this assay is therefore lower and obtained gene concentrations are order-of-magnitude rather than exact numbers (*SI Figure 3-1*).

VALIDATION OF ACCURACY, EFFICIENCY AND PRECISION

The LoQ is 2.5E+00 gene copies/ µL DNA extract for all genes, with exception of genes within multiplex assay 4 (semi-quantitative), as this was the lowest concentration at which replicates showed a CV ≤ 35%. The LoD is 5E-01 for all genes in the multiplex assays 1 and 3 as well as for *tetB* and *bla*$_{SHV}$. The LoD is 1.0E+00 for all genes within multiplex assay 5 as well as for *IntI1*. Standard curves for all multiplex assays including the efficiency, R^2, the slope and the y-intercept can be seen under *SI Figures 3-2 – 3-6*. Concentrations to determine the LoD started from 5.0E-01 and increased by 5.0E-01 steps.

Intra – and interassay variation were determined by calculating the CV based on the ΔC_t for each gene within the five multiplex assays. Intra-assay variation was < 1.5% for all genes (range: 0.26 % – 1.4 %), while inter-assay variation was < 2.5 % (range: 0.49 % – 2.3 %). Amplification efficiencies for the different genes in the multiplex assays ranged from 86 % - 104 %, with genes within individual multiplex assays never differing more than 10 % in their efficiencies during one qPCR experiment (*SI A.3-2*). As efficiencies did not differ more than 10 % between the genes within a multiplex during individual qPCR reactions, amplification of these genes is directly comparable.

3.4.2 Temporal variation of ARG concentrations

Multiplex qPCR assays were applied to river Rhine samples to monitor temporal variation of ARGs at two sites (L6 and L8).

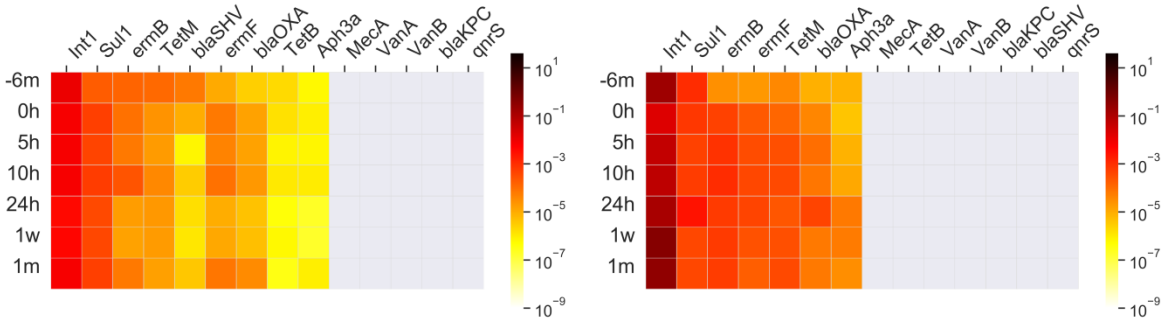


Figure 3-3: ARG Concentration Variance; Temporal Variance at Sampling Locations L6 (left) and L8 (right), scale: gene copy number per 16S rRNA shown

Intl1, sul1, ermB, ermF, TetM, blaOXA and *apha3* were detected at both sites, while *blaSHV* and *tetB* were detected consistently at L6, and consistently not detected at L8 (**Figure 3-3**). The temporal variance within samples at one location was largely not statistically significant ($p \geq 0.05$). Overall, it can be said that the observed ARG profiles are representative and consistent for both sampling locations (**Figure 3-3**).

3.4.3 Spatial variation for ARG Presence and Concentrations

Out of 14 target genes, 5 were detected at the most upstream location (L1), 7 genes at L2 and 10 genes at L3. At more downstream locations, the number of detected genes was 7, with exceptions at L6 and L7 (9 and 8 genes, respectively) (**Figure 3-4**).

The sampling location least polluted in regard to gene presence was L1 with only 5 detected genes. Concerning ARG concentrations, L6 was least polluted with total relative concentrations of 8.21E-04 ARG copies/16S rRNA gene. L3 was most polluted in terms of gene presence with 10 detected genes and L2 concerning ARG concentrations with a total relative ARG concentration of 1.47E-02 ARG copies/16S rRNA gene (**Figure 3-4**). Concentrations of 4 (out of 5) genes (*Intl*1, *sul*1, *tet*M and *erm*F) increased between L1 and L2.

Temporal fluctuations were significantly smaller than spatial variances and standard deviations ($p \leq 0.05$; *SI Table 3-4* for variances and p-value). Exceptions to this were the *erm*B gene ($p_{L6}=0.053$, $p_{L8}=0.101$) at both locations and bla_{SHV} at L6 ($p=0.341$), where the temporal variances were not significantly smaller than spatial variances. Larger variances of the bla_{SHV} gene at L6 can be explained by concentrations around the LoQ. In some samples bla_{SHV} was detected but could not be quantified, which led to larger calculated variances.

Overall, gene concentrations fluctuated with no clear trend across sampling locations (**Figure 3-4**). ter Laak et al. [348,349] state that anthropogenic pressure increases further downstream and the cumulative upstream population impacting any given location increases with further downstream locations. Due to these reasons, we assume increasing

anthropogenic pollution at more downstream locations. It is to be noted that more complex dynamics are at work, as chemical and genetic anthropogenic pollutants are subjected to decay and/or transformation in aquatic environments and might in- or decrease the impact potential of chemical compounds [151,350–353]. The hypothesis that ARG or *Intl*1 concentrations increase with anthropogenic pressure, could not be verified.

ARG *concentrations* do not seem to be an indicator for concentrations of these ARG downstream, as concentrations fluctuate between locations. Upstream gene *presence* seems to be an indicator of downstream gene presence for some genes, as shown for *aph*(III)a and *bla*$_{OXA}$, but does not necessarily determine downstream gene presence for all genes.

A lack of visible *spatial* trends has been previously observed in large rivers [354–356]. Previous studies that detected only partial consistency of ARG profiles were largely conducted on smaller rivers [211,357,358], but *temporal* consistency at individual locations in ARG presence and relative concentration has been shown in larger rivers [354]. Similarly, ARG concentrations fluctuated in surface water samples instead of showing a steady increase with increasing downstream locations, in a spatial study of the large Pearl river [354]. The large catchment area and volume of the Rhine river (flow average: 2900 m^3/s) and differences in local discharges (proximity of discharge to sampling location, presence of hospitals, agricultural practices) are possible explanations for the consistency of ARG profiles at individual sampling locations over time, as well as for the differences between ARG presence and concentration between locations.

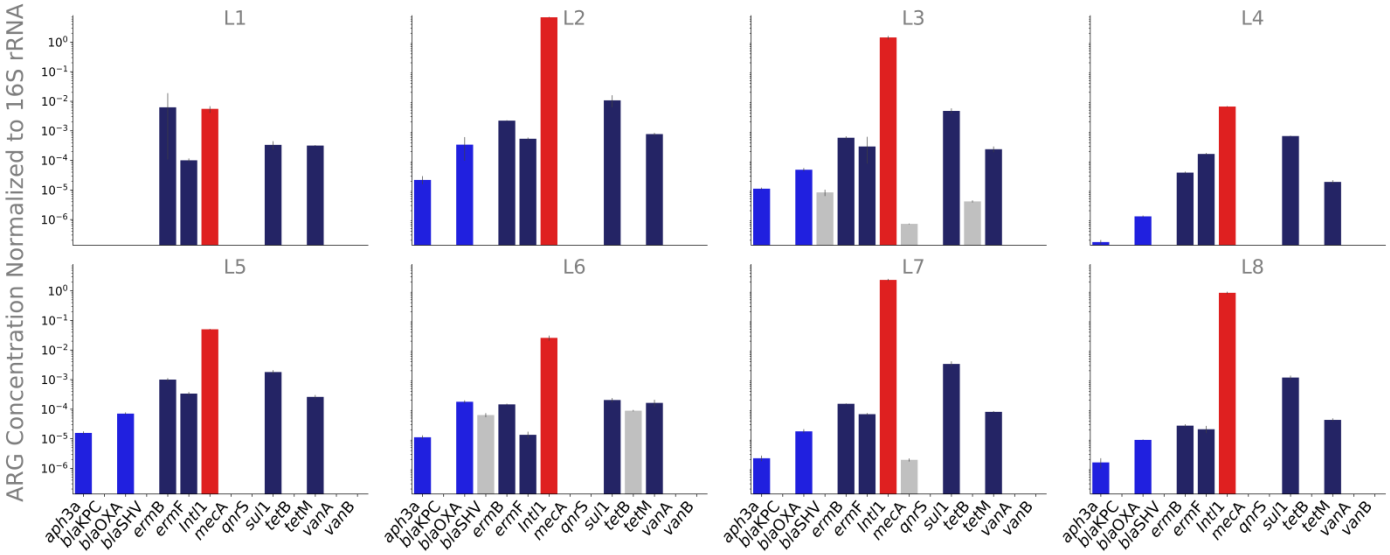


Figure 3-4: ARG Concentrations normalized to 16S rRNA at different Rhine sampling locations (mean±std); dark blue – common genes (all locations); blue – common genes (post-Lake Constance locations), grey – location-specific genes; red – *Intl*1;

Out of all monitored genes, *Intl*1 and *sul*1 were consistently detected in the highest and second-highest concentrations, with one exception at the starting location (L1) where *Intl*1 and *ermB* were detected at similar concentrations.

*Intl*1 and *sul*1 genes have previously been shown to be frequently found at high concentrations in aquatic environments [147,359–361]. In our study, *Intl*1 concentrations were

surprisingly high, reaching relative levels of $>10^0$ gene copies/16S rRNA copy at L2 and L7. Similarly, *sul*1 concentrations were unusually high at L2 when compared to the other locations (**Figure 3-4**) as well as compared to previously published concentrations in river water related samples [211,355,362], although comparable concentrations have previously been observed in river samples [363].

Individual Rhine sampling locations had unique and distinctive ARG profiles which varied from each other in gene presence and concentration. Five ARGs were detected at all sampling locations, including (**Figure 3-4**). Two additional ARGs were detected at all locations downstream from L1 (*aph*(III)a, bla_{OXA}). Significant sources of these genes seem to be present only downstream from L1, potential contributors include wastewater and agricultural discharges in Lake Constance or the Aare river.

Besides the omnipresent Rhine genes, a second set of ARGs (bla_{SHV}, *mec*A and *tet*B) were detected sporadically at three sampling locations (L3, L6 and L7). One (L7), two (L6) or three additional genes (L3) were detected (**Figure 3-4**). bla_{KPC}, *qnr*S, *van*A and *van*B were not detected in any of the samples. These genes could be of interest as potential indicator genes for specific, emerging sources of pollution within the Rhine. Examples for such specific sources of pollution are: insufficiently treated or untreated wastewater, untreated run-off from agriculture or animal husbandry or impact from hospital wastewater. bla_{KPC} and *van*A have only rarely been detected in the environment but have previously been associated with hospital wastewaters

[259–263,268,269,276,277,344], which could make them genes of priority for monitoring, to detect potential leakage from insufficiently treated hospital wastewater.

3.4.4 Estimations of ARG Pollution Depend on the Parameter Monitored

ARG pollution in river water can be expressed and compared using different parameters. The number of ARGs detected, the (total) ARG concentration (relative to the 16S rRNA gene), the number of microorganisms carrying resistance genes or *Intl*1 concentrations can all be used as indicators. In this study, the number of ARGs detected, (relative) ARG concentrations, *Intl*1 concentrations and *sul*1 concentrations were investigated (**Table 3-2**)

Table 3-2: Presence and Relative Concentration of ARGs and Intl1; green - least polluted sample based on parameter, red – most polluted sample based on parameter; gene concentrations values in gene copies/16S rRNA

	L1	L2	L3	L4	L5	L6	L7	L8
Number of genes (incl. *Intl*1)	5	7	10	7	7	9	8	7
Gene Concentration (incl. *Intl1*)	1.76E-02	6.90E+00	1.44E+00	7.61E-03	5.39E-02	2.35E+00	2.89E-02	8.59E-01
ARG Concentration (excl. *Intl*1)	6.97E-03	1.47E-02	5.89E-03	8.98E-04	3.42E-03	8.21E-04	3.71E-03	1.14E-03

*Intl*1 concentration	6.70E-03	6.90E+00	1.50E+00	6.60E-03	5.00E-02	2.60E-02	2.40E+00	8.40E-01
*sul*1 concentration	3.35E-04	1.07E-02	4.72E-03	6.69E-04	1.70E-03	1.99E-04	3.38E-03	1.04E-03

The lowest and highest number of ARGs could be detected in L1 and L3, respectively. The lowest and highest ARG concentrations were detected in L6 and L2, respectively. *Intl*1, which has previously been suggested as an indicator for environmental pollution with ARGs of anthropogenic origin [333], showed high correlations with total ARG concentrations but low correlations with the number of ARGs detected per sample as well as with ARG concentrations of individual genes (**Figure 3-5**). *Intl*1 concentrations do not continuously increase and fluctuate widely instead. In most samples (with exception of L1), *Intl*1 is present at significantly higher concentrations (usually > 2 log units higher) than the other detected ARGs, which has been previously recorded in river water [354].

Recent developments in next generation sequencing (NGS) and bioinformatics may be used to track the sources of environmental contamination, allowing to differentiate ARG profiles from different ecosystems. This might provide a better indication than the individual parameters discussed above. However, many ARGs appear to be present in multiple sources and it may be difficult to spatially differentiate the individual sources in a large river such as the Rhine [364].

*INTL*1 DOES NOT CORRELATE WITH OVERALL ARG CONCENTRATIONS BUT WITH *SUL*1 CONCENTRATIONS

Indicators and proxies have been suggested to estimate the level of (ARG) pollution of anthropogenic origin. Two examples are *Intl* [333] and *sul*1 [211]. Missing agreements on how to classify and define ARG pollution exactly (e.g through ARG presence, the proportion of resistant bacteria in a sample, relative concentration or absolute concentration) generally reduce the usefulness of such indicators. A high correlation was observed between *Intl*1 and *sul*1 (R^2=0.93) (**Figure 3-5**, middle). Strong correlations between *Intl*1 and *sul*1 have been previously described [354,365–367] and are likely, at least partly, a result of the presence of *sul*1 in the conserved region of *Intl*1 [354]. *Sul*1 is further often the most abundant (or among the most abundant) resistance genes in environmental samples, including samples in this study.

High correlations of *Intl*1 with ARG concentrations were observed but could be attributed largely to *sul*1 concentrations; correlations disappeared when excluding *sul*1 from the regression (**Figure 3-5**). The results obtained during this study suggest that the strength of *Intl*1 as an indicator for ARG pollution strongly depends on the parameter used to determine ARG pollution and that it might not be the best indicator or at the very least show that the strength of this indicator is strongly dependent on additional factors. Another such example is a study conducted by Zhang et al. [356] during which *Intl*1 was found in very low abundance when compared to the *tet*A and *tet*C in samples from a highly polluted lake in China. It is to be noted, that the selection of certain genes of interest for qPCR analysis might represent a slight bias, as other ARGs are not taken into account.

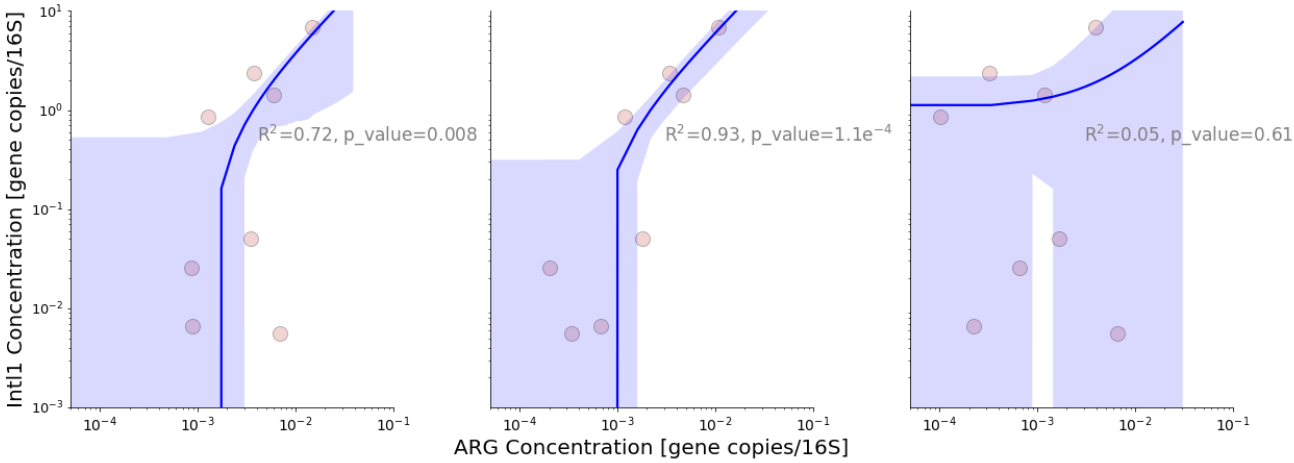


Figure 3-5: Regression Plot Showing Correlation Between Intl1 and Total ARG Concentrations; shown on x-axis: left – total relative ARG concentration; middle – sul1 concentration; right - total relative ARG concentration excluding sul1; linear least-squares regression used for calculation of R2 and p-value

ANTIBIOTIC RESISTANCE GENE CORRELATIONS WITH OTHER FACTORS

Detected ARG classes coincided with the most used antibiotics in the region [penicillins, tetracyclines, sulfonamides and macrolides] [278,345]. Antibiotic sales and usage within a region has previously been shown to influence antibiotic concentrations in aquatic environments [348], so that this data can potentially be used as an estimate for expected variation of antibiotic concentrations between countries.

Antibiotic usage and the preferred class of antibiotic used varied widely between the three countries [278,345]. Nevertheless, ARG concentrations in the different countries showed little to no correlation with antibiotic use (*SI Table 3-5*), as has previously been observed [368] There were two exceptions (*SI Table 3-5)*: sulfonamide concentrations correlated weakly to moderately with *Intl*1 and *sul*1 (R^2=0.25 and R^2=0.17, respectively); and aminoglycoside concentrations did interestingly not only correlate with *aph*(III)a (R^2=0.58), but also with *bla*$_{OXA}$ (R^2=0.58), *erm*B (R^2=0.68), *erm*F (R^2=0.42) and *tet*M (R^2=0.68). *bla*$_{OXA}$, *erm*B, *erm*F and *tet*M resistance should not be impacted by aminoglycoside activity as their targets in the cells differ, so we don't see direct causality behind the observed correlation. Further, individual ARG concentrations correlated most with *aph*(III)a gene concentrations. The highest correlations could be observed between *aph*(III)a and, *tet*M, *bla*$_{OXA}$ and *erm*B (R^2 = 0.66) (**Table 3-3**). A possible explanation is co-resistance mediated by transfer on MGEs also carrying *aph*(III)a, such as the conjugative transposon Tn1545 [369]. This would account for correlation with aminoglycoside usage and *aph*(III)a concentrations, both. Co-resistance due to MGEs is well-documented [297–299,370,371] and other Tn1545-like transposons (Tn6263 and Tn6331) have previously been found to confer aminoglycoside/macrolide co-resistance [372].

For a more in-depth picture, correlation analyses on regional parameters (provincial or municipal level), including non-class-segregated antibiotic sales, farming intensity, nitrate pollution, population density and untreated wastewater discharge, were conducted (see *SI A.3-3*). Only farming intensity (represented by farm animals per km^2) positively correlated with all discussed AR parameters (R^2 = 0.11 – 0.52; see *SI A.3-4*).

While ARG concentrations do not necessarily correlate with antibiotic concentrations [373], pharmaceutical pollution, including disinfectant and heavy metal pollution can be an influencing factor, especially in *Intl*1 selection [300,374–378]. L2 and L3 are locations with an extensive pharmaceutical industry (incl. L2 – Novartis, Basilea etc, L3 – Merck, Bayer, Steigerwald etc.).

Discharges from antibiotics manufacturing have previously been shown to be able to increase ARG concentrations locally [374] and have been mentioned as being higher-risk hotspots [195]. It is not known whether pollution from the pharmaceutical industry [379] is responsible for the observed increases in ARG and Intl1 presence (L3) and abundance (L2) at these locations. Other influencing factors are antibiotic use/sales and farming intensity. While L2 is in a region with intense farming, L3 is characterized by low antibiotic sales and little farming (see *SI A.3-3*).

Table 3-3: Coefficient of Determination (R2) for Correlations between various ARG concentrations and mean correlation per ARG; conditions: ≥5 common quantitative datapoints

	aph(III)a	*Intl*1	*sul*1	*tet*M	*bla*$_{OXA}$	*erm*B	*erm*F
aph(III)a	1.00	0.18	0.27	0.66	0.66	0.66	0.27
*Intl*1	-	1.00	0.62	0.33	0.06	0.01	0.13
*sul*1	-	-	1.00	0.41	0.11	0.02	0.33
*tet*M	-	-	-	1.00	0.38	0.25	0.25
bla$_{OXA}$	-	-	-	-	1.00	0.38	0.11
*erm*B	-	-	-	-	-	1.00	0.18
*erm*F	-	-	-	-	-	-	1.00
data points	7	8	8	8	7	8	8
mean	0.53	0.33	0.4	0.47	0.39	0.36	0.33

3.5. Conclusion

Multiplex qPCR assays are an efficient method to monitor antibiotic resistance genes in the environment. While second- and third generation sequencing are the high-throughput methods of choice for gene detection today, they are not considered quantitative. Other quantitative high-throughput methods like microassays and high-throughput qPCR are prone to issues, including sensitivity issues. Multiplex qPCR assays increase the throughput while maintaining the accuracy and quantitative nature of classic qPCR assays. Higher throughput methods are of special interest in environmental antibiotic resistance research as there is a continuously increasing number of genes of interest.

Antibiotic resistance in the Rhine river may be influenced by anthropogenic pollution to a certain extent as the AR rise between L1 and L3 suggests, but does not continuously increase over the course of the Rhine. Factors influencing local ARG concentrations can be river size, as data suggests that smaller rivers are more directly impacted by anthropogenic pollution, but also proximity and type of discharges of wastewater treatment plants, presence of hospitals, agricultural/farming discharges and run-off. A general link between the most used antibiotics in the Rhine catchment (penicillins, tetracyclines, sulfonamides and macrolides) and presence of ARG classes was observed

*Intl*1 concentrations are a good indicator for *sul*1 concentrations but not for overall anthropogenic ARG pollution. It is necessary to determine which factors are most relevant for the quantification of ARG pollution. Investigating correlations between *Intl*1 concentrations and data of NGS of ARG presence might be of further interest as the selection bias caused by gene selection for qPCR analysis would be minimized. The combined presence of certain ARGs on MGE could explain the observed correlation between those specific ARGs and are also relevant in health risk assessment [380].

3.6. Supplementary Material

SI Table 3-1: Primers, Probe and Target Sequences for ARGs in Multiplex qPCR Assays; greyed out sequences were tested but not used in final assays due to interaction issues or suboptimal efficiency; * = SYBR Green II assay not used in multiplex

Gene	Primers	Probe	Dye Used	Amplicon length [bp]
16S rRNA*	FW: CCTACGGGAGGCAGCAG RV: TTACCGCGGCTGCTGGCAC	-	SYBR	193
Aph(III)a	FW: ACATATCGGATTGTCCCTATACGAA RV: TCGGCCAGATCGTTATTCAGTA	TAGCTTAGACAGCCGCTTA	Tex615	82
bla_{KPC}	FW: TCGAACAGGACTTTGGCGGCT RV: GGACAGCTCCGCCACCGTCATG	CGCTGGTTCCGTGGTCA	HEX	260
bla_{OXA}	FW: CACTTACAGGAAACTTGGGGTCG RV: GATCACCATTCTAAACACACT	ACATCAAGCATAAAAGCCAAGA	FAM	79
bla_{SHV}	FW: TCGCCTGTGTATTATCTCCC RV: CATTGTGGTGATTTATCTGCG	TTGAGCAAATTAAACAAAGCGA	FAM	768
ermB	FW: GATACCGTTTACGAAATTGG RV: GAATCGAGACTTGAGTGTGC	ACAGGTAAAGGGCATTTAACGA	Tex615	364
ermF	FW: CGACACAGCTTTGGTTGAAC RV: GGACCTAACTCATAGACAAG	AATTATTTTCTGATGCCCGA	FAM	309
IC	FW: ATGACAGCCACTCCTCCG RV: GAAGACTGTTTGGTTCGTTCC	AGCAGAGACCCATTCCCTCAGAGC	TexRed	149
IntI1	FW: CGAACGAGTGGCGGAGGGTG RV: TGGGTGCCAAGCTCTCGGGTA	TCGTGATGCCTGCTTGTTCTACGGCA	Tex615	312
MecA	FW: CTTCCACATACCATCTTCTTTAAC RV: GTTGTAGTTGTCGGGTTTGG	ACGTTGCGATCAATGTTACCGT	HEX	336
qnrS	FW: GACGTGCTAACTTGCGTG RV: TGGCATTGTTGGAAACTT	TACGACATTCGTCAACTGCAAGT	FAM	118
Sul1	FW: CGCACCGGAAACATCGCTGCAC RV: TGAAGTTCCGCCGCAAGGCTCG	CGAACCTTCAAAAGCTGAAGTCGGCGT	HEX	162
TetB	FW: ACTGCCGTTTTTTCGCC RV: CCTTATCATGCCAGTCTTGC	TATTCTTCCTGCCACAAAGGCTTGGA	HEX	774
TetM	FW: GCAATTCTACTGATTTCTGC RV: CTGTTTGATTACAATTTCCGC	AAAGATGGCGTACAAGCACA	FAM	186
VanA	FW: TCTGCAATAGAGATAGCCGC RV: GGAGTAGCTATCCCAGCATT	ATACGAGCCGTTATACAT	HEX	377
VanB	FW: CCGCCATCCTCCTGCAAAAAA RV: GTGACAAACCGGAGGCGAGGA	ACACGAGCAAGCCCTCTGCA	FAM	433
AmpC	FW: TTCTATCAAMACTGGCARCC RV: CCYTTTTATGTACCCAYGA	-	-	547
$bla_{CTX-M15}$	FW: CTATGGCACCACCAACGATA RV: ACGGCTTTCTGCCTTAGGTT	TCTGGTCACTTACTTCACCCAGCCT	Cy5	103
bla_{TEM-1}	FW: GAGTATTCAACATTTTCGT RV: TCACTGATTAAGCATTGGT	TATTCCCTTTTTTGCGGCAT	Tex615	857
VanA	FW: TCTGCAATAGAGATAGCCGC RV: GGAGTAGCTATCCCAGCATT	TCGTTGACATACATCGTTGCGA	HEX	377
VanB	FW: CCGCCATCCTCCTGCAAAAAA RV: GTGACAAACCGGAGGCGAGGA	ACACGAGCAAGCCCTCTGCA	Cy5	433

SI Table 3-2: Optimal annealing temperature and temperature range for SYBR Green II and TaqMan assays obtained after gradient temperature qPCR tests

Gene	Optimal Annealing Temperature [°C]		Optimal Temperature Range [°C]		Tested Temperature Range [°C]
	SYBR	**TaqMan**	**SYBR**	**TaqMan**	
***aph*(III)a**	61	56.4	56 - 61	56.4 - 61.7	55 - 63
***bla*$_{KPC}$**	-	60.9	-	55 -62	55 - 62
***bla*$_{OXA}$**	55	55	55 - 60	55 - 58.5	55 - 63
***bla*$_{SHV}$**	59.4	59.4	59 - 60.2	59 - 60.2	58 - 65
***erm*B**		56.4		55 -60	55 - 62
***erm*F**		59.5		56.5 – 59.5	55 - 62
IC	58.6	59.80	58 - 60	58 - 60	56 - 60
***Intl*1**	64	64	59.5 - 64	59.5 - 64	59 - 65
***mec*A**	57.2	60.1	55 - 58.4	55 - 60.1	55 - 63
***qnr*S**	56.8	57.6	56 -59	56 -58.6	59 - 60
***Sul*1**	59.4	59.4	56 - 60	56 - 60	56 - 60
***tet*B**	-	60.2	-	59 – 61.5	59 - 60
***tet*M**	56	56.4	56 - 58.5	55.5 -59	55 - 63
***van*A**	56.4	59.8 / 61	56 - 62	56 -62	55 - 63
***van*B**	-	59.8 / 61	-	55.5 -63	55 - 63

SI Table 3-3: Multiplex qPCR Cycling Conditions/Program

Step	Temperature [°C]	Duration [mm:ss]
1	95	05:00
2	95	00:30
3	Optimal Annealing (see *SI Table 2*)	00:30
4	72 + Plate Read	00:40
5	GOTO Step 2, 45 more times **END**	

SI Table 3-4: Significance of difference between temporal measurements at L6 and L8 (p-val) as well as temporal variance and spatial variance ("all") measured using one-sided F-test; p ≥ 0.05 highlighted

	Location				
	Lek (Utrecht)		Lobith		all (spatial data)
Gene	p-val (F-test)	variance	p-val (F-test)	variance	variance
Int1	<2.2E-16	0.126	<2.2E-16	0.083	2.312
Sul1	1.32E-06	0.105	<2.2E-16	0.03	1.016
ermB	0.101	0.175	0.053	0.276	0.676
ermF	2.53E-14	0.032	3.20E-08	0.055	0.45
TetM	0.03	0.11	6.80E-06	0.07	0.258
blaOXA	1.43E-07	0.13	1.42E-07	0.188	0.755
Aph3a	0.045	0.149	<2.2E-16	0.038	0.577
TetB	-	-	0.047	0.461	0.933
blaSHV	-	-	0.341	0.359	0.178

	L8		L6		all (spatial data)
Gene	**p-val**	variance	**p-val**	variance	variance
***Int*1**	<2.2E-16	0.126	<2.2E-16	0.083	2.312
***sul*1**	1.32E-06	0.105	<2.2E-16	0.03	1.016
***erm*B**	0.101	0.175	0.053	0.276	0.676
***erm*F**	2.53E-14	0.032	3.20E-08	0.055	0.45
***tet*M**	0.03	0.11	6.80E-06	0.07	0.258
***bla*$_{OXA}$**	1.43E-07	0.13	1.42E-07	0.188	0.755
***aph*3a**	0.045	0.149	<2.2E-16	0.038	0.577
***tet*B**	-	-	0.047	0.461	0.933
***bla*$_{SHV}$**	-	-	0.341	0.359	0.178

SI Table 3-5: Correlation between the concentration of detected antibiotic resistance genes and antibiotics shown as the coefficient of determination (R2), condition: ≥5 common quantitative data points; genes not meeting the condition criteria in grey; *AMG – Aminoglycosides, ML – Macrolides, PEN – Penicillins, QNL – Quinolones, SUL – Sulfonamides, TET - Tetracyclines*

	Location				
	Lek (Utrecht)		Lobith		all (spatial data)
Gene	p-val (F-test)	variance	p-val (F-test)	variance	variance
Int1	<2.2E-16	0.126	<2.2E-16	0.083	2.312
Sul1	1.32E-06	0.105	<2.2E-16	0.03	1.016
ermB	0.101	0.175	0.053	0.276	0.676
ermF	2.53E-14	0.032	3.20E-08	0.055	0.45
TetM	0.03	0.11	6.80E-06	0.07	0.258
blaOXA	1.43E-07	0.13	1.42E-07	0.188	0.755
Aph3a	0.045	0.149	<2.2E-16	0.038	0.577
TetB	-	-	0.047	0.461	0.933
blaSHV	-	-	0.341	0.359	0.178

Gene	**aph3a**	**bla$_{OXA}$**	**bla$_{SHV}$**	**ermB**	**ermF**	**Int1**	**mecA**	**sul1**	**tetB**	**tetM**
AMG Use	0.58	0.58		0.68	0.42	-0.25	-1	0		0.68
ML Use	-0.13	-0. 13		-0.30	-0.10	-0.25	-1	-0.12		-0.30
PEN Use	-0.12	-0.12		-0.34	-0.10	-0.25	-1	-0.17		-0.34
QNL Use	-0.10	-0.10		-0.32	-0.10	-0.23	-1	-0.18		-0.32
SUL Use	0.12	0.12		0.34	0.10	0.25	1	0.17		0.34
TET Use	-0.58	-0.58		-0.68	-0.42	0.25	1	0		-0.68

SI A.3-1: Meteorological Conditions

No rain was recorded by sampling collaborators for 03.05.2017 during or before sampling. Sampling was organized for the early – mid-morning at all sampling locations (rain events recorded on references websites might have occurred after sampling). Very light to medium-light rain was recorded the day prior to the sampling event (1.57 mm/day – 8.8 mm/day).

Relevant officially recorded historical meteorological data can be found on the following websites:

https://www.meteoschweiz.admin.ch/product/output/climate-data/climate-time-series-processing/SAE/dailyevol_SAE_2017_G.pdf (L1) https://www.meteoschweiz.admin.ch/home/klima/schweizer-klima-im-detail/jahresverlauf-an-stationen.html?filters=2017_2017 (L2)
https://www.wetterkontor.de/de/wetter/deutschland/rueckblick.asp?id=L886&datum=05.05.2017&t=2 (L3)
https://www.wetterkontor.de/de/wetter/deutschland/rueckblick.asp?id=103&datum=05.05.2017&t=2 (L4)
https://www.wetterkontor.de/de/wetter/deutschland/rueckblick.asp?id=103&datum=05.05.2017&t=2 (L5)
https://www.wetterkontor.de/de/wetter/deutschland/rueckblick.asp?id=H203&datum=05.05.2017&t=2 (L6)
https://www.worldweatheronline.com/arnhem-weather-history/gelderland/nl.aspx (L7)
https://www.worldweatheronline.com/nieuwegein-weather-history/utrecht/nl.aspx (L8)

SI A.3-2: Efficiency ranges per multiplex assay

Over the course of 5 experiments efficiencies in the following ranges were detected: SIQ [99 % - 103 %], TSI [96 % - 105%], MOA [91 % - 102%], EEK [86 % - 99 %], BAM [93 % -104 %]).

SI A.3-3: Agricultural, farming and environmental data

Data was collected either at municipal level ("city") or at province-level ("region"). As data available was largely not stated as normalized data per km², the authors gathered data about the area of the different locations ("Region Area","City Area") and calculated the values per are (/km²) to make them comparable across different-sized cities and regions, where necessary.

Code	Name	Region name	Region area	City area	Population [region]	Population density region [inhabitants/km2]	Total number of large livestock in region	Large livestock per km2 [region]	Manure storage facilities per city	Manure storage facilities / km2 [city]	Nitrate Excess in ug/l	Antibiotic sales per region in tons	Total Wastewater generation and discharge by river basin district (RBD)	Total Wastewater generation and discharge by river basin district (RBD) per km2	Total Wastewater discharged without treatment per RBD	Total Wastewater discharged without treatment per RBD / km2 [region]
L1	Diepoldsau	Ostschweiz	11521	11.23	1174990	101.99	622270	54.01	n/a	n/a	n/a	n/a	118.61	0.01	1.56	0.0001
L2	Basel	Nordwestschweiz	1958	23.85	1158508	591.68	1120470	572.25	13	0.55	10	n/a	76.69	0.4	4.4	0.002
L3	Darmstadt	Hessen	21115	122.23	6265809	296.75	1072700	50.80	2735	22.38	15	5	809.45	0.04	126.85	0.006

	L4	L5	L6	L7	L8
	Cologne	DD	Lobith	Arnhem	Utrecht
	Nordrheinwestfalen	Nordrheinwestfalen	Gelderland	Gelderland	Utrecht
	34110	34110	5137	5137	1386
	405.15	217.41	80.11	101.54	99.21
	17912134	17912134	9969356	2026578	1284504
	525.13	525.13	1940.70	394.51	926.7705628
	8467500	8467500	2797000	2797000	473000
	248.24	248.24	544.48	544.48	341.2698413
	2838	2955	n/a	770	2020
	7.00	13.59	n/a	7.58	20.36085072
	35	45	150	n/a	n/a
	5	5	30	n/a	n/a
	2328.66	2328.66	1200.655	1200.655	72.65
	0.07	0.07	0.04	0.04	0.052417027
	433.86	433.86	220.305	220.31	6.75
	0.01	0.01	0.006	0.006	0.005

Information was obtained from the following sources:

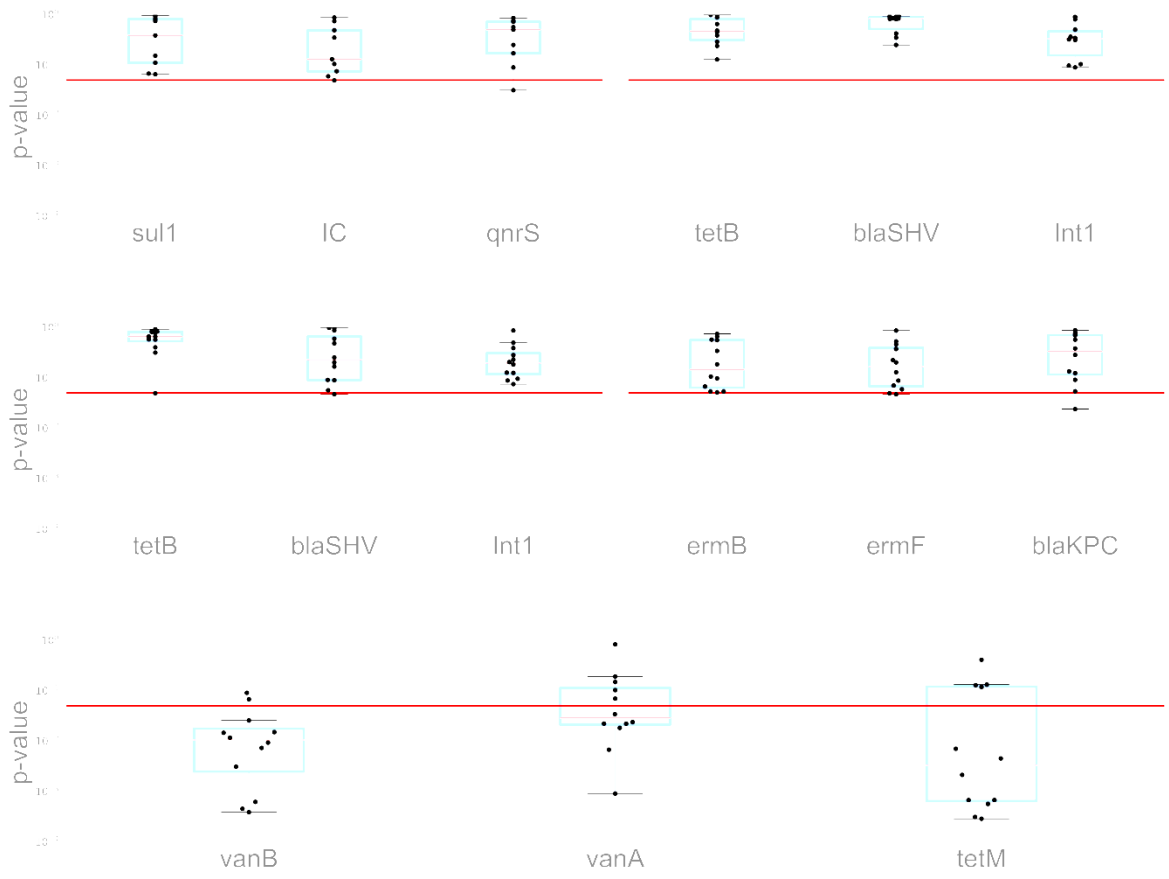
- https://ec.europa.eu/eurostat/web/regions/data/database
- https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=aei_fm_ms&lang=en [manure storage facility by region]
- https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=agr_r_animal&lang=en [farm animal number by region]
- https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_wwgen_rb&lang=en [WW (treated and untreated) discharged by region)]
- https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_watpop_rb&lang=en [Pop. connected to public water supply by region]
- https://www.bfs.admin.ch/bfs/de/home/statistiken/kataloge-datenbanken/daten.assetdetail.8346720.html [farm animals by region Switzerland]
- https://www.umweltbundesamt.de/publikationen/antibiotika-antibiotikaresistenzen-in-der-umwelt [regional antibiotic sales for Germany and border regions + graphic presentation/maps added to SI material]
- https://www.umweltbundesamt.de/publikationen/antibiotika-antiparasitika-im-grundwasser-unter [additional background info regarding correlations between farm animals and antibiotic concentrations in groundwater]

SI A.3-4: Correlations between AR pollution parameters and collected regional data; R2 shown

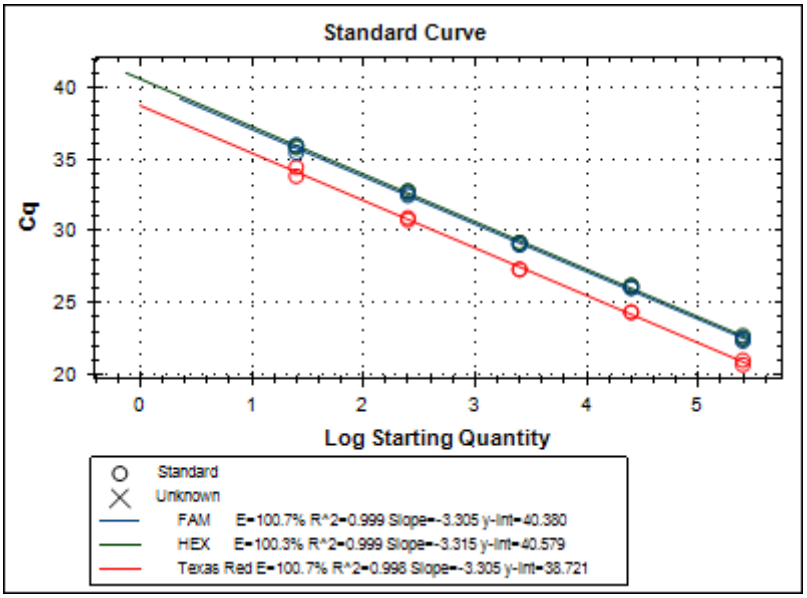
Based on regional data collected (see *SI A.3*) a correlation analysis was conducted between that data and the antibiotic resistance pollution parameters discussed in the present work. Reliable p-values (significance) could not be calculated due to the low number of observations.

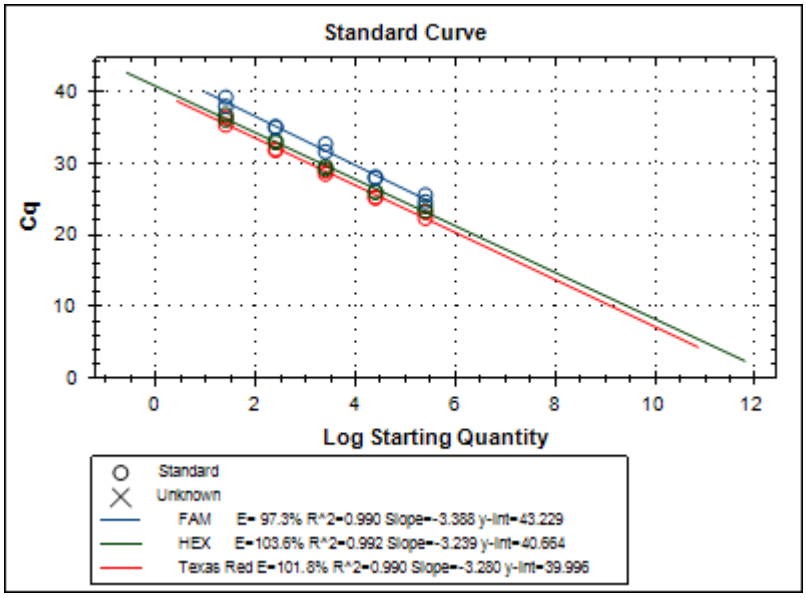
	Number of genes	**Gene Concentration (incl. *Intl*1)**	**ARG Concentration (excl. *Intl*1)**	***Intl*1 concentration**	***sul*1 concentration**
population [region]	0.12	-0.34	-0.48	-0.5	-0.38
population density region [inhabitants/km2]	0.38	0.23	-0.4	-0.15	-0.24
total number of large livestock in region	-0.07	-0.37	-0.41	-0.38	-0.31
large livestock per km2 [region]	0.17	0.52	0.11	0.52	0.35
manure facilities per city	0.19	-0.7	-0.7	-0.89	-0.75
manure facilities / km2 [city]	0.53	-0.5	-0.51	-0.6	-0.5
Nitrate Excess in µg/L	0.28	-0.16	-0.59	-0.49	-0.61
Antibiotic sales per region in tons	0.23	0.74	-0.52	-0.42	-0.57
Total Wastewater generation and discharge by river basin district (RBD)	0.17	-0.46	-0.52	-0.46	-0.37
Total Wastewater generation and discharge by river basin district (RBD) per km2	0.16	-0.16	-0.42	-0.16	-0.1
Total Wastewater discharged without treatment per RBD	0.16	-0.45	-0.53	-0.45	-0.37
Total Wastewater discharged without treatment per RBD / km2 [region]	0.22	-0.41	-0.56	-0.41	-0.32

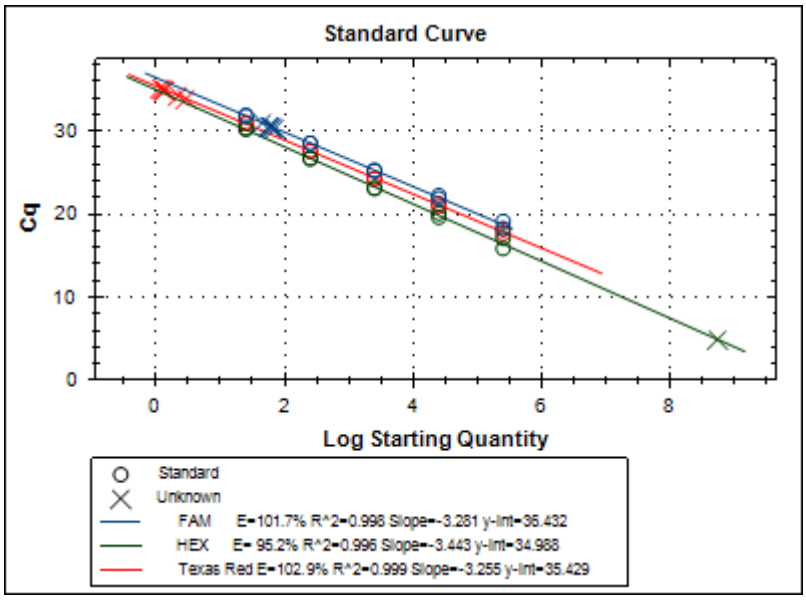
SI Figure 3-1: Difference between gene concentrations obtained by SYBR Green II assay and by multiplex qPCR assays; red line: p = 0.05; black dots = p-value obtained for each precision /quantification experiment per gene (12 total with varying concentrations), 1st row: multiplex 1 (left) and multiplex 2 (right), 2nd row: multiplex 3 (left) and multiplex 4 (right), 3rd row: multiplex 5

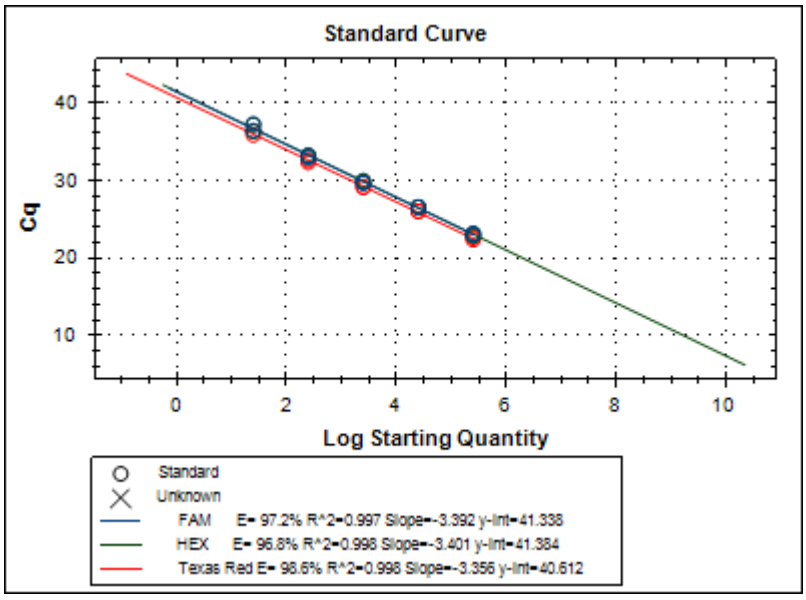


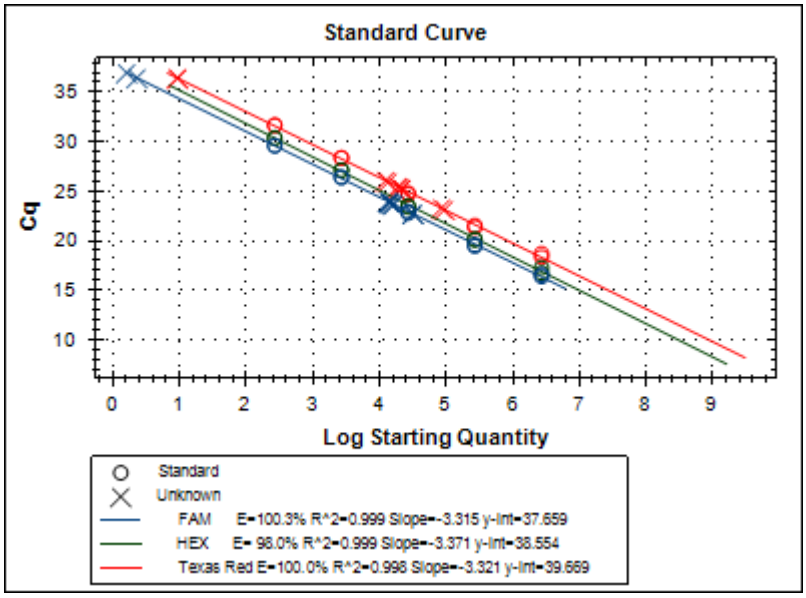
SI Figures 3-2 – 3-6: Multiplex Assay efficiencies, R2, slopes and y-intercepts for standard curves; from top to bottom: multiplex 1 - 5




Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
FAM E= 97.3% R^2=0.990 Slope=-3.388 y-Int=43.229
HEX E=103.6% R^2=0.992 Slope=-3.239 y-Int=40.664
Texas Red E=101.8% R^2=0.990 Slope=-3.280 y-Int=39.996


Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
FAM E=101.7% R^2=0.998 Slope=-3.281 y-Int=36.432
HEX E= 95.2% R^2=0.996 Slope=-3.443 y-Int=34.988
Texas Red E=102.9% R^2=0.999 Slope=-3.255 y-Int=35.429


Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
FAM E= 97.2% R^2=0.997 Slope=-3.392 y-Int=41.338
HEX E= 96.8% R^2=0.998 Slope=-3.401 y-Int=41.384
Texas Red E= 98.6% R^2=0.998 Slope=-3.356 y-Int=40.612


Standard Curve
Cq
35
30
25
20
15
10
0
1
2
3
4
5
6
7
8
9
Log Starting Quantity
Standard
Unknown
FAM E=100.3% R^2=0.999 Slope=-3.315 y-Int=37.659
HEX E= 98.0% R^2=0.999 Slope=-3.371 y-Int=38.554
Texas Red E=100.0% R^2=0.998 Slope=-3.321 y-Int=39.669

4. The Impact of Sub-inhibitory and Inhibitory Tetracycline Concentrations on Horizontal Gene Transfer and Bacterial Wastewater Effluent Community Composition

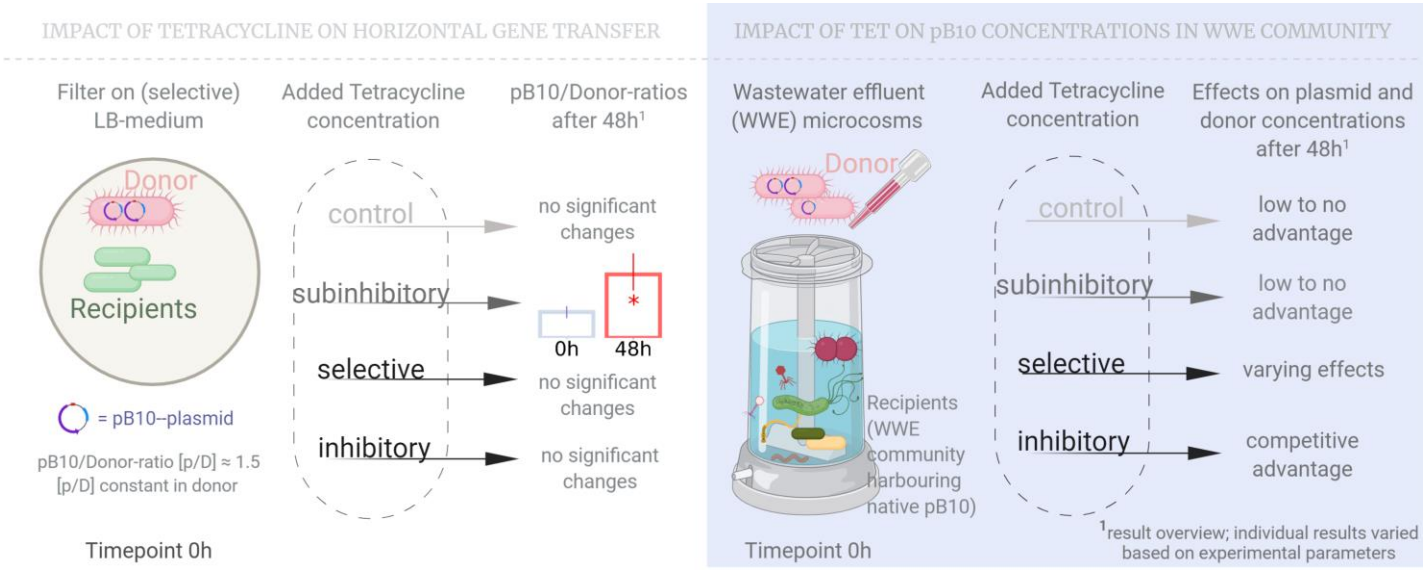


Figure 4-1: Graphical Abstract; Chapter 4

4.1. Abstract

The present study investigates the transfer of an antibiotic resistance gene-carrying plasmid under selective pressure and the effect of different compound concentrations on the plasmid and donor within a bacterial wastewater effluent (WWE) community. Different concentrations of tetracycline or Ca^{2+} were used, to test their effect on horizontal gene transfer (HGT) using a donor (*Escherichia coli* DH5α) carrying the plasmid. Experiments were conducted on filters on nutrition medium as well as in microcosms using WWE as medium. *Acinetobacter baylyi* or WWE community bacteria were used as recipients. Sub-inhibitory tetracycline concentrations were chosen to mimic concentrations found in the environment or wastewater treatment plants (WWTPs), while higher concentrations were in the inhibitory range.

We show that very low sub-inhibitory antibiotic concentrations can be a driving factor of HGT, with ARG concentrations increasing up to over 10-fold more than in controls. We further show that selective and inhibitory concentrations require prior resistance to have an effect on HGT. In filter experiments with A.*baylyi* sub-inhibitory tetracycline concentrations consistently increase transfer, in contrast to selective and inhibitory concentrations, which inhibited growth of non-resistant recipients to an extent that impeded transfer. Filter experiments with a native WWE community showed distinct effects for different sub-inhibitory concentrations: very low sub-inhibitory concentrations (1.5 ng/ml) had the most pronounced effect while higher sub-inhibitory concentrations (15 ng/ml) did not exhibit an effect. Further, higher donor concentrations could be shown to increase pB10/donor-ratios using selective tetracycline concentrations in these experiments.

Microcosm experiments rendered varying results across experimental replicates, likely due to a more complex matrix with changing parameters, including varying levels of native antibiotic concentrations. The WWE community harbored native pB10 plasmid, which further increased complexity. Sub-inhibitory tetracycline concentrations did not show any effect, but selective concentrations had a driving impact on plasmid propagation. Donor numbers strongly increased only at high selective concentrations, indicating a selective advantage when compared to a majority of the WWE community. Ca^{2+} counteracted reductions in resistance levels when added at high concentrations (25 mM) and might be conducive to HGT.

On base of these results, we highlight that modern wastewater systems can present a risk factor for increased potential of ARG propagation, beyond the confines of their immediate surrounding. This is the case as they connect high selective pressure, high-resistance wastewaters with sub-inhibitory concentration, low-resistance natural environments, which could be shown to be a potentantial poentiator of propagation of ARG-carrying plamids.

4.2. Introduction

Wastewater treatment plants (WWTPs) are considered one of the major nodes in the spread of substances of emerging concern into the environment , including antibiotic resistance genes (ARGs), pharmaceuticals and microorganisms[145,324,381–384]. WWTPs may act as "hotspots" for antibiotic resistance (AR) [145] and induce ARG proliferation due to conditions considered as favorable for AR dissemination, including: an elevated concentration of selective agents, such as antimicrobials and heavy metals [123,137,282,297,385,386]; elevated stress levels and accompanying levels of induced horizontal gene transfer (HGT) [145,387] and high bacterial densities and nutrient availability [145,146].

HGT can be seen as a major driver of antibiotic resistance [388]. Transfer of ARGs between environmental and clinical strains has been observed [389] and research indicates that some clinically relevant ARGs originate in environmental microorganisms [390]. Three mechanisms of HGT have been described, free DNA uptake (transformation), DNA transfer by bacteriophages (transduction) and transfer of mobile genetic elements (e.g. plasmids) by pili structures (conjugation) [391]. While it is known that inhibitory concentrations of selective agents (e.g. antibiotics, heavy metals) can increase AR, less is known about the effect and mechanisms behind sub-inhibitory concentrations under environmental settings. However, there have been studies suggesting that sub-inhibitory concentrations similarly increase AR with underlying mechanisms like the activation of SOS-responses, cross-resistance, increased biofilm formation and elevated conjugation levels [392–400]. Nevertheless, minimum inhibitory concentrations (MIC) driving HGT are unknown for a large number of compounds [401], a fact that is important as not all compounds have the same effect of AR.

Pollutants can persist in surface waters after being discharged by WWTPs [324]. While pollutant concentrations decrease with increasing distance to WWTPs, antibiotics are frequently present in surface water bodies at sub-inhibitory concentrations, up to the range of ng/ml [402]. While an increasing amount of research is being done to determine levels of antibiotic resistance in anthropogenic, clinical and environmental settings, these findings alone are not sufficient to estimate the risks for adverse effects on human health associated with these levels [403]. A few factors that play a crucial role in determining risk are: the impact of anthropogenic pollution on ARG levels [318,404], antimicrobial source tracking [405] and the possibility of reciprocal ARG transfer between environmental bacteria and pathogens/commensals [402,403,406].

Several attempts were made to reveal the main drivers and limitations of ARGs dissemination processes in WWTPs and environmental settings. Bonot and Merlin [213] investigated the dissemination of pB10 multi-resistance plasmid in river sediment microcosms. They observed that pB10 was maintained at a steady-state level while the *E. coli* donor was quickly disappearing, which tends to show that most of the transconjugants formed at an early stage of the experiment. Similar observations were made by Bellanger et al. [407], additionally showing that transfer of this plasmid is strongly matrix dependent. The identification of environmental stressors and selective agents driving plasmid transfer in environmental settings would allow taking action to better control plasmid-based dissemination of ARGs.

The present study investigates the transfer of ARGs from a donor to *Acinetobacter baylyi (A.baylyi, also known as A.calcoaceticus)* or to an effluent wastewater bacterial community

and examines the role of various "stressors" on potential HGT of the pB10 plasmid as well as the role of sub-inhibitory concentrations of tetracycline regarding said transfer. We further show the impact of different tetracycline and Ca^{2+} concentrations on a WWE bacterial community and resistance-carrying plasmids, with or without the influence of an external resistance-carrying microorganism. *A.baylyi* is an environmental organism but has been identified as a "pathogen for opportunistic infection" [408]. The pB10 plasmid contains antibiotic resistance genes, including tetracycline resistance. Tetracycline [393,395,396,409] and Ca^{2+} [410,411] have been shown to increase antibiotic resistance and/or conjugative processes and were selected as "stressors" for our study. An increase in antibiotic resistance and potential HGT was examined under various conditions, including: 1) multiple stressor concentrations ranging from low sub-inhibitory concentrations over selective concentrations to high inhibitory concentration (2) different environments (filter system vs. microcosm system) and (3) different donor – recipient ratios.

4.3. Methods and Materials

4.3.1 Bacterial Strains

Escherichia coli DH5α (pB10) was used as a donor in all experiments [213]. *A.baylyi* was used as a recipient in filter transfer experiments; it did not harbor the pB10-plasmid. Inocula preparation is described in *SI A.4-3*.

4.3.2 Plasmid

The pB10 plasmid was used to investigate transfer. It is an IncP-1β antibiotic multi-resistance plasmid conferring resistance to 4 antibiotics (tetracycline, sulfonamide, amoxicillin and streptomycin) and mercury [412]. Tetracycline resistance in conferred by *tet*(A) which encodes for efflux pumps. 10 µg/ml was used as selective tetracycline concentrations. 0.0015, 0.015 and 0.4 µg/ml (corresponding to 1.5, 15 and 400 ng/mL) were termed sub-inhibitory concentrations and 40 µg/ml and 100 µg/ml as inhibitory concentration [413]. To avoid confusion caused by different units, all tetracycline concentrations will be given in µg/ml.

4.3.3 Media

Luria-Bertani broth (Sigma, USA) was used as growth medium.

Wastewater effluent (WWE) after biological treatment was obtained from a full-scale WWTP (located in Burgerland, Austria) with a population equivalent of 7250. Parameters for raw wastewater (influent) and effluent on a day of sampling can be found in *SI Table 4-3*. Bacterial cell numbers were determined by 16S rRNA TaqMan qPCR.

4.3.4 Pre-experiments

To determine conditions and set up controls, pre-experiments were conducted.

GROWTH CURVES

Growth rates and the time necessary to reach the stationary phase, were measured at different temperatures (20 °C and 37 °C; both strains) and tetracycline concentrations (0, 0.015, 10, 40, 100 and 250 µg/ml; donor strain).

All growth curve experiments were conducted in Luria-Bertani (LB) broth (Sigma, USA). OD_{600} was measured with Eppendorf BioSpectrometer® to evaluate growth rates.

GROWTH INHIBITION

To investigate the effect of antibiotic concentrations on donor and recipient growth, both strains were grown on LB-agar plates supplemented with tetracycline and growth inhibition was recorded after 24h and 48h (*SI Table 4-4*). Tetracycline concentrations used: 0, 0.0015, 0.015, 0.15, 1, 10 µg/ml and 100, 250 µg/ml (only donor).

PLASMID AMPLIFICATION IN DONOR CELLS

Plasmid concentrations in donor cells were observed over time at different tetracycline concentrations (0, 0.0015, 0.015, 0.4, 10, 40 and 100 µg/ml). The aim was to exclude the possibility of plasmid amplification in the donor cells and thus biased p/D-ratios due to varying plasmid levels in donor cells. Experiments were conducted in LB broth (Sigma, USA). Gene concentrations were quantified at time point 0 and after 48 h.

FREE PLASMID PERSISTENCE

Free plasmid persistence over time was investigated to obtain a baseline for subsequent free plasmid DNA transfer experiments. Persistence was tested in WWE microcosms spiked with free plasmid DNA. Samples for qPCR analysis were taken at time point 0 and after 22, 27 and 48 hours. Samples were filtered with sterile syringe filters (0.45 µm, cellulose acetate, VWR) and frozen at - 20 °C.

4.3.5 TRANSFER EXPERIMENTS

Experiments were conducted in duplicates unless otherwise stated. Each experiment was repeated at least once with WWE batches taken in different weeks and using newly-grown inocula. There were 4 replicates per experiment for each experimental condition, unless otherwise stated.

With the exception of free plasmid experiments, no discrimination between conjugation, transformation or transfection was made as the effect on total HGT was observed.

FILTER TRANSFER EXPERIMENTS

Donor and recipient cells (recipient: *A.baylyi* or WWE community bacteria) were pipetted onto a membrane filter (0.45 µm cellulose acetate, Pall Corporation, USA). Donor and A.*baylyi* inocula were pipetted onto the filter, whereas wastewater effluent was filtered to obtain WWE community bacteria. The filter was placed on LB agar containing different tetracycline concentrations and incubated for 48 h at room temperature (RT) or 37 °C. RT was 19°C to 21°C. Tetracycline concentrations were as follows:

Table 4-1: Conditions and parameters across filter experiments; * - 2 replicates

Recipient	Tetracycline concentrations used [µg/ml]	Replicates	Temp.	Donor-recipient proportions used [donor : recipient]
A.*baylyi*	0, 0.015, 10 , 100*	6 (RT); 2 (37°C);	RT, 37°C	1:1, 1:20
WWE	0, 0.0015*, 0.015, 10	4 (RT); 2 (37°C);	RT, 37°C	1:1, 1:20

MICROCOSM TRANSFER EXPERIMENTS

Donor cells or free plasmid DNA were added to 400-mL batch bioreactors. Tetracycline and Ca^{2+} (Calcium chloride, Sigma-Aldrich, Japan) were added at varying concentrations. Bioreactors were sealed to avoid contamination from external microorganisms but permitted gas exchange. A magnetic stirrer (60 rpm) ensured homogeneity. WWE was used as medium and the bacterial community as potential recipients. Non-donor control experiments were performed to be able to make statements about the role of the donor, as WWE naturally contained pB10-plasmids.

Table 4-2: Conditions and parameters across microcosm experiments; * - 2 replicates

Tetracycline concentrations used [µg/ml]	Ca^{2+} added [µg/ml]	Replicates per condition	Temp.	Donor-recipient proportions used [donor : recipient]
0, 0.0015*, 0.4, 10, 40*, 100	0	4	RT	0:1 (non-donor control); 1:1
0	0, 250, 1000	2	RT	0:1 (non-donor control); 1:1

Additionally, free plasmid DNA experiments were conducted using WWE to investigate contribution of transduction to overall HGT.

Free plasmid DNA (1E+06 pB10 copies/mL) and tetracycline (0, 1 or 10 µg/ml) were added.

4.3.6 Gene Quantification – qPCR

Sequence-specific primers and probes for the donor (*E.coli* DH5α) and for the pB10 plasmid were used [213]. Both genes as well as 16S rRNA [414,415], were quantified by qPCR. All reactions were performed in the Light-Cycler 480 (Roche Applied Science, Vienna). Primers and cycling conditions are listed in *SI Tables 4-1, 4-2 + 4-5 and SI 4-2* and were previously described [213]. qPCR efficiencies ranged between 96 % - 104 % and were therefore directly comparable.

4.3.7 Data Analysis

Statistical Analysis

Statistical analysis was performed using SciPy v.1.4.1. Dependent t-tests were conducted to assess the significance of differences between time points. $P \leq 0.05$ was considered statistically significant and significance of parameter de-/increase during the experiment is indicated as follows: *$* – p≤0.05$*, *$** – p≤0.01$*; *$*** – p≤0.001$*. Additionally, values of $0.05 \leq p \leq 0.06$ are shown in figures.

Analytical Parameters

Based on qPCR results, plasmid-per-donor-ratios (p/D-ratios) and/or relative plasmid concentrations (pB10 copies/16S rRNA copies) were calculated. p/D-ratios were used to capture HGT in filter transfer experiments while relative plasmid concentrations were used to investigate the impact of different compound concentrations on resistance in the bacterial WWE community. Additionally, pB10, *E.coli* DH5α and 16S rRNA concentrations (gene copies/mL) were analyzed for more information on the underlying mechanisms.

4.4. Results and Discussion

4.4.1 Pre-Experiments

Bacterial Growth

Recipients (*A.baylyi)* were more robust to different temperatures (*SI Figure 4-1*) and started growing sooner than donors (*E.coli* DH5α*)* at lower temperatures, indicating a competitive advantage at those temperatures.

PLASMID AMPLIFICATION UNDER SELECTIVE PRESSURE

Plasmid copy number was reported to increase in bacteria under selective pressure [416]. Therefore, the potential increases of plasmid copy number in donor cells, which could lead to overestimation of plasmid transfer events, was investigated.

Donor cells harbored one to two pB10 copies each at t_{0h} (red line, **Figure 4-2**). Plasmid concentration in donor cells did not significantly change due to selective pressure (or absence hereof) from tetracycline concentrations (within 48h, **Figure 4-2**). Therefore, we account that plasmid amplification within donor cells should not have a significant impact on p/D- ratios in transfer experiments. p/D-ratio increases up to 2 will not be attributed to HGT.

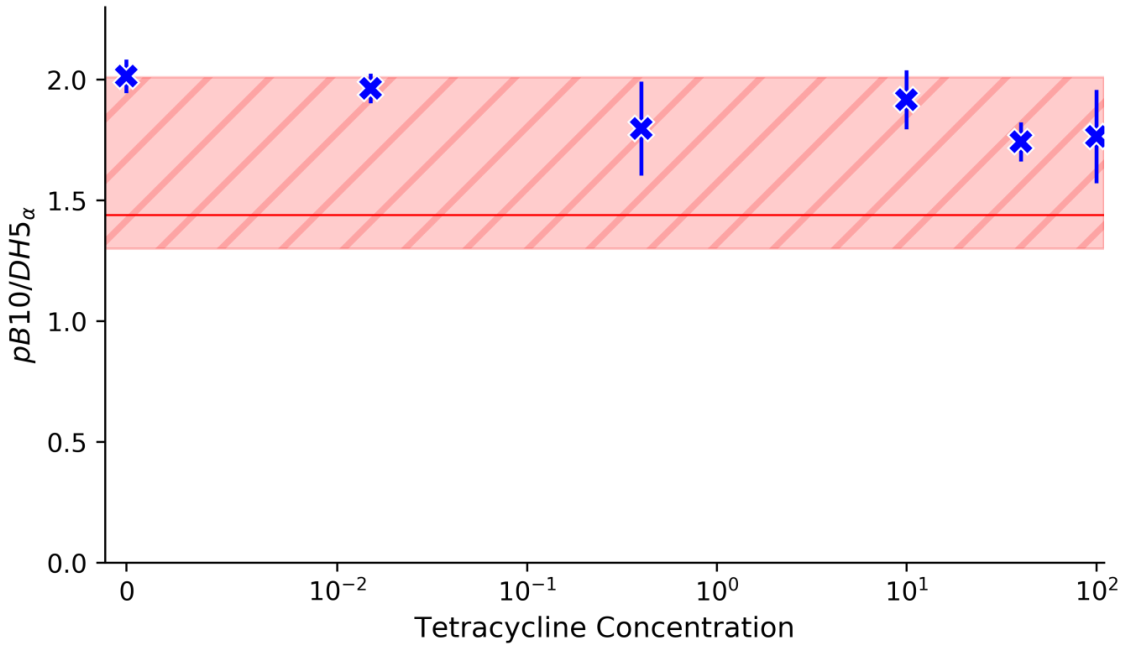


Figure 4-2:Number of plasmids (pB10) per donor cell (DH5a), shown at t=0h (red line) and after 48h (blue crosses) of incubation at different antibiotic concentrations, striped highlighted area=recorded range

FREE PLASMID PERSISTENCE

Free plasmid concentrations sharply decreased within the first 22h, as indicated by qPCR results. The total decrease of free plasmid DNA was > 99% within 48h in WWE (*SI Figure 4-2*). Comparable total degradation of free (pB10) plasmid was recorded [213,417].

4.4.2 FILTER TRANSFER EXPERIMENTS

DONOR – RECIPIENT EXPERIMENTS

To investigate the general potential of pB10 to transfer to unrelated strains, experiments with A.*baylyi* as recipient were conducted. At *t=0* p/D-ratios ranged from 1.25 – 1.75 (*Figures 4-3 – 4-5*) across all experiments. Significant p/D-ratio increases during the

experiment were attributed to HGT, as plasmid amplification/reduction in the donor was ruled out (*chapter 4.4.1*).

IMPACT OF TEMPERATURE ON PB10 TRANSFER

Initial experiments were conducted at 37°C and RT, to investigate if benefitting recipient-growth is conducive to HGT.

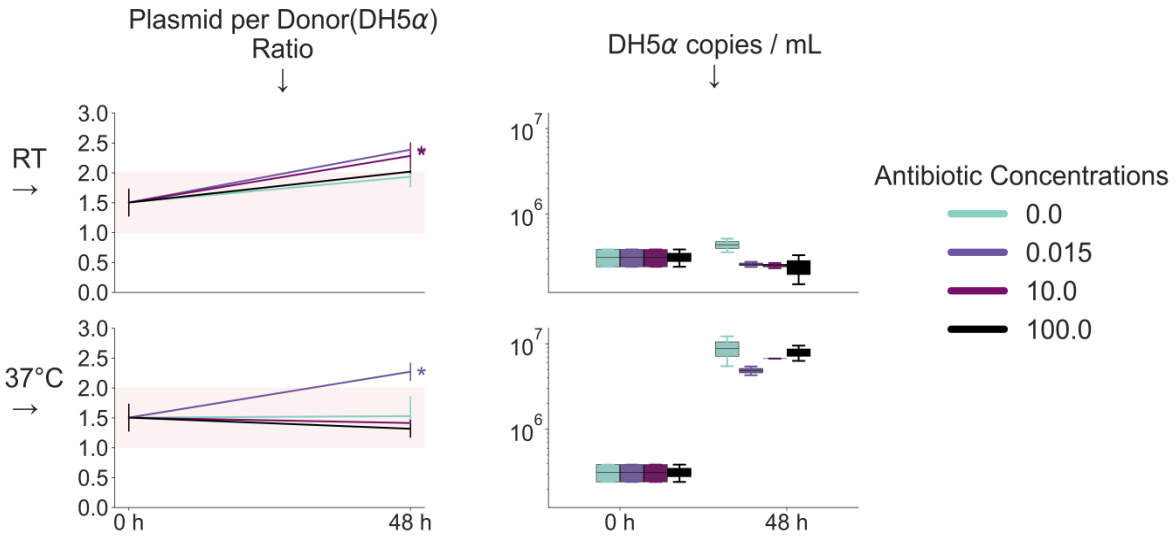


Figure 4-3: Changes in pB10/DH5a concentrations after 48h for D:R-ratios of 1:1 depending on temperature, only data from experiment 1 shown for comparison of different temperatures (recipient: A.*baylyi*); at room temperature (top column) and 37°C (bottom column); red highlighted area – p/D ratios found in donor; antibiotic used: tetracycline

For experiments with donor-to-recipient-proportions of 1:1 ($D:R_{1:1}$, **Figure 4-3**) no significant changes in p/D-ratios could be detected after 48h for tetracycline concentrations of 0 µg/ml (both temperatures) and for tetracycline concentrations of 10 and 100 µg/ml at 37°C. p/D-increases could be detected for Tet_{10} at RT, as well as for $Tet_{0.015}$ at 37°C. For $Tet_{0.015}$, p/D-ratios after 48h were further significantly higher than in control experiments (Tet_0) (**Figure 4-3**).

For $D:R_{1:20}$ (*Fig.4.4*) no significant changes in p/D-ratios were detected at 37 °C. Significant changes were detected for all tetracycline concentrations at RT. Tet_0 and $Tet_{0.015}$ showed the most pronounced increase.

Lower temperatures are likely conducive to HGT under these conditions, because the recipient outgrows the donor. *A.baylyi* shows faster growth at lower temperatures (SI.*Fig.1*) and has previously been shown to grow at a wide range of temperatures (20°C–45°C) [418,419]. To more closely investigate and verify that these conditions are conducive to HGT, all subsequent transfer experiments were conducted at RT. Subsequent *A.baylyi* filter experiments were conducted with tetracycline concentrations between 0 – 10 µg/mL.

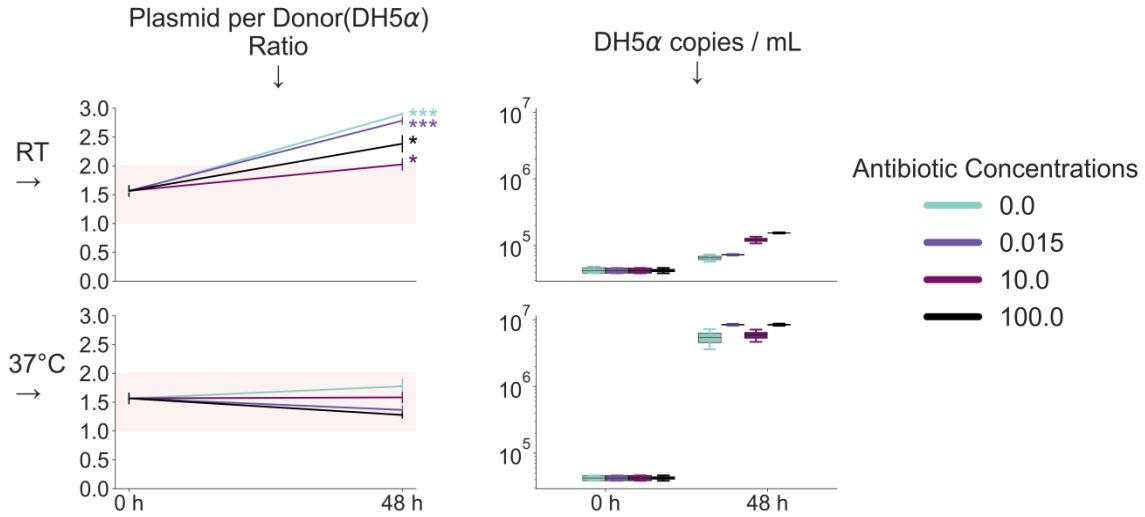


Figure 4-4: Changes in pB10/DH5a concentrations after 48h for D:R-ratios of 1:20 depending on temperature, only data from experiment 1 shown for comparison of different temperatures (recipient: A.*baylyi)* ; at RT (top column) and 37°C ; antibiotic used: tetracycline; red highlighted area (column 1) – p/D-ratios natively found in donor

Results could largely be reproduced (**Figure 4-5**). Sub-inhibitory tetracycline concentrations ($Tet_{0.0015}$ + $Tet_{0.015}$) increased p/D-ratios for both tested D:R-proportions, showing transfer of the pB10 plasmid to *A. Baylyi*. p/D-ratios were also increased at $D:R_{1:20}$ when no antibiotic was added. No other experimental conditions showed an increase (**Figure 4-5**). Further, $D:R_{1:1}$ could effect a decrease in donor concentrations (*SI Table 4-6*), whereas a $D:R_{1:20}$ was always associated with an increase of donor concentrations (**Figure 4-5**).

Certain conditions were beneficial to HGT: (1) conditions with lower concentrations of resistance-carrying donors than of potential recipients and (2) conditions with sub-inhibitory tetracycline concentrations. Selective concentrations (Tet_{10}) did not provoke an increase in p/D-ratios, this is likely due to a strong and fast inhibition of the recipient organism's growth (*SI Table 4-3*).

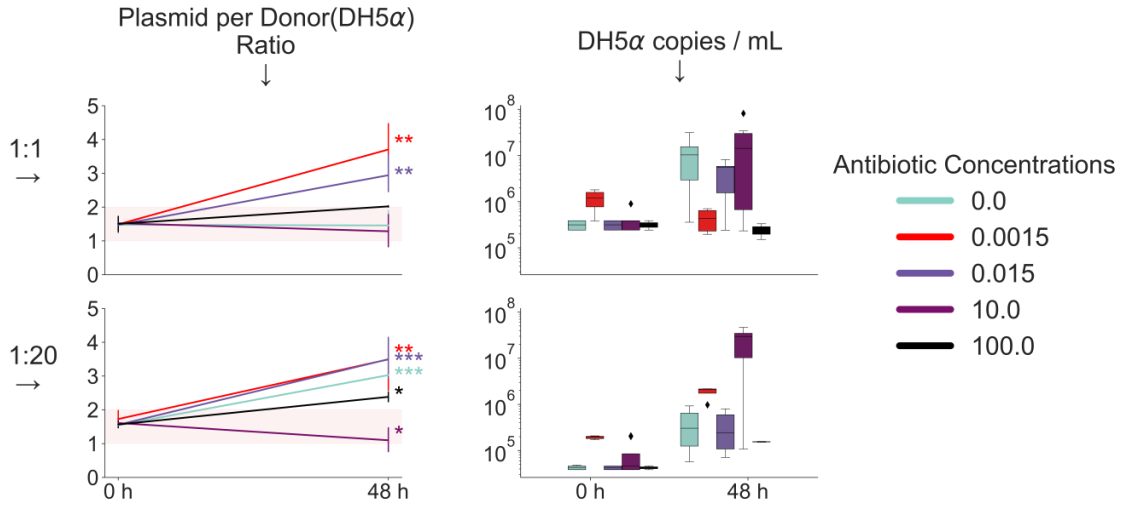


Figure 4-5: Changes of pB10/DH5a Concentrations after 48h at RT under different conditions (recipient: A.*baylyi*); experiments with donor-recipient-proportions of 1:1 (top row) and 1:20 (bottom row); red highlighted area (column 1) – p/D-ratios natively found in donor; antibiotic used: tetracycline

A larger recipient-ratio confers additional benefits under certain conditions. This is likely due to nutrient limitations leading to exploitative competition which might further lead to inference competition [420–422]. Recipient organisms will subsequently increase their chances for growth and plasmid uptake. Conditions under which resistance-carrying donors are underrepresented make it harder for the donors to thrive when selective pressure is low/null ($D:R_{1:20}$, $Tet_{0.0015}$), while higher tetracycline concentrations ameliorate conditions for donors even when underrepresented ($D:R_{1:20,}$ Tet_{10}).

DONOR – WASTEWATER EFFLUENT COMMUNITY EXPERIMENTS

The bacterial WWE community harbored pB10 plasmids (**Figure 4-7**, *top row*). This was expected as this plasmid was originally isolated from wastewater sludge [412]. To capture the impact on the bacterial WWE community, 16S rRNA concentrations were additionally taken into account in these experiments.

It is unclear if the target plasmids in WWE bacteria are located on E.coli cells. As native E.coli DH5α cells were not detected in the WWE, it is clear that the plasmids were located on organisms other than the donor.

At t_{0h}, p/D-ratios were 2E+00±2E-01 and 3E+00±5E-01 and relative pB10 concentrations 4E-01±3E-01 copies/16S rRNA and 5E-02±2E-02 copies/16S rRNA for experiments with donor-to-effluent-bacteria (D:EFF) proportions of 1:1and 1:20, respectively (**Figure 4-5**). p/D-ratios increased for all parameters tested, while relative pB10 concentration increased for $DEFF_{1:1}/Tet_{0.0015}$ and decreased for $DEFF_{1:1}/Tet_0$ (p=0.051) (**Figure 4-6**).

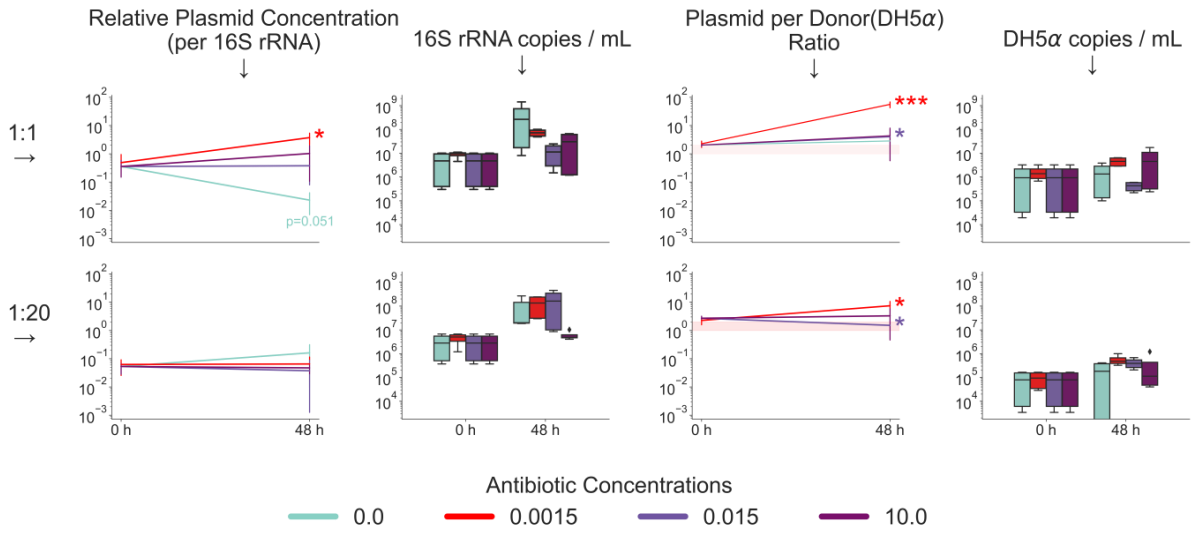


Figure 4-6: Changes in relative pB10 concentrations and pB10/DH5a ratios over 48h at RT under different conditions (recipient: WWE community bacteria) ; experiments with donor – effluent-bacteria – ratios of 1:1 (top row) and 1:20 (bottom row); red highlighted area (column 3) – p/D-ratios natively found in donor; antibiotic used: tetracycline

Low sub-inhibitory tetracycline concentrations increased plasmid concentrations under conditions where donor and effluent cells were present at comparable concentrations (**Figure 4-6**, top row). Two mechanisms could explain this observation: 1) selective pressure drove HGT of the pB10 plasmid [281,292,393,423], or 2) benefitted by selective pressure organisms carrying the pB10 plasmid outgrew donor cells carrying the pB10 plasmid [424–426].

To investigate which of these two mechanisms drove increase of pB10 concentrations, microcosm experiments were conducted with non-donor controls for each condition.

In experiments without p/D–increase, levels decreased to/stayed at concentrations characteristic within donor cells (*SI Table 4-7*). This might be an indicator that pB10 plasmids do not always confer benefits under selective pressure within the native bacterial WWE community. Bacterial WWE communities vary largely and even small changes within a community might have pronouncedly different reactions to antibiotic compounds. ARGs have differing fitness-costs, ranging from "costly" mutation to mutations with no apparent cost [427,428]. Wastewater (effluent) communities harbor a large number of tetracycline genes [429–431], which may be more "cost-effective" compared to the fitness cost of pB10. This hypothesis is strengthened by the large size of pB10, as fitness costs associated with necessary replication increase with plasmid size. Under conditions where effluent bacteria outnumber donor cells, these donor cells might then be at a disadvantage even under tetracycline-induced selective pressure.

Interestingly, very low sub-inhibitory tetracycline concentrations seem to have the largest effect. Even in experiments where effluent bacteria outnumbered donor cells, a pronounced p/D-increase could be observed (**Figure 4-6**; $DR_{1:20}$, $Tet_{0.0015/0.015}$). A potential explanation is, that environmental bacterial communities use sub-inhibitory antibiotic concentrations (including tetracycline [18,20]) to various ends [13,19,21], including signaling that could cause "diverse biological responses in bacteria" [15] including hormetic effects. Sub-inhibitory concentrations of tetracycline were found to increase HGT [395], further cementing the importance of sub-inhibitory antibiotic concentrations in risk assessment.

4.4.3 Microcosm Experiments

Donor – Community Experiments

In a first experiment, the dynamics between donor cells and WWE bacteria and the (disruptive) impact of tetracycline were explored. In the repetition of the experiment, non-donor controls were employed, to capture potential HGT. In a follow-up experiment, Ca^{2+} was added to Tet_0-microcosms to examine potential effects on HGT.

Donor concentrations significantly decreased for sub-inhibitory tetracycline concentrations. p/D-ratios significantly decreased for Tet_0 and $Tet_{0.0015}$ (**Figure 4-7**), they increased for $Tet_{0.4}$ and Tet_{10} only in one replicate (*SI Table 4-8*).

The WWE community harbored pB10-plasmids and had a competitive advantage over the added donor at lower selective pressure between 0 – 10 µg/ml of tetracycline. Any p/D-ratio-increases, under these conditions, were solely driven by the decrease of donor cells and a proportionally lower decrease of pB10 plasmid (*SI Table 4-8*). The competitive advantage of the native bacterial community was reduced at higher levels of tetracycline (Tet_{10}, Tet_{100}), where the selective advantage of the non-native donor seems to outweigh the competitive advantage of the native WWE community. Correspondingly, relative pB10 concentrations after 48h only differed significantly between donor and non-donor control microcosms for Tet_{100}, indicating potential HGT between donor cells and the WWE bacterial community.

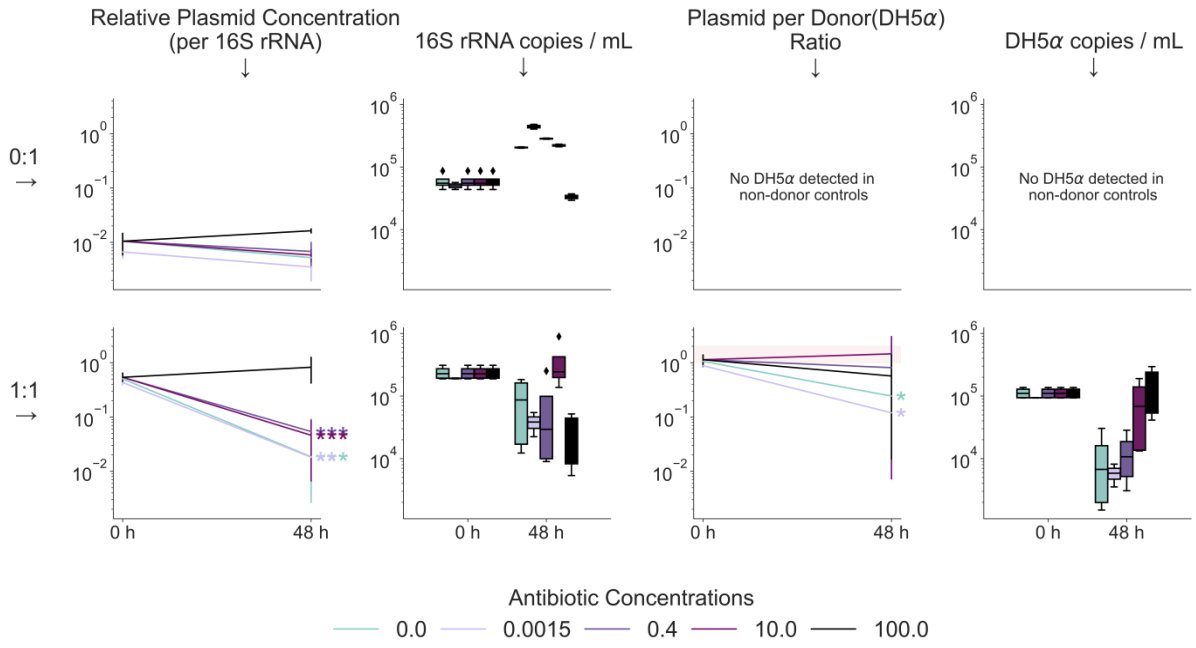


Figure 4-7: Changes of pB10/DH5a Concentrations at t=0h and after 48h at RT under different conditions; experiments with donor – effluent-bacteria – ratios of 0:1 (top row) and 1:1 (bottom row); red highlighted area (column 3) – p/D-ratios natively found in donor

Results could largely be reproduced for relative plasmid concentrations, while results for p/D-ratios varied between replicates (*SI Table 4-8*), indicating that interactions between donors and WWE community bacteria depend largely on community variation and chemical WWE composition. Wastewater quality and compound concentrations, including antibiotic concentrations have large temporal fluctuations, in the short term as well as seasonally [432–434] due to community events [435] and extreme meteorological conditions [436]. It is therefore not surprising that the impact of selective agents will vary depending on the WWE composition. Bellanger et al. [407] have previously observed strong matrix effects on pB10 plasmid transfer.

Compared to filter assays (**Figures 4-3 – 4-6**), sub-inhibitory concentrations had a lesser effect in microcosm assays (**Figure 4-7**). One reason may be well-known interactions of tetracycline with bivalent cations, which are ubiquitous in WWE [437–440], thus lowering the selective potential of tetracycline, especially in sludge-related environments [441]. Further, pB10 transfer frequencies may differ significantly between (solid surface) filter experiments and (stirred liquid) microcosm experiments, as the increased and prolonged cell-cell contact might be a decisive factor. This is especially true for a large plasmid, like pB10, which needs a longer time of established contact to transfer. Finally, tetracycline concentrations in WWE are usually in the sub-inhibitory range and relatively low (< 4 µg/ml) [442–445] when compared to concentrations in untreated wastewater, influent or sludge (up to 48 µg/ml) [442,445–447]. It therefore seems likely that the WWE community developed resistances to the relatively high levels of tetracycline, in/before the WWTP, that were retained during the short passage into the effluent.

Inhibitory concentrations, on the other hand, had a larger effect in microcosm assays. Various reasons should be found among the factors introduced to the transfer experiments by the increased complexity of microcosm assays. Some of these factors include a decrease of the relative advantage for bacteria carrying the pB10 plasmid as a large number of other factors and (potentially counter-acting) selective agents come into play, as discussed in

chapter 4.4.2. . To investigate some of the potential factors, follow-up assays at three calcium (Ca^{2+}) concentrations were performed. Further, to investigate the importance of cell-cell interactions, follow-up experiments using solely free pB10-plasmid DNA were conducted.

THE IMPACT OF CALCIUM

Increasing Ca^{2+}-concentrations inhibited the decrease of p/D-ratios over 48h (**Figure 4-8***, bottom row*) and increased relative pB10 concentrations in non-donor microcosms at Ca^{2+}_{25mM}.

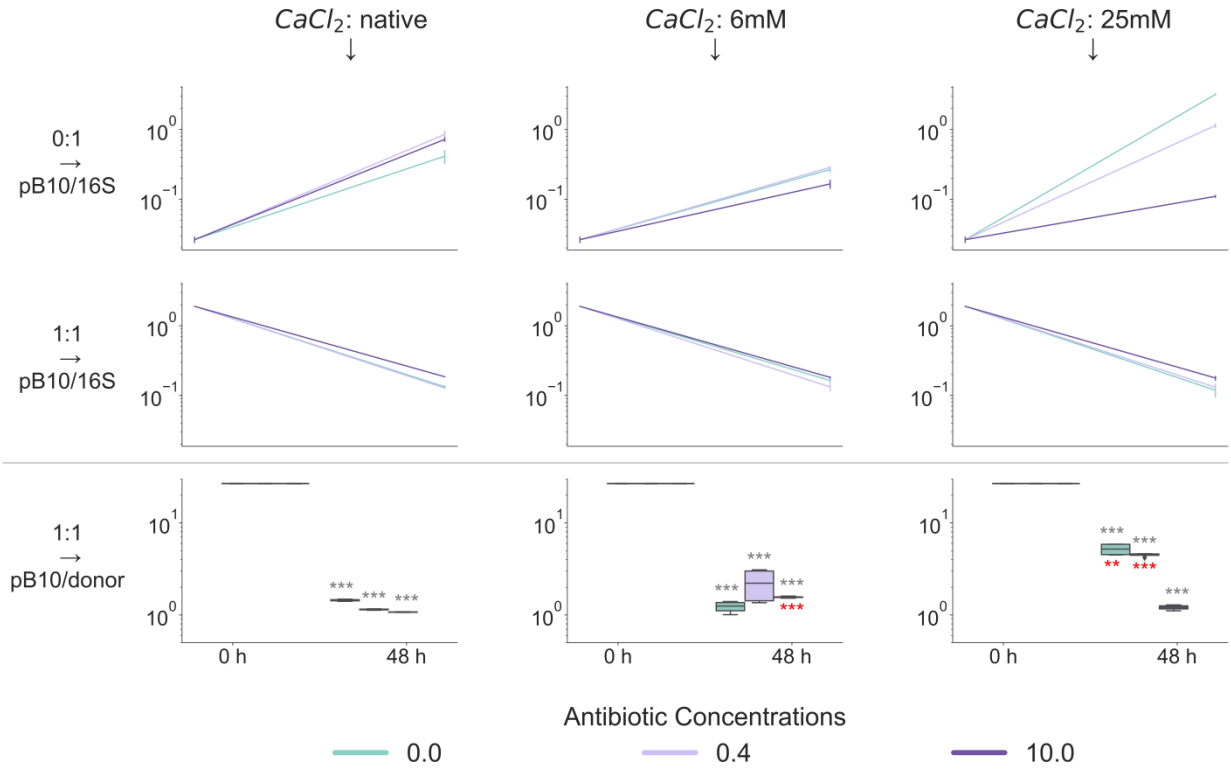


Figure 4-8: Impact of different calcium concentrations on WWE community, DH5α and pB10 over 48h (boxplots) at RT under different conditions; no added donor (top row), donor added (bottom row); grey stars indicate significance of de-/increase of p/D ratio during experiment (t0 compared to t48); red stars indicate difference between native-calcium control and according microcosm with added calcium;

Ca^{2+} concentrations caused p/D-ratios to increase in 3 out of 6 cases for $D:EFF_{1:1}$ microcosms when compared to the non-Ca^{2+} control (**Figure 4-8**, red stars). Ca^{2+} has been shown to be able to increase AR and can act as a regulatory agent for antibiotic activity or bacterial signaling [410,411]. For $D:EFF_{1:1}$ microcosms added Ca^{2+} did not show an effect on relative plasmid concentrations(**Figure 4-8***, central row*). Allelic exchanges might have caused the donor pB10-plasmid to be integrated into the bacterial genome [448] and thus not be susceptible to increased HGT influenced by Ca^{2+}.

pB10 concentrations were reduced over the course of the experiment. DH5α concentrations were not affected by this decrease. These results could indicate the presence of a compound that selected against the pB10 plasmid in general. As p/D-ratios significantly decreased in $D:EFF_{1:1}$-microcosms it is possible that a selection against the pB10 plasmid was at work and that the pB10 plasmid was more beneficial in native bacteria when compared to the donor cells. Another possible reason might be strongly elevated tetracycline concentrations in this WWE batch. Increased donor concentrations could be another

indicator for tetracycline concentration elevated to levels above selective concentrations. Consequently neither sub-inhibitory nor inhibitory concentrations (0.4 µg/ml, 10 µg/ml) effected increases of p/D-concentrations.

Calcium increased p/D-ratios (and potentially HGT) compared to non-calcium controls. Lower Ca^{2+} concentrations (Ca^{2+}_{6mM}) seem to have less impact and their driving effect seems to need additional agents causing selective pressure (e.g. increased tetracycline concentrations). These results indicate that elevated Ca^{2+} concentrations could acerbate the risk mitigated by HGT in environments with strongly increased broad-range plasmid concentration, especially if pathogens are present [449–451].

4.4.4 Free DNA Experiments

For microcosms without added free plasmid (**Figure 4-9**, top row) pB10/16S concentrations stayed unchanged. For microcosms with added free plasmid (**Figure 4-9**, bottom row) pB10/16S concentrations stayed constant. One exception was Tet_{10}^{spiked} where pB10/16S concentrations were significantly reduced.

Native pB10 concentrations declined when no selective pressure is exerted, but stayed the same when selective pressure is exerted by tetracycline. pB10-spiked microcosms on the other hand are able to retain their pB10/16S concentrations even without added selective pressure (**Figure 4-9**, bottom row, Tet_0). Based on free plasmid persistence pre-experiments results (*SI Figure 4-2*), free pB10 concentrations are reduced by >99.9 % over the experiment time frame in sterile filtrated WWE flow-through.

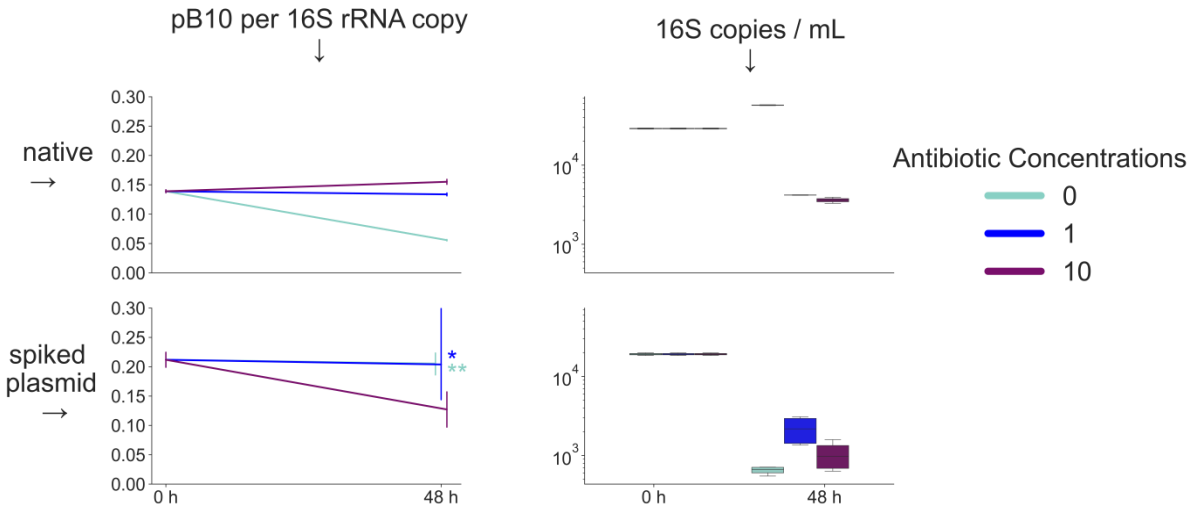


Figure 4-9: pB10/16S Concentrations at t=0 and after 48h (boxplots) at RT under different conditions – BIO medium; no free plasmid added (top row), free plasmid added (bottom row); red stars indicate significance of de-/increase of p/D ratio during experiment (t0 compared to t48);

Interestingly, higher concentrations of selective agent reduce pB10/16S concentrations significantly. This might be an indication that free plasmid DNA is susceptible to deterioration/degradation by enzymes or certain chemicals [417], including tetracycline. As free DNA degrades at a much faster rate (*SI Figure 4-2*), pB10/16S levels after microcosm

experiments indicate that the free pB10 plasmid was taken up by the wastewater bacterial community.

4.5. Conclusion

Sub-inhibitory tetracycline concentrations had an unexpectedly large effect on resistance levels and were shown to drive HGT of an ARG-carrying plasmid. Higher selective tetracycline concentrations, on the other hand, had varying effects in WWEs but had the potential to effect an increase in resistance. Modern urban wastewater systems connect these two conditions, with high antibiotic concentrations in wastewater and WWTPs and drastically reduced concentrations (mostly in the low sub-inhibitory range) in the receiving environment. Additionally, environmental water bodies are characterized by a lower density of bacteria and temperatures that are not optimal for the growth of most pathogens. Our experiments showed that these conditions benefit HGT in the absence of pre-existing resistance in potential recipients.

While the complex, non-homogenous nature of the WWE resulted in varying effects, it is clear that sources with strongly elevated antibiotic concentrations (e.g. antibiotic production locations, highly antibiotic-polluted urban environments and water bodies) pose a threat and can increase the risk, resulting WWEs pose to receiving environments. WWEs can provide (multi-)resistant donors to receiving water bodies with environmental conditions creating a HGT-conducive environment.

Many studies, focus largely on inhibitory antibiotic concentrations, unnaturally high donor-ratios and, in case of resistance-carrying pathogens, temperatures that do not occur in most natural environments. Such conditions do not necessarily render results transferable to real-life conditions. Sub-inhibitory antibiotic concentration of different compounds and on different bacterial communities will very likely have vastly different effects, with bacterial signaling being one of the mechanism responsible for the induction of HGT. HGT rates in nature might therefore be significantly higher than previously estimated. Even slightly elevated rates of HGT are problematic, in so far that humans and pathogens can concur in natural environments. An increased potential for HGT of ARGs could increase the number of antibiotic resistant pathogens and therefore increase the rate of propagation, infection with ARBs and subsequently the risk for society.

Investigations into HGT in "non-hotspot" natural environments are important. Further, drinking water and its distribution system need to be investigated more closely, as calcium concentrations vary widely at different locations, and communities with high drinking water calcium concentrations could have elevated HGT levels.

Summarizing, it can be said that a larger focus needs to be placed on the effects of (low) sub-inhibitory antibiotic concentrations, as these concentrations are common in anthropogenic and environmental settings and can act as a driving factor for HGT of ARGs.

4.6. Supplementary Material

SI Table 4-1: Primers, Probe and Target Sequences for gene detection;

Gene	Primers	Probe	Dye Used	Amplicon length [bp]
16S rRNA*	FW: 5'-CCTACGGGAGGCAGCAG-3' RV: 5'-TTACCGCGGCTGCTGGCAC-3'	–	SYBR Green II	193[i]
pB10	FW: 5'-CAATACCGAAGAAAGCATGCG-3'	5' -CCTCCACGGTGCGCGCTG-3'	FAM	135[ii]
	RV: 5' -AGATATGGGTATAGAACAGCCGTCC-3'			
DH5α	FW: 5' -ACCGGGTACATCATTTCC-3'	5'-TCTGATTGGTGCGCTGGTGGTCTGG-3'	FAM	140[iii]
	RV: 5'-GCCCCGGTAAGAATGAT-3'			

[I] G. Muyzer, E. C. de Waal, and A. G. Uitterlinden, 'Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S RRNA.', Applied and Environmental Microbiology, 59.3 (1993), 695–700; Mangala A. Nadkarni and others, 'Determination of Bacterial Load by Real-Time PCR Using a Broad-Range (Universal) Probe and Primers Set', Microbiology, 148.1 (2002), 257–66 <https://doi.org/10.1099/00221287-148-1-257>.
[II] Sébastien Bonot and Christophe Merlin, 'Monitoring the Dissemination of the Broad-Host-Range Plasmid PB10 in Sediment Microcosms by Quantitative PCR', Applied and Environmental Microbiology, 76.1 (2010), 378–82 <https://doi.org/10.1128/AEM.01125-09>.
[III] Bonot and Merlin, p. 10.

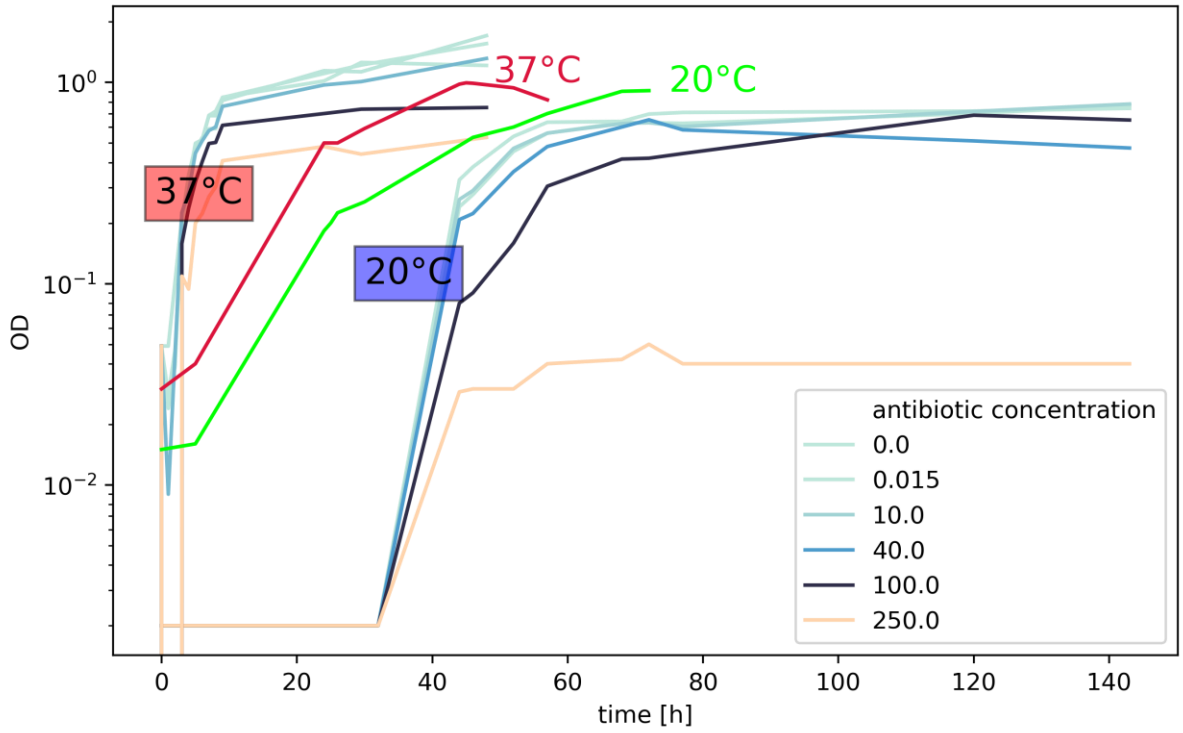
SI Table 4-2: 16S rRNA qPCR Cycling Conditions;

Step	Temperature [°C]	Time [sec]	Cycles
Pre-denaturation	95	600	1
Amplification	95	10	45
	60	60	
	72	10	
Cooling	40	10	1

SI Table 4-3: Raw Wastewater Influent (IN) and Effluent (EFF) Parameter Values;

	COD, mg/L	DOC, mg/L	P-PO4, mg/L	N-NH4, mg/L	NOx-N, mg/L	N-NO2, mg/L	SS, mg/L	Av. CFU/ml E.coli	Av. CFU/ml coliforms	Av. CFU/ml heterotrophs	Av. 16S copies/ml
IN	238	-	1.9	19.2	2.2	0.2	146.7	1.45E+04	8.35E+04	1.30E+05	5.22E+08
EFF	13	4.8	0.2	0.1	0.6	0.1	2.2	5.00E+01	2.85E+02	1.90E+03	3.68E+06

SI Figure 4-1: Growth curves for donor (blue) and recipient (pink, green) over time at different antibiotic concentrations;

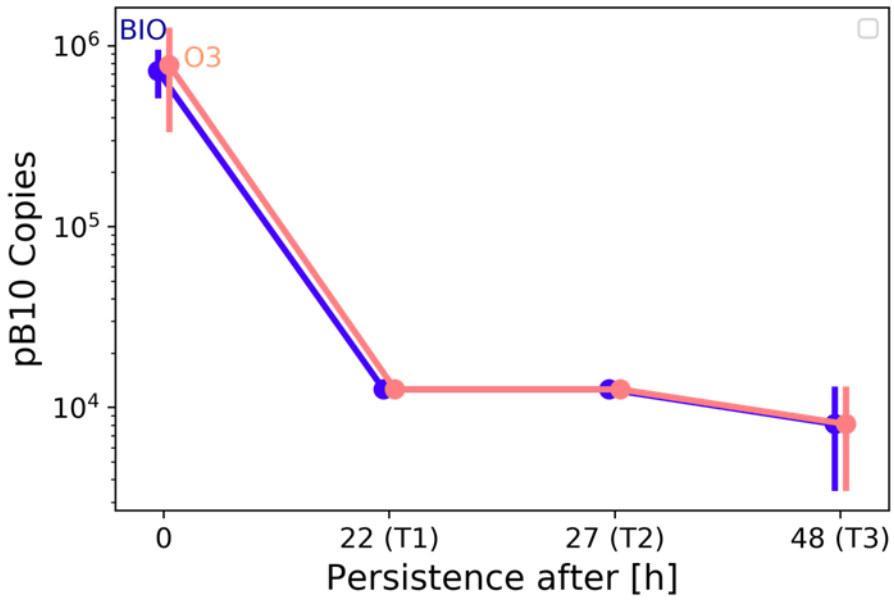


SI Table 4-4: A.baylyi (recipient) growth at different antibiotic concentrations and temperatures; +++ = growth, ++[+] = growth (but visibly less than at RT), · = no growth, ++ = slight growth, + = minimum growth

Strain	Temperature	Antibiotic Concentration [µg/ml]	Growth	
			24 h	48 h
A.*baylyi*	RT	0	+++	+++
		0.0015	+++	+++
		0.015	+++	+++
		0.15	+++	+++
		1	++	+++
		10	–	–
	37°C	0	++[+]	+++
		0.0015	++[+]	+++
		0.015	++[+]	+++
		0.15	++	++
		1	+	++
		10	–	–

Note: E.coli$_{DH5α}$ (donor) grew at all tested antibiotic concentrations (range 0 µg/ml - 250 µg/ml) with inhibited growth only at 250 µg/ml at RT

SI Figure 4-2: Free plasmid DNA persistence over 48 h in BIO or O3 medium;



SI A.4-2: 16S rRNA qPCR Protocol

16S rRNA copy numbers were quantified by TaqMan assay, designed and optimized by Ingenetix GmbH (Vienna, Austria) in a 10 µL reaction mixture, containing 1 x LightCycler® 480 Probes Master (Roche, Germany), 1 x TaqMan assay and 2 µL of a sample, according to the protocol in Table 1. Standard curves were prepared for each run by 10-fold dilution of a standard, ranging from 10^8 to 10^3 copies. The amplification efficiency ranged from 90% to 105%.

SI Table 4-5: Multiplex qPCR Cycling Conditions/Program;

4.7. Step	*4.8. Temperature [˚C]*	*4.9. Duration [mm:ss]*
1	95	05:00
2	95	00:30
3	Optimal Annealing Temp. (see *SI Table 2*)	00:30
4	72 + Plate Read	00:40
5	GOTO Step 2, 45 more times END	

SI A.4-3 Inocula Preparation

Inocula for the donor and recipient strains were freshly prepared prior to experiments . Inocula for donor and recipient cells were prepared 24h after plating cells at 37°C (or 48h after plating cells at RT) to obtain cells in their stationary phase.

24 h prior to inocula preparation, the strains were plated on Luria Bertani (LB) Agar (Sigma, USA) and incubated at 37 °C. Donor cells were grown on LB medium containing 10 µg/ml tetracycline (tetracycline hydrochloride, Sigma-Aldrich, China).

30 mL of LB medium were inoculated with a sterile loop and the inocula were incubated for 24 h at 37 °C, to ensure that donor and recipient were both in the same growth phase (exponential).

SI Table 4-6: Overview of Results for different A.baylyi Filter Experimental Replicates (Runs);

Run	D:R	Time	Temperature	Tetracycline Conc.	Count	DH5 cop./mL	DH5 cop./mL	pB10 copies/mL	pB10 copies/mL	pB10/DH5	pB10/DH5
						mean	std	mean	std	mean	std
1		0		*	4	3.14E+05	8.31E+04	4.56E+05	4.39E+04	1.50E+00	2.58E-01
	1: 1	48	RT	0	2	4.37E+05	1.11E+05	8.58E+05	3.17E+05	1.93E+00	2.34E-01
			37	0	2	8.83E+06	4.76E+06	1.24E+07	3.21E+06	1.53E+00	4.61E-01
			RT	0.015	2	2.59E+05	2.49E+04	6.16E+05	1.68E+04	2.39E+00	1.65E-01
			37	0.015	2	4.85E+06	8.04E+05	1.09E+07	8.32E+05	2.27E+00	2.05E-01
			RT	10	2	2.50E+05	2.47E+04	5.68E+05	2.04E+04	2.28E+00	3.07E-01
			37	10	2	6.70E+06	1.60E+03	9.47E+06	4.64E+05	1.41E+00	6.89E-02
			RT	100	2	2.41E+05	1.27E+05	4.89E+05	2.63E+05	2.02E+00	2.78E-02
			37	100	2	7.90E+06	2.26E+06	1.02E+07	1.43E+06	1.32E+00	1.97E-01
	1:20	0		*	4	4.28E+04	5.15E+03	6.66E+04	1.15E+04	1.55E+00	9.09E-02
		48	RT	0	2	6.53E+04	1.16E+04	1.89E+05	3.14E+04	2.90E+00	3.23E-02
			37	0	2	5.42E+06	2.57E+06	9.41E+06	3.65E+06	1.78E+00	1.70E-01
			RT	0.015	2	7.27E+0	2.72E+0	2.02E+05	1.39E+04	2.78E+	8.65E-

						4	3			00	02
			37	0.015	2	8.43E+06	4.08E+05	1.15E+07	2.97E+05	1.37E+00	1.01E-01
			RT	10	2	1.22E+05	1.98E+04	2.48E+05	5.70E+04	2.02E+00	1.40E-01
			37	10	2	5.88E+06	1.74E+06	9.20E+06	1.99E+06	1.58E+00	1.30E-01
			RT	100	2	1.55E+05	4.57E+03	3.69E+05	4.22E+04	2.38E+00	2.02E-01
			37	100	2	8.44E+06	4.61E+05	1.08E+07	3.83E+04	1.28E+00	7.43E-02
2		0		*	4	3.14E+05	8.31E+04	4.21E+05	8.08E+03	1.42E+00	4.02E-01
	1: 1	48	RT	0	2	1.72E+07	9.86E+06	1.68E+07	6.19E+06	1.22E+00	6.79E-01
			RT	0.015	2	6.28E+06	1.27E+06	1.97E+07	3.60E+06	3.22E+00	8.21E-01
			RT	10	2	4.27E+07	2.62E+07	3.76E+07	1.91E+06	1.08E+00	4.31E-01
		0		*	4	4.23E+04	4.50E+03	6.45E+04	1.15E+02	1.54E+00	1.66E-01
	1:20	48	RT	0	4	5.71E+05	3.17E+05	1.59E+06	5.68E+05	3.09E+00	9.21E-01
			RT	0.015	4	4.97E+05	2.96E+05	1.75E+06	7.74E+05	3.85E+00	7.84E-01
			RT	10	4	3.78E+07	9.21E+06	2.93E+07	5.89E+06	7.85E-01	9.43E-02
3		0		*	4	1.15E+06	6.34E+05	1.61E+06	7.34E+05	1.49E+00	2.25E-01
	1: 1	48	RT	0.0015	4	4.40E+05	2.59E+05	1.46E+06	5.59E+05	3.70E+00	9.11E-01
			RT	10	4	1.52E+06	1.02E+06	7.86E+05	1.07E+05	6.96E-01	5.36E-01
		0		*	4	1.91E+05	1.65E+04	3.30E+05	5.99E+04	1.73E+00	3.00E-01
	1:20	48	RT	0.0015	4	1.83E+06	5.74E+05	6.72E+06	3.04E+06	3.49E+00	8.72E-01
			RT	10	4	2.21E+07	1.20E+07	1.62E+07	3.92E+06	8.02E-01	2.59E-01

* - batches at t=0 were prepared for each D:R-proportion without initially added tetracycline and four samples for DNA extraction aliquoted onto separately prepared filter → t=0 values are similar for experiments with same D:R-proportions across different tetracycline concentrations; homogeneity of batches were insured by shaking before Aliquoting batches by pipetting onto filters (which were then placed on tetracycline-containing media of different tetracycline-concentrations)

SI Figure 4-3: Overview of Results for different WWE Filter Experimental Replicates ("Runs");

Run	D:EFF	Time	Tetracycline Concentration	Count	16S cop./mL	16S cop./mL	DH5 cop./mL	DH5 cop./mL	pB10 cop./mL	pB10 cop./mL	pB10/16S	pB10/16S	pB10/donor	pB10/donor
					mean	std	mean	std	mean	std	mean	std	mean	std
1	1:01	0*	0	4	3.65E+05	9.19E+04	2.87E+04	1.32E+04	5.50E+04	1.84E+04	1.49E-01	1.28E-02	1.98E+00	2.66E-01
		48	0	2	1.40E+07	8.49E+06	1.23E+05	3.25E+04	4.60E+05	5.66E+04	3.88E-02	1.94E-02	3.81E+00	5.48E-01
			0.015	2	2.45E+06	1.34E+06	2.50E+05	4.24E+04	1.29E+06	4.10E+05	6.74E-01	5.37E-01	5.09E+00	7.76E-01
			10	2	1.23E+06	3.54E+04	2.90E+05	7.07E+04	2.39E+06	8.63E+05	1.94E+00	6.48E-01	8.12E+00	9.95E-01
	1:20	0*	0	4	4.60E+05	1.27E+05	5.20E+03	2.55E+03	1.64E+04	1.07E+04	4.04E-02	3.46E-02	3.01E+00	5.94E-01
		48	0	2	1.93E+07	1.06E+06	0.00E+00	0.00E+00	4.50E+06	1.84E+06	2.37E-01	1.09E-01	inf	
			0.015	2	9.50E+06	1.41E+06	4.80E+05	2.83E+05	6.70E+05	2.97E+05	7.37E-02	4.22E-02	1.47E+00	2.47E-01
			10	2	4.88E+06	3.54E+04	4.50E+04	7.07E+03	2.60E+05	0.00E+00	5.33E-02	3.87E-04	5.85E+00	9.19E-01
2	1:01	0*	0	4	9.55E+06	7.07E+05	2.54E+06	9.97E+05	5.28E+06	1.81E+06	5.61E-01	2.31E-01	2.11E+00	1.14E-01
		48	0	2	9.70E+08	6.51E+08	3.20E+06	9.33E+05	5.61E+06	9.76E+05	7.03E-03	3.71E-03	1.88E+00	8.53E-01
			0.0015**	4	7.33E+07	2.59E+07	4.62E+06	2.00E+06	2.38E+08	4.95E+07	3.74E+00	1.92E+00	5.61E+01	1.52E+01
			0.015	2	2.19E+07	3.89E+06	5.90E+05	1.41E+04	1.70E+06	2.11E+05	7.97E-02	2.38E-02	2.87E+00	2.88E-01
			10	2	6.28E+07	4.60E+06	1.31E+07	6.14E+06	6.71E+06	4.67E+05	1.07E-01	1.53E-02	5.68E-01	2.31E-01
	1:20	0*	0	4	5.88E+06	1.17E+06	1.59E+05	1.02E+04	3.82E+05	4.24E+04	6.56E-02	5.81E-03	2.40E+00	1.13E-01
					4.49E+06	2.46E+06	9.58E+04	7.33E+04	2.28E+05	1.83E+05	6.24E-02	4.09E-02	2.26E+00	8.78E-01
		48	0	2	2.68E+08		3.89E+05	3.25E+04	2.22E+06	3.39E+05	9.18E-03		5.69E+00	3.97E-01
			0.0015**	4	1.36E+08	1.22E+08	5.74E+05	3.00E+05	3.59E+06	3.95E+05	6.50E-02	5.89E-02	7.47E+00	3.45E+00
			0.015	2	3.86E+08	1.02E+08	3.54E+05	2.06E+05	4.65E+05	2.69E+04	1.26E-03	4.02E-04	1.56E+00	8.32E-01
			10	2	7.10E+06	4.31E+06	6.96E+05	7.36E+05	2.32E+05	2.26E+04	4.13E-02	2.83E-02	7.16E-01	7.25E-01

* - batches at t=0 were prepared for each D:EFF-proportion without initially added tetracycline and four sample for DNA taken from separately prepared filter → t=0 values are similar for experiments with same D:EFF-proportions across different tetracycline concentrations; homogeneity of utilized batches were insured by shaking before aliquoting from batches onto filter (which were then placed on tetracycline-containing media of different tetracycline-concentrations)

** - as sub-inhibitory concentrations of 0.0015 µg/mL were added in second run, these microcosms were conducted in quadruplets instead of duplicates in run2

SI Figure 4-4: Overview of Results for different Microcosm Experimental Runs;

Run	D:EFF	Time	Tetracycline Concentration	Count	16S copies/mL	16S cop./mL	DH5 cop./mL	DH5 cop./mL	pB10 cop./mL	pB10 cop./mL	pB10/16S	pB10/16S	pB10/donor	pB10/donor
					mean	std	mean	std	mean	std	mean	std	mean	std
1	0:1	0	*	2	7.02E+04	2.33E+04	0.00E+00	0.00E+00	9.61E+02	8.28E+01	1.43E-02	3.57E-03	n/a	n/a
	1:1	0	*	2	2.85E+05	3.52E+04	1.32E+05	7.78E+03	1.62E+05	6.06E+02	5.72E-01	7.27E-02	1.23E+00	6.82E-02
		48	0	2	1.54E+04	4.45E+03	2.09E+04	1.35E+04	5.39E+02	3.54E+02	3.30E-02	1.34E-02	2.57E-02	2.85E-04
			0.4	2	9.63E+03	9.50E+02	2.20E+04	9.16E+03	3.45E+02	1.94E+01	3.61E-02	5.58E-03	1.70E-02	6.22E-03
			10	2	2.03E+05	9.29E+04	1.57E+05	4.74E+04	1.20E+03	3.84E+01	6.67E-03	3.24E-03	8.01E-03	2.18E-03
			40	2	1.30E+04	8.94E+03	1.57E+05	4.74E+04	1.20E+03	3.84E+01	1.22E-01	8.66E-02	8.01E-03	2.18E-03
			100	2	7.28E+03	2.71E+03	2.59E+05	5.09E+04	4.34E+03	7.27E+02	6.61E-01	3.46E-01	1.68E-02	4.98E-04
2	0:1	0	*	2	5.02E+04	9.19E+03	0.00E+00	0.00E+00	3.23E+02	5.12E+01	6.64E-03	2.24E-03	n/a	n/a
		48	0	2	2.05E+05	7.07E+03	0.00E+00	0.00E+00	1.06E+03	3.27E+02	5.21E-03	1.77E-03	n/a	n/a
			0.0015	2	4.43E+05	5.37E+04	0.00E+00	0.00E+00	1.50E+03	7.99E+02	3.52E-03	2.23E-03	n/a	n/a
			0.4	2	2.84E+05	8.49E+03	0.00E+00	0.00E+00	1.95E+03	1.34E+03	6.79E-03	4.53E-03	n/a	n/a
			10	2	2.22E+05	1.48E+04	0.00E+00	0.00E+00	1.28E+03	3.68E+02	5.83E-03	2.05E-03	n/a	n/a
			100	2	3.33E+04	5.57E+03	0.00E+00	0.00E+00	5.49E+02	1.58E+02	1.63E-02	2.01E-03	n/a	n/a
	1:1	0	*	2	1.92E+05	4.24E+03	9.37E+04	8.91E+02	8.23E+04	8.32E+03	4.30E-01	5.30E-02	8.78E-01	8.04E-02
		48	0	2	1.69E+05	2.05E+04	1.83E+03	4.53E+02	7.21E+02	7.34E+02	4.03E-03	3.86E-03	4.59E-01	5.16E-01
			0.0015	2	3.84E+04	2.27E+04	5.84E+03	3.27E+03	8.48E+02	9.28E+02	1.81E-02	1.35E-02	1.19E-01	9.21E-02
			0.4	2	1.50E+05	1.43E+05	4.47E+03	1.99E+03	6.96E+03	2.38E+03	7.17E-02	5.27E-02	1.60E+00	1.82E-01
			10	2	5.55E+05	4.77E+05	1.35E+04	3.90E+02	3.87E+04	2.06E+04	8.53E-02	3.63E-02	2.89E+00	1.61E+00
			100	2	4.68E+04	6.93E+03	4.93E+04	1.15E+04	4.87E+04	4.29E+04	9.83E-01	7.70E-01	1.12E+00	1.13E+00

* - batches were prepared for each D:EFF-proportion without initially adding tetracycline and at t=0 two separate samples for DNA extraction taken per batch → t=0 values are similar for experiments with same D:EFF-proportions across different tetracycline concentrations; homogeneity of utilized batches were insured by shaking before aliquoting from batches into 400 mL of WWE for the separate microcosms

5. Comparison of qPCR and Different Next-Generation Sequencing Analysis Methods for the Analysis of Antibiotic Resistance in Environmental Samples

Keywords: Bioinformatic analysis; Shotgun metagenome sequencing; Method comparison; Whole genome sequencing

5.1. Abstract

The present study investigates the impact of different analysis methods as well as various bioinformatic parameters on antibiotic resistance gene (ARG) detection and quantification. qPCR results were compared to Illumina NextSeq shotgun sequencing analysis results . Whole-genome sequencing (WGS) data was analyzed using a machine learning (ML) model (deepARG), a custom non-ML pipeline or custom-made '*in-silico* qPCR' scripts. Parameters evaluated include assembly, databases usage, analysis methods and DNA concentrations.

We show that ML analysis of unassembled WGS data has an advantage over all other methods for ARG detection and results are more similar to qPCR results. Pre-analysis assembly of paired WGS reads had the largest negative impact on results, even surpassing the impact of the analysis method used, an effect that may be most pronounced in results from low to moderate coverage data. The lowest impact parameter, with results compared to qPCR results, was the choice of database. ARG prediction in samples with low DNA concentrations could be shown to be less accurate. Further, WGS analysis was able to predict ARGs that were screened for but not detected by qPCR.

More than half of WGS-measured abundances varied less than 1-fold from qPCR results. Over-quantification and ARG concentrations were inversely correlated and some very low-abundance ARGs were shown to be severely over-quantified across all analysis methods, when copared to qPCR results. A combination of WGS and qPCR can reduce the large selection bias introduced by qPCR and add relevance to qPCR results, as these can be analyzed within a more integrated context. WGS analysis could serve as a basis to select adequate genes of interest for qPCR analysis based on a research question.

5.2. Introduction

The study of microbial communities, in the environment as well as in anthropogenic and clinical settings is crucially important in research fields such as water reuse, antibiotic resistance propagation and pharmaceutical compound discovery and environmental microorganisms are being examined more thoroughly than ever before. However, there are challenges when working with environmental samples. One of the main limitations is that the majority of environmental organisms are non-cultivable [221], leading to the increased reliance on molecular methods, such as real-time quantitative polymerase chain reaction (qPCR) or next-generation sequencing (NGS) including whole-genome sequencing.

qPCR has been held as the gold standard in gene detection and quantification in environmental research [230]. DNA is extracted from environmental samples and screened for target genes via qPCR [452–454]. Nevertheless, regular qPCR is low-throughput in regard to the number of genes being analyzed simultaneously, even when multiplexed. A selection bias is introduced by the need to select genes and choose probes and primers. Even more importantly: it provides information about a very limited number of targets. High-throughput PCR technologies, e.g. microarrays and HT–qPCR (high-throughput qPCR), increase the number of targets that can be assayed, but come with their own challenges, including high result variability and lower specificity [212].

In the past years, next-generation sequencing (NGS) methods have been on the rise and have often completely substituted qPCR in many environmental microbiome studies. NGS without benchmarking to standards or combination with additional methods such a 5′ hydrolysis with digital droplet PCR [455], is considered to be semi-quantitative [216,456], partly due to its susceptibility to technical bias [215]. Results from NGS and qPCR studies are being compared with each other in scientific discussions [457–461] without fully taking into consideration the impact of the different methods, or biases and differences that can arise from their pronouncedly distinct nature [462]. There is a growing number of bioinformatic tools for metagenomic analysis [463–467], where two methods that try to solve the same problem might differ remarkably from one another based on the parameters they use.

Traditional NGS data analysis is characterized by three sequential steps: (1) assembly of reads into larger sequences (contigs), (2) gene prediction and (3) annotation or clustering of domains, functions or pathways [468]. Most tools designed for the accurate prediction of genes from assembled NGS data aim for high sequence identity (cut-off values >80-90%), low e-values and high alignment length [468,469]. The prediction of a gene is usually determined by the 'best hit' of a sequence query in a sequence database. However, this traditional data analysis framework has limitations, especially during assembly. While small metagenomes are handled well by most assembly tools, the assembly of larger, complex metagenomes is challenging and requires ultra-deep sequencing and correspondingly higher computational power [470,471]. Further, repeated sequences make the localization of relative positions in the genome difficult [470]. Moreover, the presence of highly conserved genomic regions might cause inter-genomic assembly errors in the case of complex metagenome samples [472]. Finally, standard assembly techniques might not capture the genomic diversity of microbial communities with different species abundance levels, if the tools were developed by using single species data from cultivated samples [472].

Machine learning-based pipelines have recently emerged as an alternative approach to mine WGS data for ARG analysis [469,473–475]. While traditional pipelines depend on the accurate assembly of reads, machine learning-based methods can predict genes from short WGS reads, allowing to skip the cumbersome and error-prone assembly step. Another advantage of ML-methods over assembly-based approaches is the independence from sequence identity cut-off values which consequently decreases the number of false negatives by not relying solely on the "best hit" for accurate gene prediction [469]. Furthermore, ML-methods can predict novel genes from metagenomes, since the algorithm can learn and detect unique features of gene categories for unknown gene discovery. Of course, model training is limited to using known gene categories [469]. The uniqueness of gene categories (*SI Table 5-2*) is also the reason why ML-methods tend to perform better in filtering out false positives since they take similarities across gene categories into account and can distinguish between random sequence similarities and truly distinct categories [469]. One drawback of ML-approaches is the dependence on well-curated, large databases. Size and quality of the database are crucial for the model training, as small databases with less information about gene categories will affect prediction accuracy, especially for low-abundance genes [469]. Another disadvantage is the time required for ML-model training.

The present work compares molecular methods and bioinformatic tools for the analysis of antibiotic resistance genes (ARGs) in wastewater samples. qPCR results are compared to the results of different metagenomic approaches, including a machine-learning (ML)-based method (deepARG) using raw or assembled reads, an assembly-based non-ML pipeline using different databases to compare their impact, as well as a custom-made "*in-silico* qPCR" script. This study aims to compare the detection and the abundance characterization of 13 specific ARGs in wastewater samples between qPCR and different WGS methods. It further aims to investigate the impact of various factors, including assembly, database use and low ARG concentrations of different bioinformatic WGS analysis methods, on the accuracy (compared to qPCR results) and relative quantifiability of metagenomic analysis. A number of studies comparing gene detection and quantification methods exist for miRNA analysis platforms [476–478], cancer research [479–482], prenatal screening [483,484] and phylogenetic profiling [485]. However, to date the present study represents, to the best of our knowledge, the first comparison of the performance of analysis methods to detect and quantify antibiotic resistance genes from environmental metagenomes as well as an evaluation of result quality for usage in subsequent models, comparing the outcomes of traditional pipelines, ML-based methods and qPCR to each other.

5.3. Methods and Materials

5.3.1 Sampling

Wastewater (WW) samples were taken from various locations along the wastewater infrastructure of two Dutch cities as previously described [344]. Samples included communal and hospital wastewater as well as WW treatment plant influent and effluent (secondary or tertiary treatment). A total of 11 samples were used for analysis (*SI Table 5-1*).

5.3.2 DNA Extraction and qPCR

DNA was extracted within 24 hours of sampling, using the MoBio PowerSoil kit. 13 ARGs were quantified using qPCR. These 13 "target genes" included: *aph*(III)a, bla_{KPC}, bla_{OXA}, bla_{SHV}, *erm*B, *erm*F, *mec*A, *qnr*S, *sul*1, *tet*B, *tet*M, *van*A and v*an*B; they were selected for their relevance in the environment. DNA extraction, qPCR procedure and qPCR data analysis were previously described [344].The same DNA extracts were used for qPCR and WGS analysis.

Target gene primers for in-vitro and in-silico qPCR analysis were largely universal primers capturing more than one subclass for each ARG. The exact ARGs targeted are listed in *SI Table 5-2*.

The term "target gene" is defined as the 13 genes listed in this chapter, which where quantified by qPCR and will thus be referred to for the remainder of this paper.

5.3.3 Whole-genome Sequencing

Whole-genome sequencing was conducted by Baseclear B.V. (Sylviusweg 74, 2333 BE Leiden) using Nextera XT library preparation and Illumina paired-end NovaSeq sequencing (minimum sample depth of 3 Gbp). To avoid biases due to these varying parameters (such as DNA extraction and qPCR or amplification errors) in this study, WGS analysis was conducted from the same WGS datasets, in order to investigate only the impact of different analysis methods on the output.

5.3.4 Assembly

Paired reads were assembled into contigs using metaSPAdes v 3.13.1 [472] using default parameters.

5.3.5 Custom non-ML based Analysis

Analysis was performed using an in-house pipeline**,** which included metagenome assembly, gene prediction, clustering and gene annotation using MOCAT2 (PMC4978931) and USEARCH (min alignment 80%, min. query coverage 85%) [486]. To investigate the impact of

databases on the predicted sequences, two types of analyses were performed: (1) predicted genes were annotated using the CARD database (BC1), and (2) predicted genes were annotated using the deepARG database, including CARD, ARDB and UNIPROT (BC2).

5.3.6 Machine Learning-based Analysis

Analysis was performed using deepARG-ss or deepARG-ls [469]. Unassembled reads (D1) and assembled reads (contigs) (D2) were analyzed. Detected ARGs were ensured to be individual genes as opposed to ARG-classes by manual inspection via the given accession number.

5.3.7 In-Silico qPCR Analysis

A Python script was written to perform "*in-silico* qPCR"-analysis (I1). This script simulated regular qPCR in silico, in order to be able to investigate the presence of target genes within the raw WGS dataset and help estimate the impact of advanced bioinformatic tools on WGS data analysis results. To this end, paired WGS sequences were iterated over and forward and reverse primer sequences were computationally attached to the sequences of interest. This script is only a rough approximation to in-vitro qPCR, as the assembled DNA fragments used are relatively short and this method will not be able to detect an ARG when primer sequences are located at two different assembled WGS fragments. A Github repository has been created to store, pull and use the *in-silico* qPCR script: https://github.com/lasupernova/insilico_qPCR.git

For the remainder of this work, the term qPCR will always refer to the regular in-vitro qPCR and only the term "*in-silico* qPCR" will refer to the script described here.

5.3.8 Data Analysis

Different types of analysis were performed: **1)** WGS data was analyzed for ARG presence and ARG abundance; **2)** information on [a] presence/absence and on [b] relative abundance of 13 target genes was extracted from WGS analysis results and compared to detection and relative abundance results obtained by qPCR analysis.

Unless otherwise stated, the terms lower and higher detection and under-/over-quantification of ARGs refers to WGS results compared to qPCR results as a baseline. Unless otherwise stated the term "abundance" refers to relative ARG abundances.

1) WGS analysis

Detected ARGs, as obtained by different WGS analysis methods (D1, D2, BC1, BC2), were added to a list to produce an overview of ARG presence in each of the samples. Genes could be detected more than once from WGS reads so that each ARG was detected with a different abundance or number of hits.

The number of hits per detected ARG was normalized using the total number of reads per sample (WGS) or 16S rRNA concentrations (qPCR) in order to make relative abundance results comparable across samples. ARG abundances, expressed as fractions of different genes from the complete dataset, were then compared to each other to be able to make

statements about which genes were more or less abundant in the samples based on different analysis methods.

2) Analysis of Target Genes

Information on the 13 target genes was extracted from WGS results by their accession number and gene names. Detection of target genes across different WGS analysis methods was compared to detection by qPCR (see *5.4.2*). To measure ARG abundance, the number of hits for every detected target gene was added to a list for each of the 13 target genes.

For a better overview, detection results of the individual samples were combined and the fraction of samples in which individual ARGs with higher or lower detection was shown (see *5.4.2*, **Figure 5-2**).

To compare target relative gene abundance from WGS analysis to qPCR results, the combined number of hits (of the 13 target genes) was added up per sample and defined as 100%. The fraction, of each of the 13 individual target genes, was then calculated (e.g. if the total number of hits adds up to 500 and sul1 contributes to these with 100 hits, sul1 makes up 20% of the abundance of the genes of interests isolated from the WGS dataset). Similarly, gene concentration detected by qPCR were added up and the fraction for each individual gene detected by qPCR was calculated. The fractions obtained by qPCR and by the WGS analysis methods were then compared to each other. The accuracy of each WGS analysis method to quantify ARG abundance (compared to qPCR results) was then determined either by direct comparison of the fractions and by calculating the discrepancy of the WGS results from the results obtained by qPCR (e.g. the sul1 percentage for BC1 was 64% compared to 85% for qPCR · the absolute difference is 21 %, which translates into a 25 % discrepancy of the BC1 result relative to qPCR results). When results for individual samples were combined, the standard deviation of the resulting values was added to the mean (see *5.4.3*). This was calculated for each of the 11 wastewater samples (*SI Figure 5-1*) and sample-individual results were subsequently combined across samples per analysis method, ARG and water treatment type (**Figure 5-1**).

Computational simulation of varying sequencing depth

Practical analysis of varying sequencing depth was outside of the scope of this work. To be able to make statements about the impact of the sequencing depth on analysis results, sequencing depth was computationally reduced by running Monte-Carlo-simulations as described in *SI Table 5-10*.

Statistical analysis

Statistical analysis was performed using SciPy v.1.5.0. Unless otherwise stated hypothesis testing was performed via Student's t-test using Python's scipy.stats library. $P \leq 0.05$ was considered statistically significant. Hierarchical clustering was performed by calculating cophenetic distances between observations (Ward's linkage method), using the scipy.cluster.hierarchy and scipy.spatial.distance modules (SCIPY.CLUSTER.HIERARCHY.COPHENET(Z, PDIST(SIMARRAY))).

5.4. Results and Discussion

The impact of different analysis methods and bioinformatic parameters on gene detection (*5.4.1+5.4.2*), relative abundance (*5.4.3*) and distortion of results due to potential biases were investigated. Additionally, the hypothesis that bioinformatic methods accurately detect ARG from raw WGS data was tested.

5.4.1 Antibiotic Resistance Gene Prediction is Strongly Impacted by Analysis Method and Assembly

ARG detection by WGS was measured by analyzing WGS data for the presence of the 13 qPCR target genes in the 11 wastewater samples and comparing WGS prediction to the test results of qPCR (see *SI Table 5-3*). Methods differed widely in their ability to detect ARGs in these samples (**Figure 5-1**, left). D1 (ML-method, no assembly) prediction results were most similar to qPCR results (**Figure 5-1**, right – hierarchical clustering). D2 (ML-method, assembly), which methodologically only differed from D1 by using contigs as opposed to unassembled WGS reads, detected the lowest number of ARGs of all six evaluated methods (**Figure 5-1**, left – p-value). Differences between WGS data analysis methods caused by sequencing errors [479] can be ruled out, as the same sequencing data was used across methods. Sequencing errors could, however lead to differences between WGS and qPCR results.

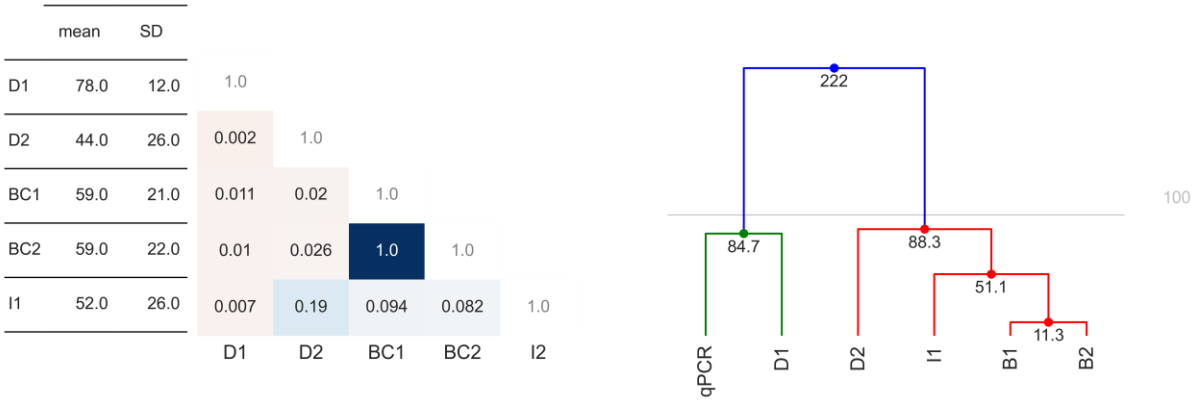


Figure 5-1 Comparison gene detection results of different analysis methods; Left. Mean and standard deviation of the presence of 13 ARG in 11 wastewater samples by WGS methods compared to qPCR (percentage) and according p-values expressing significance of resulting differences between analysis methods, Right. Hierarchical distance between different analysis methods (incl. qPCR).

The choice of analysis method ($p_{D\text{-}BC}$<0.026) and assembly ($p_{D1\text{-}D2}$=0.002) both had significant impacts on ARG prediction (**Figure 5-1**, *left*). Analysis of unassembled WGS data has previously been shown to be superior to analysis of assembled WGS data; this advantage was most pronounced for longer genes or amplicons [487]. Assembly-based analysis is likely at a disadvantage at the relatively low sequencing coverage used in this study, as the relatively low abundance of ARGs within the genome can hinder gap-less assembly and single nucleotide polymorphism detection accuracy, leading to impeded mapping of the assembled reads [475,488]. Using longer read-sequences (such as produced by third-generation sequencers) might help avoid biases in this regards [475]. metaSPAdes performs de novo assembly, which increases potential negative impact of low to moderate coverage on the

results and has been shown to not be the best options for low coverage assembly [472,489,490]. Nevertheless, algorithms usually perform better with increasing coverage, so that it may be interesting to conduct a follow-up study comparing results for different sequencing depths [491–494]. Using specialized low-coverage assemblers is yet another approach to reduce potential biases [489,491,495,496]. ML-methods do not rely on mapping to the same extent as non-ML methods; therefore, it is probable that potential disadvantages from larger target sequences have a lower impact on D1 results in the present study. Metagenomic assembly itself can be error-prone [497] and results can vary strongly [498]. Further, repetitive sequences often flank AR islands acquired by horizontal gene transfer (HGT) [499]. These cannot be correctly assembled and are discarded before assembly resulting in a loss of information [500,501], which can be crucial for correctly identifying AR profiles [502].

The choice of database ($p_{BC1\text{-}BC2}$=1.00) did not significantly impact ARG detection. These results are based on the premise of well-curated databases as points of comparison. The CARD database [503] and UniProt [504] are large, regularly updated databases. ARDB is large but not maintained anymore and all of its data can also be found in the CARD database [505]. Small, non-curated databases would show a larger advantage from being enhanced with additional data and/or added databases [506,507]. Previous research has shown that databases have an impact on results [181]. The low impact of databases on results in the present study, is likely further due to the fact that databases with focus on ARGs were chosen, indicating the importance of choosing not only well-curated databases but also databases suited to the research question instead of more generic ones. Follow-up studies including smaller, non-curated or non-ARG specific databases will be needed to verify this. Database use might, however, well be relevant for subsequent risk assessment (e.g. notation on if ARG is located on mobile genetic elements (MGEs)) [181].

5.4.2 ARG Detecttion Compared Across Methods

Detection of 13 Target ARGs – Zooming into Details

In general, lower detection of ARGs by WGS methods occurred more frequently than higher detection (compared to qPCR results, **Figure 5-2**). ARGs that were detected at very low concentrations (*qnr*S) by qPCR or which were not detected by qPCR (*van*B) were generally higher detected from WGS data. While for the ARG most abundantly detected by qPCR (*sul*1), presence as detected by WGS methods was most comparable to qPCR detection (**Figure 5-2**). Lower detection by WGS methods was associated with low gene abundances (**Figure 5-2***). Similarly, lower detection increased in "cleaner" samples, with lower bacterial loads and genetic content (*SI Table 5-1*), indicating that lower detection could be decreased by increased sequencing depth. Signal-processing, base calling limitations and disproportionate GC-content are reasons which have previously been specified [508] and are relevant for gene detection in a group of genes as diverse as ARGs, as sequences and especially GC-contents vary widely across different ARGs. D1 performed best, with only one lower detected gene in samples with high genetic content.

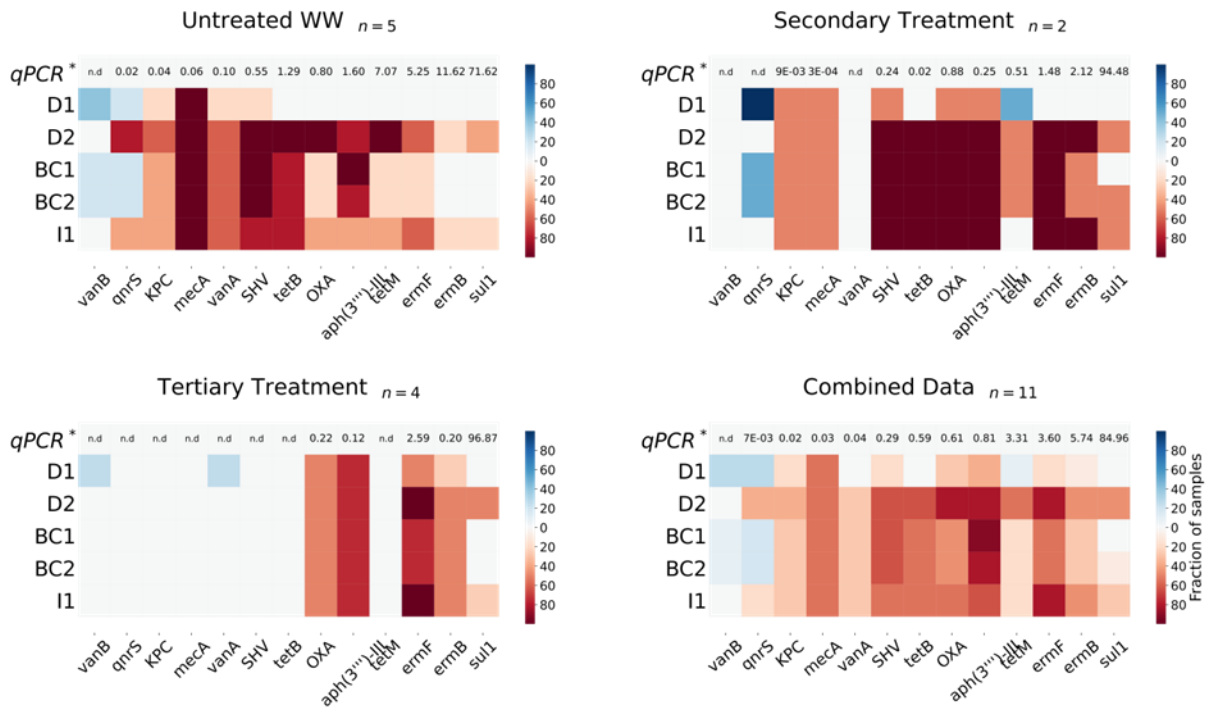


Figure 5-2: Over- and underdetection of individual genes of interest across different NGS analysis methods when compared to qPCR results; Mis-detection of target ARGs shown in percent of samples with mis-detection; blue – overdetection/(potentially false) positives, red – underdetection/(potentially false) negatives; n – number of samples in subgroup; * - relative abundance as detected by qPCR [%]; n.d.="not detected".

Using WGS methods, *van*B was solely detected in hospital wastewater samples and *qnr*S solely in WWTP influent or effluent (*SI Figure 5-1*). Supported by the non-detection of *van*B by *in-silico* qPCR (*I1*), this is an indicator that non-targeted gene subclasses can go undetected by qPCR in hospital wastewaters and WWTPs. WGS has previously been shown to be superior to qPCR in terms of gene detection [480] and to have a similar analytical sensitivity detecting mutations [477,482] as qPCR under certain conditions. "Higher detected" genes could thus also be qPCR false negatives, which seems to be the case for *qnr*S. This gene was detected by I1 by extracting the exact qPCR primer sequences from the WGS dataset. (Cross-)Validation of the different methods is therefore of utmost importance [477]. Correlations between rate of mis-detection and how well researched an ARG was, could not be shown (*SI Table 5-12*).

It has previously been shown that NGS results can be more accurate and unbiased than qPCR, as resistance-conferring mutations will not stay undetected due to dependence on specific probes or other biases from molecular methods [509].

The 13 target genes only comprised a minuscule fraction of the total number of detected ARGs by WGS and were largely not present within the 30 most abundant ARGs (**Figure 5-3**). The most abundant ARGs within the qPCR dataset were largely also most abundant within the WGS results among the target genes (**Figure 5-3**). One exception was *qnr*S, which WGS frequently detected in higher abundance than qPCR (*SI Figure 5-2*). Nevertheless, qPCR analysis is inherently focused on a selection of target sequences, while WGS results provide a more holistic picture, regardless of the analysis method used. For instance, *sul*1 represented 3.0 %, 5.5 % and 6.2 % of total ARGs predicted using method D1, BC1 and BC2, respectively, compared to over 80 % for qPCR, due to the selection of a minor fraction of target genes in qPCR analysis. To reduce the impact of the created selection bias, it is important to select reference genes appropriate to the respective research question [510,511]. Generically using generic potential proxies for ARG pollution, such as *sul*1, does not necessarily render representative results [512]. Additionally, result distortion

can result from qPCR inhibition and varying gene detection sensitivities, as has previously been observed [512,513].

General Gene Detection- The Larger Picture

WGS analysis analyzes the entirety of ARGs present in a sample and is able to predict a much larger number of ARGs than the few target ARGs selected for qPCR. This results in a more accurate, holistic picture of real conditions within the sample, while qPCR results reveal a much smaller "slice of reality". Nevertheless, differences between the ARGs detected by different WGS analysis methods could be observed.

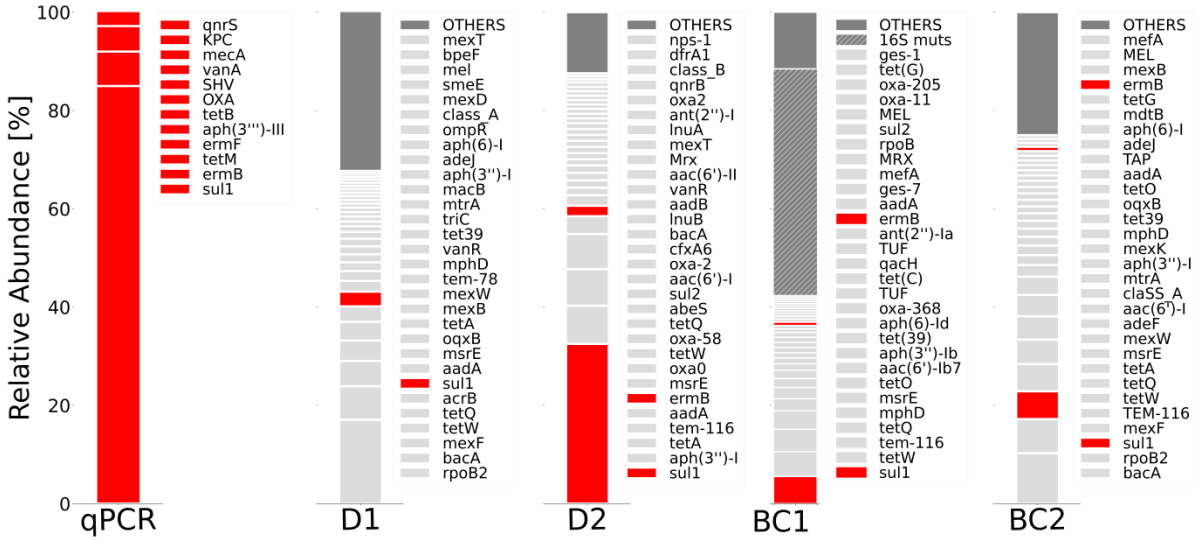


Figure 5-3: Overview of relative abundance for all ARGs detected by different analysis methods; 30 most abundant genes per analysis method shown; BC1 = results with 16S rRNA mutations grouped and excluded from 30 most abundant genes as they constitute a group of ARGs not individual genes; exact individual mutations associated with different 16S rRNA mutations listed as "BC1-a" can be seen in *SI Table 5-11*.

BC1 consistently detected a notably high number of 16S rRNA mutations conferring AR than other methods. The hypothesis, that this originates in an over-representation of 16S rRNA mutations in the CARD database was refuted, as the proportion of this type of mutation is not conspicuously high. Only 50 16S rRNA entries are present in the database, whereas the 4 most abundant beta-lactamase genes constituted 30% of all database entries. 139 entries were detected for the CTX gene class, 166 entries for TEM genes and a striking 438 entries for OXA genes (*SI Table 5-5*), all ARGs, which were, detected with lower frequency and abundance than 16S rRNA mutations. A large part of the differences between the results of BC1 and the other analysis methods was shown to originate from the vast number of detected 16S rRNA mutations in BC1. Excluding these mutations, BC1 results were more comparable to BC2 and D results (*SI Figure 5-3*).

D1 and BC2 exhibited an elevated abundance of *rpo*B2 (**Figure 5-3**), a gene closely related to the RNA polymerase beta-subunit encoding gene *rpo*B; the two genes show 88.8% similarity at nucleotide level [514]. *rpo*B, a (usually) single-copy gene, is present in all bacteria and has been suggested as a superior alternative to 16S rRNA for universal bacterial identification [515–520]. Gene duplication resulting in *rpo*B2 is one of the few cases where *rpo*B-genes are not single copy genes and always lead to rifampicin-resistant bacteria [521], whereas mutations within single copy *rpo*B can confer resistance to rifampicin but usually do not contribute to resistance [522]. As results from D1 predict nearly twice as much *rpo*B2 as results from BC2, it stands to reason that resistance based on ubiquitous housekeeping genes, such

as *rpo*B2, could be over-estimated by ML-models due to similarity with *rpo*B, as has previously been described [181].

Generally, a larger number of ARGs was predicted via ML-methods, with up to 10 times more ARGs detected when using D1, compared to other WGS analysis methods (*SI Table 5-6*). While false positives due to sequencing errors are one possible explanation [475], ARG-specific ML-models, such as deepARG, take the nature of (point-)mutations in ARGs into consideration and incorporate computational solutions, such as setting minimum read thresholds [469,475], in order to avoid false positives. A more likely explanation is assembly-independence as discussed above, which enables detection of (point-)mutations resulting in AR. D2 was similarly able to predict a larger number of ARGs when compared to non-ML methods. Further, D2 detected more ARGs than D1 in "cleaner" samples (tertiary treated WW samples), showing a benefit of assembly for sensitivity for low-abundance ARGs when using ML-methods (*SI Tables 5-6+5-7*).

One issue with both NGS and qPCR is, that they do not automatically provide phenotypic information and no information on if ARGs are located on MGEs can be derived (depending on the database used). Such information can be important for risk assessment [181].

5.4.3 Relative ARG Abundance Measurements Compared Across Methods

NGS methods quantified 50 % of target genes with abundances within the range of -50% to +100% of qPCR abundance results, while 86% of them were quantified with <1 log of difference to relative abundances measured by qPCR (**Figure 5-4**). *sul*1, *tet*M and *erm*B were quantified with the greatest accuracy across all methods and were among the most abundant ARGs quantified by qPCR (1st, 2nd and 5th most abundant, respectively; **Figure 5-3** + *SI Table 5-9*). D1 did generally tend to overestimate *tet*-genes more than other WGS analysis methods, especially in hospital wastewater samples, when looking at results at the level of individual samples as opposed to mean values shown in **Figure 5-4** (*SI Figure 5-2*). One reason that contributed to this, was faulty annotation of related *tet*-genes (e.g. most notably *tet*O), as was revealed upon systematic, manual inspection of D1 results. Interestingly, over-quantification of these genes did not equate to increased higher detection (false positive results) when compared to qPCR results (**Figure 5-2**).

The two least abundant ARGs in qPCR results (*bla*KPC and *qnr*S), were most over-quantified by all WGS analysis methods. Out of the correctly detected target genes, BC1 was able to most accurately quantify the abundance (comparing median values to exclude extreme outliers, which distort mean values). D1 was able to quantify genes more accurately than D2 or BC2 (*SI Table 5-8*). BC1 had more extreme outliers, but D1 had a larger number of outliers as the low-abundance genes that were detected by D1 were usually severely over-quantified (*SI Table 5-8 + SI Figure 5-2*).

Figure 5-4: Relative abundance (Fractions) of ARG shown by analysis method and Target ARG; Top. Mean±std for results combined from all samples shown, Bottom. Percentage difference between qPCR results and different WGS analysis method results (absolute values).

Unexpectedly, *in-silico* qPCR (I1) was able to detect gene abundances with an accuracy that was comparable to more sophisticated approaches (D1+2, BC1+2). Correlations of relative abundances of qPCR and WGS results [477,523] and high detection accuracy of and distinction between low-abundance gene variants [481,524] have previously been observed. Taking into account both gene detection and relative abundance accuracy, ML-analysis of unassembled reads was clearly superior to analysis of assembled reads.

One important aspect outside of the scope of the present study is the impact of sequencing depth. Sequencing depth can have a significant impact on the detection of low-abundance genes [462,525,526]. An increase in sequencing depth can, in many cases, improve detection. However it has been suggested that the relative proportions of detected ARGs remain largely constant "regardless of depth" [526]. Computationally decreasing the sequencing depth of our WGS dataset caused a further increase of differences between qPCR and WGS results (both regarding ARG presence and abundance), with low-concentrations ARGs being more susceptible (*SI Table 5-10*). This is an indicator that increasing real sampling depth could benefit result accuracy and reduce the strong over-quantification of genes like *qnr*S, at least to a certain extent.

5.4.4 WGS Results – In-silico qPCR

Based on WGS results, a final hypothesis was formulated: if the utilized bioinformatic tools (D1, D2, BC1, BC2) are specific and sensitive enough for accurate ARG quantification, ARGs detected with the highest abundance from WGS results, should also be quantified with highest abundance by *in-silico* qPCR. To this end, specific primers, for the five ARGs most abundantly detected by WGS analysis, were identified from literature [527–531] and screened for

using *in-silico* qPCR. As expected, *rpo*B2 and *tet*W were detected with the highest abundance from NGS-reads by I1 (*SI Figure 5-4*) across most samples, with *rpo*B2 being detected at higher concentration than *tet*W as was previously seen by general gene detection (**Figure 5-3**). Samples with higher pollution content (untreated wastewater samples) detected these genes more often than samples with lower pollution (treated hospital wastewaters). However, *bac*A, *mex*F and *rps*L could not be detected by I1, indicating a dependency of (*in-silico*) qPCR results on the selected primers. One explanation for non-detection is that the selected primers for *bac*A, *mex*F and *rps*L do not capture all variants of these genes.

5.4.5 Relevance of Environmental qPCR Results can be Increased by Combination With WGS

The position of qPCR as "gold standard" for gene quantification and discovery has previously been questioned [476]. Summarizing, it can be said that the case for gene discovery and detection is quite clear, not only from the results in the present study, but also due to WGS not being susceptible to variability in melting temperatures or nearly identical genetic sequences which can be problematic for qPCR analysis [476]. In clinical research AR is often investigated in order to answer very narrowly defined questions, such as: "Are the mycobacterium tuberculosis pathogens in this sample rifampicin resistant?". In contrast, in environmental research the amount, variety and sort of genes in samples is often unclear. WGS analysis, especially using ML-models, will be vastly more beneficial under these circumstances. The selection of appropriate genes for qPCR analysis has been shown to be of utmost importance for a research question [532] and can thus be facilitated or improved.

5.5. Conclusion

Machine learning models are superior to non-ML models, as assembly of WGS data is disadvantageous to ARG detection from environmental samples, and has only a minor positive effect on the measurement of relative ARG abundance. Independence from assembly seems especially advantageous at low WGS sequencing depths. The choice of databases has a negligible effect on ARG detection, given the usage of well-curated databases. Machine learning models were not only superior for ARG detection, but also detected unknown or unclassified subclasses of ARGs (e.g. potential sub-variants of *van*B) with similarity to known ARGs. This is an important advantage of using NGS, as potential false negatives (from qPCR results) can be identified. ARGs measured at very low concentration needs to be considered carefully as such ARGs can be severely over-quantified by WGS methods compared to higher-concentration ARGs.

When used in isolation qPCR might not be the best method to conduct research (especially exploratory research) on antibiotic resistance in environmental settings. A combination of both, WGS and qPCR is thinkable. A small number of selected genes could be quantified by qPCR and relative WGS abundances of all detected (NGS) genes converted to total concentrations based on the qPCR results of a few genes. The strong selection biases caused by qPCR could be prevented as WGS results would contribute to the "bigger picture" and analysis of qPCR-quantified ARGs could be performed within a more realistic frame, while avoiding overestimation of low concentration genes.

5.6. Supplementary Material

SI Table 5-1: Sample information and metadata;

Sample	Type	Matrix	Details	16S copies / mL	Total WGS reads	Location	ID
HWW1	Untreated	Hospital WW	Untreated HWW Effluent before treatment in Pharmafilter	1.4E+09	12851819	1	1
MBR	Treated	Hospital WW	WW after treatment step 1 in Pharmafilter	1.1E+05	20407531	1	8
GAC	Treated	Hospital WW	WW after treatment step 2 in Pharmafilter	4.6E+06	19738700	1	9
Ozone	Treated	Hospital WW	WW after treatment step 3 in Pharmafilter	3.1E+06	19338137	1	10
UV	Treated	Hospital WW	WW after treatment step 4 in Pharmafilter – Treated Effluent	5.7E+05	21425730	1	11
CWW	Untreated	Communal WW	Untreated communal WW (no contact with HWW)	5.6E+09	25977378	1	3
HWW2	Untreated	Hospital WW	Untreated HWW Effluent	7.6E+10	21519268	2	2
W1_In	Untreated	Mixed	Untreated communal WW (including WW from hospitals)	8.7E+09	20661582	1	4
W1_Out	Treated	Mixed	Treated communal WW (including WW from commune and hospitals)	8.7E+07	18813406	1	5
W2_In	Untreated	Mixed	Untreated communal WW (including WW from hospitals)	2.4E+09	18489709	2	6
W2_Out	Treated	Mixed	Treated communal WW (including WW from commune and hospitals)	2.6E+08	21502321	2	7

SI Table 5-2: qPCR gene subclasses captured by different qPCR primers and screened for in WGS datasets;

Name	Gene-Identifiers	Acccession Number (CARD)
***aph*3a**	aph(3')-IIIa	ARO:3002647
***mec*A**	mecA	ARO:3000617
***sul*1**	sul1	ARO:3000410
***tet*B**	tet(B), tetA(B)	ARO:3000165,ARO:3000166

***tet*M**	tetM	ARO:3000186
***van*A**	vanA	ARO:3000166
***van*B**	vanB	ARO:3000013
***bla*$_{KPC}$**	KPC-6,KPC-17,KPC-24,KPC-3,KPC-19,KPC-12,KPC-9,KPC-8,KPC-14,KPC-2,KPC-4,KPC-10,KPC-16,KPC-5,KPC-7,KPC-13,KPC-22,KPC-11,KPC-15	ARO:3002316,ARO:3002327,ARO:3004496,ARO:3002313,ARO:3002329,ARO:3002322,ARO:3002319,ARO:3002318,ARO:3002324,ARO:3002312,ARO:3002314,ARO:3002320,ARO:3002326,ARO:3002315,ARO:3002317,ARO:3002323,ARO:3003180,ARO:3002321,ARO:3002325
***bla*$_{OXA}$**	OXA-33,OXA-224,OXA-4,OXA-31,OXA-1,OXA-320,OXA-47	ARO:3001427,ARO:3001806,ARO:3001399,ARO:3001425,ARO:3001396,ARO:3001793,ARO:3001781
***bla*$_{SHV}$**	SHV-153,SHV-179,SHV-183,SHV-182,SHV-180, SHV-56, SHV-121, SHV-42, SHV-89, SHV-74, SHV-24, SHV-2, SHV-7,SHV-134, SHV-185, SHV-148, SHV-186, SHV-141, SHV-11,SHV-39,SHV-60, SHV-64, SHV-103, SHV-127, SHV-165,SHV-8, SHV-101, SHV-135, SHV-31, SHV-34, SHV-137,SHV-110, SHV-100, SHV-157, SHV-5, SHV-145, SHV-163,SHV-6, SHV-15, SHV-84, SHV-161, SHV-65, SHV-30, SHV-133, SHV-21, SHV-70, SHV-61, SHV-66, SHV-59,SHV-38, SHV-158, SHV-151, SHV-144, SHV-48, SHV-86,SHV-77, SHV-69, SHV-37, SHV-27, SHV-150, SHV-35,SHV-154, SHV-95, SHV-93, SHV-123, SHV-108, SHV-187,SHV-104, SHV-83, SHV-78, SHV-44, SHV-147, SHV-106,SHV-82, SHV-76, SHV-12, SHV-13, SHV-50, SHV-156,SHV-26, SHV-105, SHV-1, SHV-149, SHV-178, SHV-46,SHV-81, SHV-62, SHV-152, SHV-25, SHV-107, SHV-33,SHV-63, SHV-80, SHV-75, SHV-102,SHV-124, SHV-22,SHV-159, SHV-125, SHV-2A, SHV-3, SHV-23, SHV-55,SHV-120, SHV-40, SHV-172, SHV-98, SHV-111, SHV-28, SHV-57, SHV-85, SHV-155, SHV-129, SHV-94, SHV-109,SHV-79, SHV-20, SHV-67, SHV-173, SHV-49, SHV-142,SHV-126, SHV-96, SHV-45, SHV-189, SHV-14, SHV-128,SHV-97, SHV-160, SHV-53, SHV-32, SHV-41, SHV-36,SHV-143, SHV-119, SHV-18, SHV-19	ARO:3001176
***erm*B**	ermB	ARO:3000375
***erm*F**	ermF	ARO:3000498
***qnr*S**	QnrS1, QnrS2,QnrS3,QnrS4,QnrS5,QnrS6,QnrS7,QnrS8,QnrS9	ARO:3002790,ARO:3002791,ARO:3002792,ARO:3002793,ARO:3002794,ARO:3002795,ARO:3002796,ARO:3002797,ARO:3002798

SI Table 5-3: Percentage of correctly detected ARGs per sample across different analysis methods (compared to qPCR results);

sample	1	2	3	4	5	6	7	8	9	10	11	mean	SD
qPCR	100	100	100	100	100	100	100	100	100	100	100	100	0
deepARG1	83	83	83	83	58	75	75	66	100	58	91	78	12
deepARG2	16	33	16	50	41	8	25	58	91	66	75	44	26
BC1	25	50	75	58	41	50	33	66	100	66	83	59	21
BC2	25	58	75	58	33	50	33	66	100	66	83	59	22
insilico 1	16	50	58	58	50	33	33	66	100	66	83	56	23
insilico 2	8	50	75	66	25	16	33	66	83	66	83	52	26
	28.8	54	63.6	62.2	41.3	38.7	38.7	64.7	95.7	64.7	83		

SI Table 5-4: Significance of differences resulting from different analysis methods expressed by p-values;

	D1	D2	BC1	BC2	I1	I2
D1	1	0.002	0.011	0.010	0.010	0.007
D2	-	1	0.020	0.026	0.007	0.190
BC1	-	-	1	1.000	0.227	0.094
BC2	-	-	-	1	0.307	0.082
I1	-	-	-	-	1	0.319
I2	-	-	-	-	-	1

SI Table 5-5: Number of Genes per ARG Class Within the CARD Database; 5 most abundant gene classes as well as classes for genes of interest shown

ARGs were not analyzed on gene family level in this study but by looking at individual detected ARGs. However, the number ARG entries per class is important in order to make statements about potential biases introduced by a different strongly varying number of gene entries per ARG class. To this end, the following list is shown.

AMR Gene Family	Number of individual genes in database	ARGs of interest associated
OXA beta-lactamase	438	*bla*$_{OXA}$
TEM beta-lactamase	166	n/a
SHV beta-lactamase	157	*bla*$_{SHV}$
CTX-M beta-lactamase	139	n/a
resistance-nodulation-cell division (RND) antibiotic efflux pump	123	n/a
16s rRNA with mutation conferring resistance	55 *	16S mutations
quinolone resistance protein (qnr)	102	*qnr*S
sulfonamide resistant sul	4	*sul*1
van ligase, glycopeptide resistance gene cluster	2	*van*A, *van*B
APH(3')	17	*aph*(3')-IIIa
Erm 23S ribosomal RNA methyltransferase	42	*erm*B, *ermF*
major facilitator superfamily (MFS) antibiotic efflux pump	112	*tet*B
tetracycline-resistant ribosomal protection protein	42	*tet*M
KPC beta-lactamase	12	*bla*$_{KPC}$
methicillin resistant PBP2	6	*mec*A

* - combined 16S rRNA mutations, including mutations conferring resistance to: aminoglycoside (42 genes), peptide antibiotics (4 genes), tetracyclines (4 genes), pactamycin (1 gene); as well as 16S rRNA Methyltransferases (4 genes)

SI Table 5-6: Number of different ARGs detected per sample by analysis method;

sample	1	2	3	4	5	6	7	8	9	10	11

qPCR	12	12	12	12	10	12	12	12	12	12	12
D1	250	284	358	328	110	264	130	107	68	90	78
D2	144	205	-	212	120	53	-	-	160	174	143
BC1_a	53	136	166	122	24	74	15	21	14	16	8
BC1_b	49	132	155	111	15	66	15	16	8	12	7
BC2	66	155	186	145	26	77	26	24	13	25	14

SI Table 5-7: Maximum number of ARGs detected per treatment type by analysis method;

	qPCR	**D1**	**D2**	**BC1_a**	**BC1_b**	**BC2**
untreated	12	296	122	110	102	125
secondary	11	120	120	19	15	26
tertiary	12	85	159	14	10	19

SI Table 5-8: Result discrepancy and variation from qPCR results across qPCR genes by analysis method; * - median absolute deviation, ** - mean of number of analysis methods that detected the gene across samples

	BC1	**BC2**	**D1**	**D2**	**I1**	**I2**
median	131.5698	708.6288	249.3957	13966.4	129.7869	187.4322
mad *	8209.689	7829.968	3216.14	401708.1	107.8558	597.5742
mean	5550.291	5902.962	2106.647	261371.7	162.5882	599.8495
std	18135.34	20188.95	5929.488	813599.4	235.4398	1895.826
count **	3.272727	3.272727	6	3.545455	2.636364	2.454545

SI Table 5-9: Result discrepancy and variation from qPCR results disregarding analysis method (=across all analysis methods) by quantified gene; * - median absolute deviation, ** - mean of number of analysis methods that detected the gene across samples

	*sul*1	SHV	*erm*B	*tet*M	OXA	*erm*F	*aph*(3''')-III	KPC	*van*A	*tet*B	*qnr*S	*mec*A	*van*B
median	26	124	206	220	376	511	746	850	4435	29745	65211.63	[6284588]	-
mad *	18	61	381	325	219	45	479	193	4249	26402	14345.27	-	-
mean	35	124	410	377	477	516	745	936	4435	29745	56555.25	[6284588]	-
std	26	216	512	522	421	105	1803	853	6009	52273	18621.83	-	-
count **	5	1	3	2	2	3	1	1	0	1	1.181818	[0.181818]	-

SI Table 5-10: ARG presence and relative abundance at different computational sequencing depths

In order to be able to make statements about the impact of sequencing depth on analysis results, the WGS results dataset (D1) was computationally reduced to outputs associated with a reduced sequencing depth. To achieve this, a Monte-Carlo-Simulation of 100 cycles was run and random subsets of the dataset were created using the **.sample()** method from the Python **pandas** library [iv]. Subsets were created with 50 %, 10 %, 5 %, 1 %, 0.5 % and 0.1 % of the dataset. The results from the 100 cycles were combined and are shown below.

% of dataset in subset sample	100	50	10	5	1	0.5	0.1
***sul*1**	31.33531	29.37319	23.90465	22.24264	21.02796	19.57348	30.0094
***tet*M**	25.20403	25.2043	25.70417	24.94073	23.91027	23.53409	20.07804
***erm*B**	20.85385	20.97679	20.67042	19.48029	19.1545	16.77685	19.80445
***erm*F**	7.010843	7.050997	7.721874	8.420604	12.25819	10.00077	5.072588
***qnr*S**	3.792273	3.765814	4.122021	4.657416	5.970582	5.04873	5.652149
***aph*(3''')-III**	3.709621	4.215978	5.754014	7.034873	7.214429	4.918986	2.499471
OXA	2.722657	2.78142	3.524687	4.24875	4.296568	3.836519	2.838951
***van*B**	1.701661	2.085022	2.737983	2.458656	1.459538	1.351951	1.203407
***tet*B**	1.54608	1.644026	2.454187	3.177604	3.043865	3.177236	1.358165
KPC	1.429395	1.745755	1.942629	1.991098	0.857291	1.209409	0
***van*A**	0.408399	0.624256	0.78519	0.688611	0.228386	0.328339	0.338095
SHV	0.285879	0.532451	0.678171	0.658732	0.578414	0.098361	0
***mec*A**	0	0	0	0	0	0	0

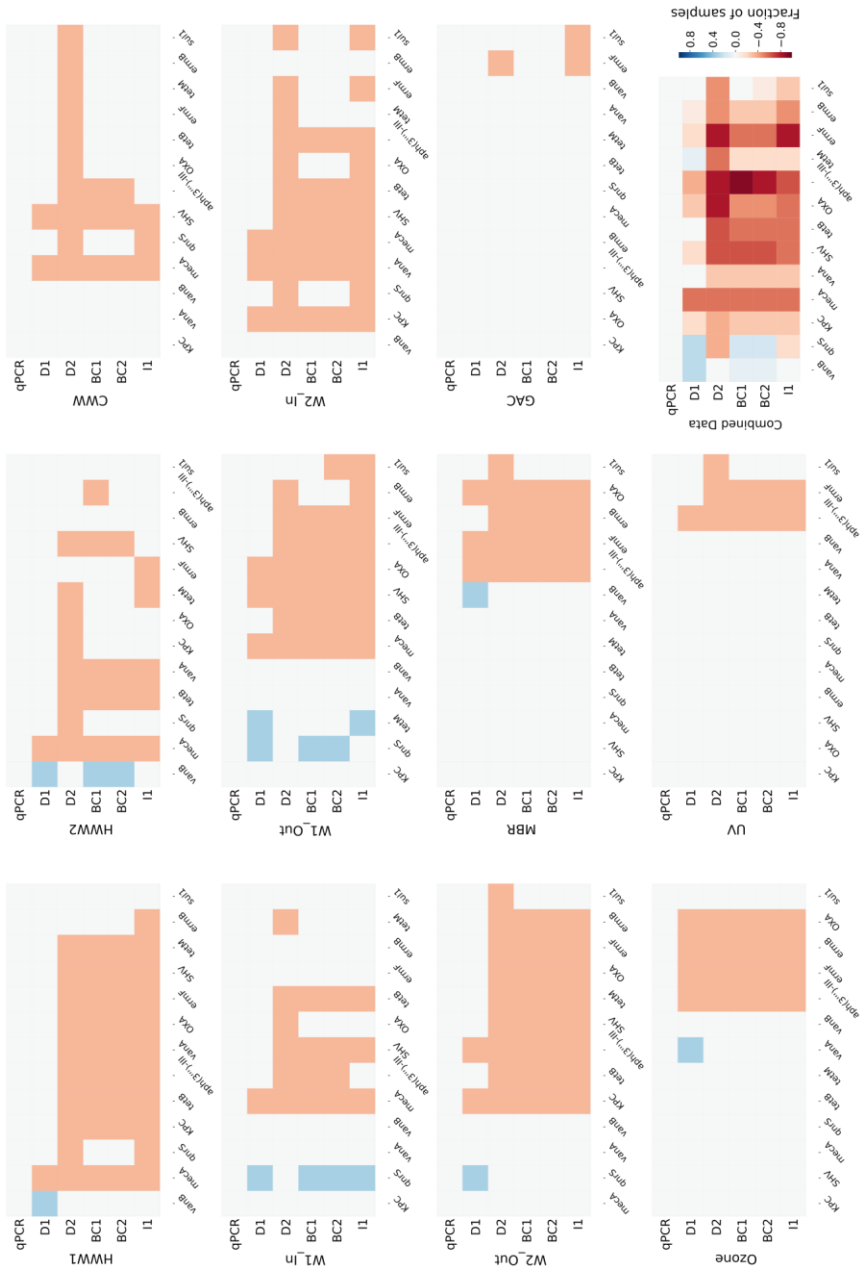
Si Table 5-11: Exact individual mutations associated with different 16S rRNA mutations and TUF mutations;

Abbreviation	Organism carrying mutation	Resistance against
16S rRNA mut. 1	Neisseria meningitidis	spectinomycin
16S rRNA mut. 2	Chlamydophila psittaci	spectinomycin
16S rRNA mut. 3	Pasteurella multocida	spectinomycin
16S rRNA mut. 4	Mycobacterium tuberculosis	streptomycin
16S rRNA mut. 5	Neisseria gonorrhoeae	spectinomycin
16S rRNA mut. 6	Mycobacterium abscessus	neomycin
16S rRNA mut. 7	Helicobacter pylori	tetracycline
tuf 1	Planobispora rosea	inhibitor
tuf 2	Escherichia coli	tobramycin
tuf 3	Clostridium difficile	tobramycin
tuf 4	Streptomyces cinnamoneus	tobramycin
tuf 5	Escherichia coli	Enacyloxin
16S rRNA mut. 8	Mycobacterium tuberculosis	amikacin
16S rRNA mut. 9	Mycobacterium chelonae	tobramycin
16S rRNA mut. 10	Mycobacterium tuberculosis	kanamycin
16S rRNA mut. 11	Mycobacterium abscessus	tobramycin

Si Table 5-12: Number of articles found via Google Scholar per gene name; data retrieval date: 10.02.2020

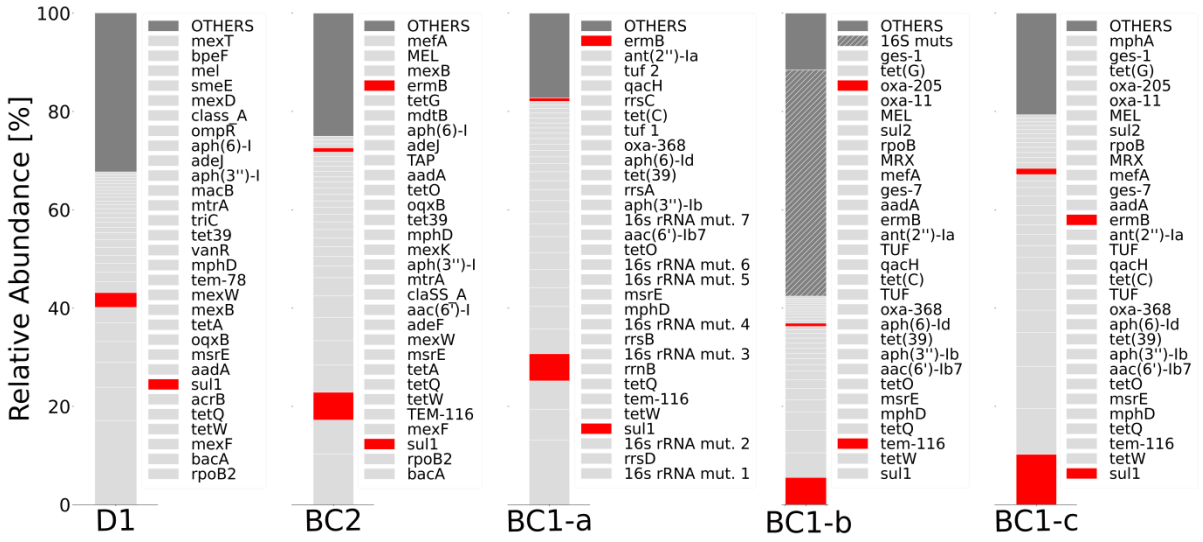
	KPC	OXA	SHV	aph(3''')-III	ermB	ermF	mecA	qnrS	sul1	tetB	tetM	vanA	vanB
articles	10400	16300	12800	3850	9060	3180	57800	5460	22400	5190	15300	45000	23700

SI Figure 5-1: ARG detection by different analysis methods across individual samples (plot 1-11) and of combined samples (plot 12);

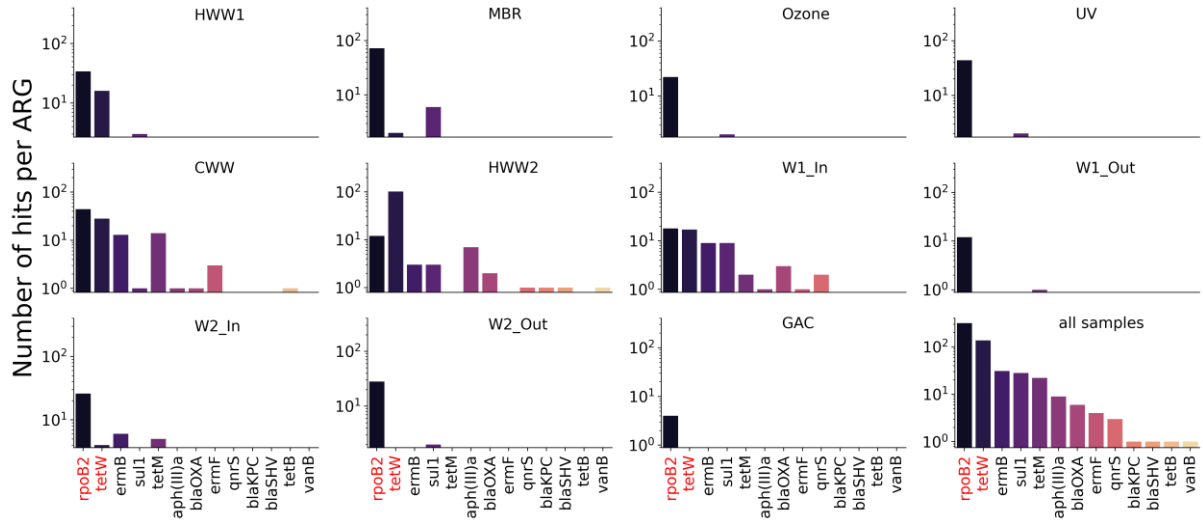


SI Figure 5-2: Relative ARG concentrations as detected by different analysis methods across individual samples (plot 1-11) and of combined samples (plot 12);

SI Figure 5-3: Comparison between BC2+D results and BC1 results including vs excluding 16S rRNA mutations conferring antibiotic resistances; BC1-a : regular (writing out all predicted ARGs), BC1-b : 16S rRNA mutations conferring antibiotic resistances grouped together (not in top 30 genes), BC1-c : 16S rRNA mutations conferring antibiotic resistances completely excluded from figure;



SI Figure 5-4: Detection of rpoB2 and tetW by insilico qPCR compared to other qPCR genes; results per sample (subplots 1 – 10) and for all samples (subplot 11); *bacA, mexF and rpsL were screened for but not detected by I1



6. Concluding Chapter: "Where do we go from here?"

In scientific circles and beyond there has been a lot of talk about the "post-antibiotic" era. Especially in light of the current public health crisis and its economic and social implications and consequences that are described in Chapter 1.1., scientists, policymakers and the general public alike are becoming more alert to the risks and dangers that antibiotic resistance poses to our health and society.

Looking at the increasing number of multi-resistant pathogens, the growing numbers of complications and death due to antibiotic resistance as well as widespread levels of resistance in the environment, there is no denying that the post-antibiotic era is already approaching rapidly and, as the most recent pandemic demonstrated impressively, we are not sufficiently prepared to manage infectious disease outbreaks using social measures and restrictions, and these come at great social and economic costs. The focus now needs to be on damage control, containment, deceleration of the spread of resistance as well as the implementation of effective measures (including the education of the general public), based on informed risk assessment.Raising awareness and increased legislation in regards to prescription, social measures to contain infections and heightened control and testing in clinical settings are one way to control the spread of antibiotic resistance. Another effective way is to control and minimize the spread from clinical and urban environments via the urban wastewater system to the environment and thus to stop the cycle of ARG propagation between natural and anthropogenic environments. On one hand, propagation within the urban wastewater system by way of horizontal gene transfer could be reduced. On the other hand, increased monitoring of wastewater systems would increase our knowledge of mechanisms behind ARG propagation in these systems and thus create more data and information to be used further.

Efficient and accurate risk assessment relies heavily on information and models. The present work aimed at providing more information, elucidating the mechanisms behind the spread of wastewater-derived antibiotic resistance genes in the environment and compared methods in order to make predictions about these mechanisms' and methods' effectiveness and adequacy to generate knowledge about the spread of antibiotic resistance genes in wastewaters and the environment. Summarizing conclusions, recommendations and future perspectives will be discussed in the present chapter.

6.1. Concluding Discussion

The aim of this thesis was to explore and understand microbial strategies and risk factors for antibiotic resistance gene propagation in wastewater and river environments. To achieve this goal, four specific sets of experiments were designed, each according to one of the targeted research questions. The encompassing approach was to evaluate antibiotic resistance and its propagation, using a combined approach of molecular, bioinformatic and engineering methods, including:

- (multiplex) qPCRs assays
- different bioinformatic pipelines for WGS data analysis
- bioreactor experiments.

In *Chapter 1,* the status quo of scientific knowledge is shown and open questions, as well as research questions for the present work, discussed. It is shown, that at the beginning of this research, the exact impact of hospital wastewaters on the downstream communal wastewater system were not completely clear. The literature and scientific publications are a rich source of empiric information about antibiotic resistance presence, distribution and concentrations in different water systems. It was, however, not clear what the exact quantitative and qualitative benefit of on-site wastewater treatment of high-risk point sources (like hospitals) were. Similarly, the influence of sub-inhibitory antibiotic concentrations on horizontal gene transfer and ARG propagation were not completely clear; the consequences resulting from mixing waters from high-concentration (in terms of antibiotic), high-resistance (in terms of ARG abundance and variety) sources with low-concentration, low-resistance environmental waters were largely unexplored. Finally, analytical differences resulting from different analysis technologies, in the field of ARG research, were widely assumed but had not been explicitly compared. Nevertheless, results gained by different analysis methods are widely directly compared to each other. Knowledge about the impact of the analysis method is decisive to be able to compare results obtained by different methods and to be able to draw accurate conclusions from similarities and differences based on such comparisons.

In *Chapter 2*, the impact of on-site hospital wastewater treatment on the receiving communal wastewater system was analyzed. It could be shown that on-site treatment of high-risk point sources can have a significant positive impact on the downstream water system (for example by quantifiably reducing the number and concentration of ARGs and antibiotics) and might help mitigate the risk of ARG propagation. This knowledge is vital in order to decide on effective legislation. The results presented in *chapter 2* used hospital wastewater to demonstrate the increased risk potential of high-risk point-sources and the benefits of on-site treatment of such sources. All of the 13 ARGs detected in hospital wastewater were significantly reduced in relative and absolute concentration by on-site treatment; nine out of these 13 ARGs could be reduces to under the limit of detection or completely eliminated. The results could well be representative for other high-risk point sources, such as antibiotic production sites. To verify this hypothesis, follow-up studies at antibiotic production sites and other high-risk point sources of antibiotics and antibiotic resistance genes should be conducted.

However, while a large number of antibiotics were monitored, only 14 genes specifically selected for their relevance in natural environments were investigated and changes in the microbial wastewater community were out of the scope of the research. Based on the results of *chapter 5* it has become clear, that the application of target-based sequencing as

well as metagenomics in this research could have added value to the experimental set-up ad given more insight into the mechanisms behind ARG propagation driven by discharges from high-risk point sources.

The hazard posed by hospital wastewaters as well as the impact on downstream communal wastewater systems, due to elevated levels of antibiotics, antibiotic resistance genes and other pharmaceutically active or infectious agents, including viruses, has been confirmed in several studies [533–540]. However effective reduction, especially of antibiotic resistance genes, is likely strongly dependent on the type of treatment. Advanced treatment technologies (such as in *Chapter 2*) seem to be far superior to secondary wastewater treatment, with improved ARG concentration reduction rates of up to 3.6-fold higher. Secondary treatment might acerbate the risk resulting from antibiotic resistance genes in hospital wastewater due to increasing the relative concentration of resistance genes on mobile genetic elements [533,539] and might not be powerful enough to sufficiently reduce antibiotic concentrations [541,542]. Secondary treatment increased relative ARG concentrations for approximately 10-30 % of all ARGs detected. Advanced treatment technologies have been shown to render varying degrees of benefits depending on treatment parameters (such as ozone concentrations or hydraulic retention times of GAC) [543].

One of the recommendations, from the research presented in *Chapter 2*, was to start implementing on-site treatment of wastewaters at high-risk point sources. Other calls for decentralized on-site treatment, stricter legislation for wastewaters from high-risk point sources and stronger monitoring (including of hospital wastewaters) have since been voiced [533,537–539,543–549].

In *Chapter 3*, it was shown that anthropogenic pollution, via urban and industrial wastewater and agricultural manure discharges, does not lead to continuously increasing levels of antibiotic resistance genes in the water body that is affected by these pollution sources. A relationship between antibiotic usage/pollution and between a number of proposed proxies for ARGs from anthropogenic origin was discussed. A direct link between antibiotic usage and antibiotic resistance could not be determined in the present work. Other studies have found varying evidence for correlations between antibiotic prescription and usage and antibiotic resistance levels. Some studies detected a definite correlation [550–552], while other studies detected no correlation or correlations only for a limited number of antibiotics with tetracycline being most prominent for correlations with tet-gene concentrations [553–556]. A number of reasons for potential false negative non-detection of correlations can be found among the experimental setup of the present work: 1) the investigated ARGs were selected due to their importance and impact for antibiotic resistance in the environment; this selection does not necessarily have to be representative of the composition of ARGs to be found in the samples (2) regional antibiotic usage was determined by data from governmental and other administrative sources, not by quantifying antibiotic concentrations from the utilized samples (3) ARGs are often strongly associated with and linked to specific bacterial phyla, leading to an increase of those ARGs upon exposure to environmental conditions which select for certain phyla [557]. The present research was mainly aimed at determining if antibiotic resistance gene concentrations increase along the river Rhine and relationships between antibiotic usage and ARG concentrations were a secondary research question. Therefore, the experimental setup resulted in the shortcomings described above, which could unveil indications and cross-resistances but has otherwise limited explanatory power to describe the relationship between antibiotic usage and ARG concentrations. The direct measurement and quantification of antibiotics using HPLC, as was done in Chapter 2, has proven to be the better option.

In *Chapter 4*, the risk and impact associated with sub-inhibitory antibiotic concentrations of tetracycline on transfer of ARG were explored. It could be identified that locations, which act as meeting point for high-resistance wastewaters and natural water bodies, present a particular risk. Several studies investigated the general impact of sub-inhibitory antibiotic concentrations [171,558–568], identifying the effects of sub-inhibitory concentrations on a number of mechanisms, including: induction of transducing phage [558], induction of antibiotic resistance [171,561,567,568] and biofilm formation [394,564,565]; all mechanisms that can increase the propagation of antibiotic resistance. Despite research revealing some ways in which sub-inhibitory antibiotic concentration might propagate antibiotic resistance (as mentioned above), not much research has been conducted to obtain more insight into the processes driven by sub-inhibitory antibiotic concentrations on antibiotic resistance and bacterial community composition in wastewater-impacted natural water environments and it is a field of research which needs to be shown greater attention. Nevertheless, in light of the findings of the studies cited above, the increased potential from AR propagation in complex bacterial communities (when compared to simple two-organisms donor-recipient systems) described in chapter 4 can be explained for example by the presence of phages in more complex samples or the presence of a larger number of microorganisms with the capability for biofilm formation, which can be stimulated by sub-inhibitory antibiotic concentrations and thus create adequate local microenvironments for the exchange and propagation of antibiotic resistance genes. Additionally to sub-inhibitory antibiotic concentrations, sub-inhibitory concentrations of other chemical compounds have been shown to have effects on antibiotic resistance propagation[560,568–571]. These processes could, in part, be driven by increased Integrase expression under the influence of sub-inhibitory compound concentrations [561] or by modified biofilm formation [564,565,570]. Hotspots of wastewater-impacted natural water environments could not easily be identified by standard monitoring methods, as these are regularly aimed towards detecting high compound concentrations. The potential risk from locations acting as "meeting point" for wastewaters and natural water bodies could therefore have long been overlooked and underestimated as a source of antibiotic resistance propagation. More studies in this direction are necessary to definitely confirm or rule out an increased risk potential.

In *Chapter 5*, the advantages and shortcomings of different molecular analysis methods (namely qPCR and multiple WGS analysis pipelines), frequently used for environmental antibiotic resistance research, were elucidated. Surprisingly, WGS results were not only very accurate for ARG detection, but also for relative ARG quantification, with over half of all gene concentrations measured by WGS not varying more than 100% from qPCR concentrations . Machine-learning models performed especially well and can reduce the need for assembly steps, which can add another level of complexity and potential source for errors to WGS analysis in non-ML analysis pipelines. Improving ML –and deep learning algorithms will further improve WGS quantification accuracy [474,572,573]. Additionally, ML-algorithms are a huge potential asset in the fight against antibiotic resistance in a number of other fields, including antibiotic creation [574], risk assessment [380], outbreak prediction [575] or prescription aid [576]. Its importance in antibiotic resistance research will likely strongly increase in the years to come.

Regarding the experimental setup from the previous chapters described in this thesis in light of the results in *Chapter 5*, it becomes clear that WGS adds tremendous value and increases the amount of available information disproportionately. In hindsight, applying WGS to most research questions in this thesis might have had added substantial merit in terms of perspective, information and generated knowledge. Although absolute gene concentrations cannot be obtained from WGS data, relative abundances and a more comprehensive context due to a significantly increased understanding of the presence of ARGs would enhance the knowledge available for thorough analysis of the data. The benefits of WGS are especially

apparent in environmental research as, unlike in clinical research, specific ARGs of interest and their distribution are often not known, during the formulation of research questions. The absence of more complete data resulting from WGS (compared to qPCR), might well lead to the oversight and neglect of important key factors, relationships and risk factors. Similarly, the correct categorization and importance of ARGs detected by qPCR would strongly benefit from WGS data, to be able to evaluate ARGs with regards to the "complete picture". The benefits of WGS compared to qPCR have been extensively described, including independence from specific primers leading to the ability to capture known and unknown genetic entities and the status of qPCR as the "gold standard" has been challenged [577–581]. Technical shortcomings of WGS, especially limited reads length number and cost, are constantly being improved with technological and computational advances [580,582]. The approach of combining initial WGS analysis with subsequent qPCR analysis of specific genes of interests for a more "fine-grained" insight (when necessary) or phenotypic methods, has been mentioned [579,583–585]. Based on the results of *chapter 5,* one of the recommendations of this thesis is to analyze environmental samples using WGS before choosing the most adequate ARGs for absolute quantification via qPCR, based on WGS data. This approach will help formulating and answering research question more targetedly, enable researchers to analyze qPCR results with greater exploratory power and increase the potential to recognize necessary gaps in knowledge, which could be, addressed by follow-up research.

While WGS and subsequent computational analysis have the potential to "super-charge" biological environmental biology and ecology research definite guidelines are missing. Guidelines and protocols, such as the MIQE guideline for qPCR [343], are crucial to ensure reproducibility and comparability of results across studies and research teams. For methods requiring bioinformatic-intense analysis, such guidelines are especially important, as a myriad of different analysis tools exist for every possible step of WGS data analysis [585,586]. As was shown in *Chapter 5*, even small differences in bioinformatic analysis pipelines can have significant effects on the generated results and guidelines on minimum sequencing depth for ARG investigation in different environmental samples, agreements on the use of machine-learning models (instead of assembly-based pipelines rendering inferior results) and defined databases as well as a consensus on the acceptable minimum alignment or minimum query coverage could be thinkable. Of course, different fields of research require different analysis and analyses methods, however producing guidelines for at least the bare minimum of consensus between researchers would go a long way in increasing the quality of research in terms of reproducibility and comparability.

6.2. Implications for Society and Recommendations

The present work shows quite clearly that point-sources of antibiotics and antibiotic resistance genes, such as untreated hospital wastewaters, increase antibiotic and ARG concentrations, and introduce hospital-related ARGs into downstream communal wastewater systems and subsequently into the environment, thus increasing exposure (e.g via irrigation)[587–592]. In humans and animals increased exposure can lead to infections with antibiotic resistant bacteria [593–596]. In the (environmental) bacterial community exposure can increase the opportunity for HGT to environmental bacteria and pathogens [597,598].

It was further clarified, that sub-inhibitory antibiotic concentrations can play a crucial role in the propagation of antibiotic resistance by means of increased potential for horizontal gene transfer. In literature, this potential was shown to be strongly matrix-dependent, so that the

exact effects cannot easily be predicted in diverse settings and under varying conditions. Nevertheless, it is clear that the contact interface of anthropogenically impacted wastewaters and environmental water bodies present an additional risk. Untreated wastewater of point sources could thus have an even larger contribution to the spread of antibiotic resistance. To better understand the implications of these results, follow-up studies in using a larger number of antibiotics could be conducted and experimental conditions could be chosen to more closely mimic specific aquatic environments or local conditions (e.g. temperature could be shown to play a role and the outcomes might differ in different climatic regions of the world).

Based on the conclusions in this section and in *section 6.1*, it can be said, that two of the main measures to reduce the spread of antibiotic resistance are:

- Implementation of on-site wastewater treatment at locations, which can be described as high-risk point sources of antibiotics and antibiotic resistance genes. Such location especially include hospitals and antibiotic production sites

- Increased environmental monitoring as well as wastewater-based epidemiology. Increased environmental monitoring could be conducted in order to identify locations which may present a special hazard due to proximity to high-risk sources of wastewater in order to be able to introduce further measures which can mitigate risk and could help to evaluate the efficiency of already implemented measures (such as on-site treatment).

Additionally, internationally coordinated behavioral changes and social conventions are crucial in order for the measures described above to take full effect. Such changes include measures, such as: stricter or adjusted prescription policies, internationally coordinated stewardships, the use of AI for improved prescription and reduced antibiotic use for veteranary purposes [599–604].

6.3. Questions for Further Research

The research described in this document, has created a number of new research questions, building on the results generated. Some of the most important and pressing ones are mentioned and the potential for further research explored.

Which conditions enable increased horizontal gene transfer at sub-inhibitory antibiotic concentrations?

One of the key questions arising from the result discussed previously is the question under which exact conditions and environments very low or sub-inhibitory antibiotic concentrations present a potential hazard. As shown by this (*Chapter 4*), the effects of sub-inhibitory antibiotic concentrations on antibiotic resistance and horizontal gene transfer are strongly matrix-dependent. Results in *chapter 4* show, that environments with increased levels of existing antibiotic resistance and environments which contain higher proportions of ARG-arrying donor organisms are susceptible to increases of horizontal gene transfer of ARGs under the influence of sub-inhibitory antibiotic concentrations. Nevertheless, the widespread presence of sub-inhibitory antibiotic concentrations in anthropogenic and natural environments makes this a non-trivial question, as even a limited number of very specific "beneficial" conditions could easily amass.

Apart from anthropogenic and environmental matrices, the question about which microorganisms are susceptible to increased horizontal gene transfer under pressure from sub-inhibitory antibiotic concentrations poses itself. Additionally, the importance of bacterial communities needs to be posed. Are bacterial communities in particular niches or with a specific taxonomic composition especially enable horizontal gene transfer events under given sub-inhibitory antibiotic concentrations? Answering these questions will be of the essence in order to make predictions about risk and for general risk assessment in different environments.

Which treatment parameters of wastewater treatment technologies optimize antibiotic resistance gene reduction and reduce risk?

In the present work and beyond, different advanced wastewater treatment technologies have been shown to have a varying impact on antibiotic resistance gene as well as antibiotic concentrations. It will be important to know which treatment technologies are most efficient, while not increasing risk. Results in *Chapter 2* have shown that treatment technologies targeting antibiotic resistance genes are not the best technologies to also reduce antibiotic concentrations. Further, it could be shown that some advanced treatment technologies are more effective at reducing the concentration antibiotic resistance genes. A combination of different technologies in parallel thus seems to be the best solution. Under such a arrangement, treatment costs and throughput limits need to be considered and balanced for realistic implementation.

To effectively implement on-site hospital wastewater treatment, gathering knowledge about which treatments to employ using which parameters for optimal result. It will further be important to know if increased stress from some treatment technologies significantly increase the risk for horizontal gene transfer for the chosen combination of technologies. It will further be important to investigate if selection of

(potentially) pathogenic microorganisms or microorganisms with a high potential for horizontal gene transfer could be selected as an unwanted side effect. Investigating the impact of this type of treatment on bacterial communities and their antibiotic resitance profiles is therefore another logical next step.

Do decreased antibiotic concentrations, resulting from advanced on-site wastewater treatment of high-risk point sources, pose an additional risk for ARG propagation?

Based on the combined results of *Chapter 2 + 4*, the question about potential additional risk associated with reduced antibiotic concentrations arises. A large number of antibiotics in hospital wastewater could significantly be reduced by advanced on-site treatment, many of which could be completely eliminated or reduced under the limit of detection. Very low (sub-inhibitory) antibiotic concentrations could be shown to favor horizontal gene transfer of antibiotic resistance genes, in *Chapter 4*. It might be interesting to reproduce the microcosm experiments described in *Chapter 4*, using the treated wastewater effluent from advanced on-site hospital wastewater treatment plants. If an increased rate of horizontal gene transfer can indeed be shown (compared to untreated wastewater), follow-up experiments might clarify the driving mechanisms. Additional questions that could be answered by follow-up experiments could: "Are different types of microorganisms more receptive to horizontal gene transfer?" or "Which of the compounds reduced in concentrations during advanced treatment increase the risk of horizontal gene transfer?" .

6.4. Perspectives

Despite the continuous, accelerated progress of antibiotic resistance, a number of technologies and "arms" against pathogens and superbugs have been forming and developing based on increased computational power and the (big) data revolution.

The technology with the biggest potential for positive impact are machine-learning models and artificial intelligence. Machine-learning models are able to predict hitherto unknown antibiotic resistance genes from metagenomic datasets, to improve risk assessment, to prediction outbreaks and to design sorely needed new molecules and compounds with antibiotic function. Given sufficient data, it is not unlikely that machine learning and artificial intelligence algorithms will be able to predict unexpected hotspots of antibiotics and antibiotics resistant bacteria, predict hospital outbreaks with MRSA and other superbugs or automatically predict if antibiotic resistance genes in (clinical) samples are located on mobile genetic elements or human pathogens, thus helping with outbreak prevention efforts and general risk assessment.

A number of countries are already implementing on-site treatment of high-risk point sources and an even greater number of countries are considering to follow suit. Technological progress of (advanced) wastewater treatment technologies could facilitate the processing of ever larger volumes of wastewaters at decreased costs in the future, thus enabling the application of these advanced wastewater treatment technologies to a majority of wastewaters, not only limited on-site treatment of high-risk point sources. A great number of issues, apart from antibiotic resistance, could be ameliorated by such large-scale application of wastewater treatment. Water is one of the most precious and (increasingly scarce) goods of the 21st century and (due to climate change, demographic change and other dilemmas of out modern times) sustainable handling of this precious good will become only more essential. We will need to employ all available tools and resources (including big data in combination with artificial intelligence and machine-learning models, advanced wastewater treatment, legislation and increased monitoring) to overcome the challenges of our current times in order to ensure long-term, global (drinking) water safety.

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8. Scientific Achievements and Contributions

8.1. List of Peer-Reviewed Publications

- **Paulus, G. K.,** Hornstra, L. M., Alygizakis, N., Slobodnik, J., Thomaidis, N., & Medema, G. (2019). The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. International journal of hygiene and environmental health, 222(4), 635-644.
- **Paulus, G. K.,** Hornstra, L. M., & Medema, G. (2020). International tempo-spatial study of antibiotic resistance genes across the Rhine river using newly developed multiplex qPCR assays. Science of The Total Environment, 706, 135733.
- Alygizakis, N. A., Besselink, H., **Paulus, G. K.,** Oswald, P., Hornstra, L. M., Oswaldova, M., & Slobodnik, J. (2019). Characterization of wastewater effluents in the Danube River Basin with chemical screening, in vitro bioassays and antibiotic resistant genes analysis. Environment international, 127, 420-429.

8.2. List of Awards and Fellowships

- Marie-Skłodowska-Curie Fellowship (H2020, 2016 – 2019)
- CSF Award Winner 2018 - Best contribution (for oral presentation at international HEARD conference, awarded by ETH Zurich)
- Recipient of the Elsevier Atlas award (for published peer-reviewed research)

8.3. International Conference Contributions

---------- only contributions with role as main presenter shown ----------

POSTER PRESENTATION

- "The Benefits of Multiplex qPCR Assays in the Field of Environmental Antibiotic Resistance", 4th International Symposium on the Environmental Dimensions of Antibiotic Resistance (EDAR), Lansing , USA, August 2017

ORAL PRESENTATION

- "The Effects of on-site Hospital WW Treatment on ARG Prevalence", Halting Antimicrobial Resistance Dissemination in Aquatic Environments (HEARD2018), Ascona, Switzerland, September 2018
- "Environmental ARG pollution monitoring across the Rhine River – A tempo-spatial study", Challenges and Solutions related to Xenobiotics and Antimicrobial Resistance in the Framework of Urban Wastewater Reuse: Towards a Blue Circle Society (XENOWAC II), Limassol, Cyprus, October 2018
- "The Impact of Different Molecular Methods on Antibiotic Resistance Gene (ARG) Quantification – Comparing qPCR to Next Generation Sequencing", ", 5th International Symposium on the environmental dimension of antibiotic resistance (EDAR 2019), Hong Kong, Hong Kong, June 2019

9. About the Author

The author was born in Darmstadt, Germany in 1989, on a beautiful, sunny winter morning. She finished highschool in summer of 2008 and enrolled at the University of Hohenheim in Stuttgart, Germany for studies in Biotechnology in 2009 where she graduated in 2012 with a Bachelor of Science degree. After a one-year creative break and many travels, she started a 6-months internship at the pharmaceutical company Merck in 2013. The author then enrolled at the Rheinische Friedrich-Wilhelms-Universität Bonn, Germany in 2014. She developed a deep passion for programming, in addition to research, and obtained a Master of Science degree in Pharmacology and Toxicology in 2016. A few days later, Gabriela started her position as PhD Candidate and Marie-Sklodowska-Curie-Fellow at KWR and at TU Delft under the supervision of Gertjan Medema (TU Delft) and Luc Hornstra (KWR).

The new challenge was to help advance the fight against antibiotic resistance.

…Rumor has it, she never skipped a day at the gym while working at her PhD.

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Luc, I will never forget one of our talks during the middle of my second year, I was not (…am still not and will likely never be) used to not being able to easily juggle my workload, even temporarily. You told me that sometimes it is ok and even important for success to prioritize. So prioritize I did and it has been a complete game changer. Your constant positive support helped to put upcoming difficulties into perspective and made it very easy to take necessary academic risks.

Gertjan, your constructive criticism has been key to becoming an independent and confident researcher. I remember my first conference abstract. I sent you a draft and after not even 30 minutes, I received an email back: "This is not a good abstract." were the only words. Another 2 minutes later, I received a follow-up with a list of things I needed to do better. I implemented the changes and my abstract was accepted without a problem. I just re-read the un-edited abstract in question and have to whole-heartedly agree: it was not a good abstract! ☺ Your way of providing efficient, to-the-point feedback has significantly helped me being critical with my own work, in the best way.

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